ORIGINAL RESEARCH

Role of sodium tungstate as a potential antiplatelet agent

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Purpose: Platelet inhibition is a key strategy in the management of atherothrombosis. However, the large variability in response to current strategies leads to the search for alternative inhibitors. The antiplatelet effect of the inorganic salt sodium tungstate (Na_2O_4W), a protein tyrosine phosphatase 1B (PTP1B) inhibitor, has been investigated in this study.

Methods: Wild-type (WT) and PTP1B knockout ($PTP1B^{-/-}$) mice were treated for 1 week with Na_2O_4W to study platelet function with the platelet function analyzer PFA-100, a cone-and-plate analyzer, a flat perfusion chamber, and thrombus formation in vivo. Human blood aliquots were incubated with Na_2O_4W for 1 hour to measure platelet function using the PFA-100 and the annular perfusion chamber. Aggregometry and thromboelastometry were also performed.

Results: In WT mice, Na_2O_4W treatment prolonged closure times in the PFA-100 and decreased the surface covered (%SC) by platelets on collagen. Thrombi formed in a thrombosis mice model were smaller in animals treated with Na_2O_4W (4.6 ± 0.7 mg vs 8.9 ± 0.7 mg; $P<0.001$). Results with Na_2O_4W were similar to those in untreated $PTP1B^{-/-}$ mice (5.0 ± 0.3 mg). Treatment of the $PTP1B^{-/-}$ mice with Na_2O_4W modified only slightly this response. In human blood, a dose-dependent effect was observed. At 200 µM, closure times in the PFA-100 were prolonged. On denuded vessels, %SC and thrombi formation (%T) decreased with Na_2O_4W. Neither the aggregating response nor the viscoelastic clot properties were affected.

Conclusion: Na_2O_4W decreases consistently the hemostatic capacity of platelets, inhibiting their adhesive and cohesive properties under flow conditions in mice and in human blood, resulting in smaller thrombi. Although Na_2O_4W may be acting on platelet PTP1B, other potential targets should not be disregarded.

Keywords: sodium tungstate, protein tyrosine phosphatase 1B, platelet adhesion, antiplatelet agents

Introduction

Atherothrombosis remains the leading cause of morbidity and mortality in Western society. Platelets play a key role in hemostasis, but they are also responsible for the pathologic thrombus formation underlying the clinical manifestations of acute atherothrombotic vascular disease.[1] Therefore, modulation of platelet activation is a main goal for the development of pharmacological strategies to prevent the occurrence of cardiovascular accidents. Platelet activation occurs through multiple pathways and current agents do not interfere with all of them.

In patients suffering from acute coronary syndromes or undergoing percutaneous coronary intervention, oral antiplatelet treatment is routinely administered to inhibit platelet-mediated thrombus formation and the subsequent vessel occlusion. While currently available oral antiplatelet agents such as aspirin and P2Y12 adenosine 5′-diphosphate (ADP) receptor antagonists reduce the incidence of ischemic events, the residual risk for morbidity and mortality remains substantially elevated.[2,3]

Interindividual response variability to aspirin and, especially, to clopidogrel makes it difficult to find the appropriate balance between the risk of thrombosis and bleeding in association with the treatment with these compounds.[4–7] Therefore, there is continuous search for new alternatives providing more rapid and consistent platelet inhibition.

Sodium tungstate (Na_2O_4W) has been demonstrated to be effective in normalizing blood glucose levels and in decreasing the body weight gain and adiposity in animal models of diabetes[8] and obesity. Na_2O_4W has been shown to increase the expression of the uncoupling protein 1 (*UCP1*) and 3 (*UCP3*) genes,[8,9] implicated in the control of adaptive thermogenesis, the production of reactive oxygen species by mitochondria, the regulation of ATP synthesis, and the regulation of fatty acid oxidation. Na_2O_4W was patented as an antiobesity agent. In three different phase I studies in healthy volunteers, Na_2O_4W demonstrated a very wide tolerance window for a 6-week period. In a phase II study in obese patients under treatment with Na_2O_4W for 6 weeks, the antiobesity effect was low although positive.[10] Tungstate shares molecular similarities with vanadate (VO_4^{3-}), both being salts of trace transition metals in biological systems, with insulin-mimetic effects.[11] Vanadate and Na_2O_4W are phosphate analogs and they act as protein tyrosine phosphatase (PTP) inhibitors. Because vanadate has been shown to inhibit PTP1B, it would be plausible to think that Na_2O_4W could also exhibit a similar effect.[12] PTP1B plays a major role in the activation of platelets[13] and is required for normal platelet thrombus formation in living mice.[14] In this regard, we have indirect in vitro evidence of a potential effect of Na_2O_4W on platelet function.

The aim of the present study was to investigate the potential effect of Na_2O_4W as an antiplatelet agent using different approaches aimed to evaluate the adhesive and cohesive properties of platelets. Primary hemostasis was evaluated in wild-type (WT) and PTP1B knockout mice, after a week of treatment with Na_2WO_4 in drinking water. In vitro studies were also carried out in human blood samples treated with Na_2O_4W.

Materials and methods
Experimental design
Studies were designed to evaluate the effect of Na_2WO_4 on platelet function. WT and PTP1B knockout (PTP1B$^{-/-}$) mice[15,16] were treated for a week with Na_2WO_4 (2 g/L in drinking water). To analyze platelet function under flow conditions, four different approaches were applied: the platelet function analyzer PFA-100, the cone-and-plate analyzer (CPA), a small flat perfusion chamber, and the inferior vein cava (IVC) ligation model (an in vivo model that evaluates the pathophysiology of deep vein thrombosis in mice).

Studies on human blood were performed in vitro with either whole blood or platelet-rich plasma (PRP) incubated with Na_2WO_4. The effect of different concentrations of Na_2WO_4 (100 µM, 200 µM, and 400 µM, for 1 hour at 37°C) on platelet function was evaluated using the PFA-100 and the annular perfusion chamber. The concentration of 200 µM was then applied for aggregometry and thromboelastometry.

Studies on mice
All the experiments were performed in 8-week-old male mice littermates, of a mixed C57BL/6J×129 background. Mice were housed under standard conditions of light (12-hour light/dark cycles) and temperature (21°C). Animals were fed ad libitum with standard chow diet (type A04 from Panlab, Barcelona, Spain) and received 2 g/L Na_2O_4W for a week. All animal procedures were approved by the Animal Ethics/ Research Committee of the University of Barcelona, and principles of laboratory animal care were followed.

Animals were anesthetized with an intraperitoneal injection of ketamine–xylazine, and intracardiac puncture was performed to obtain approximately 800 µL of blood anticoagulated with 110 mM trisodium citrate (1:10, vol/vol).

Hemostatic capacity in the PFA-100
The PFA-100 can be considered a substitute for the classic bleeding time test. The system monitors platelet interaction on membranes coated with collagen–ADP (Col–ADP) and collagen–epinephrine (Col–Epi).[17] The system comprises a microprocessor-controlled instrument and a disposable test cartridge containing a biologically active membrane. The instrument aspirates a blood sample (citrated blood) under constant vacuum from the sample reservoir through a capillary and a microscopic aperture cut into the membrane. The membrane is coated with Col–Epi or Col–ADP. The presence of these biochemical stimuli, as well as the high shear rates generated under the standardized flow conditions, result in platelet attachment, activation, and aggregation, slowly building a stable platelet plug at the aperture. The time required to obtain full occlusion of the aperture is reported as the "closure time," which is expressed in seconds.

CPA testing
The CPA was used as previously described.[18] The device tests platelet adhesion and aggregation in citrated whole blood under arterial flow conditions (1,800/second for 2 minutes). Blood is in

contact with the polystyrene surface of a well by using laminar flow. Three parameters were evaluated: the surface covered by platelets (expressed in percentage, %SC), the average size of the polystyrene-bound platelet clusters or platelet aggregates (AS, μm^2), and the number of platelet groups (OB, n).

Experiments in flat perfusion devices

The interaction of mouse platelets with collagen-coated coverslips (100 µg per coverslip) was explored in a low-volume flat perfusion device. Citrated blood samples were perfused for 5 minutes at a shear rate of 1,200/second adjusted with a peristaltic pump (Renal Systems, Minneapolis, MN, USA).

Perfused surfaces were fixed (2.5% glutaraldehyde in 0.15 M phosphate-buffered saline [PBS], 4°C, 24 hours), stained with toluidine blue (0.02%), and platelet interactions evaluated by light microscopy. Morphometric analysis was performed using a computerized system (Image J), and platelet interactions were globally expressed as the percentage of the surface covered by platelets (%SC).

In vivo thrombosis model

IVC ligation model was used as previously described.[19] Briefly, mice (n=8 for each group) were anesthetized with a 2% mixture of isoflurane–oxygen and placed in a supine position. After laparotomy, intestines were exteriorized and sterile saline was applied during the whole procedure to prevent drying. Posterior venous branches were cauterized and all visible side branches (usually one or two) were ligated. Then, gentle separation from aorta was performed and IVC was ligated using a 7-0 polypropylene suture immediately below the renal veins to obtain complete blood stasis. After surgery, peritoneum and skin were closed with a 6.0 mm-size monofilament suture. Mice were euthanized after 3 hours, and the vessel just below the renal veins and proximal to the confluence of the common iliac veins was excised. Mice showing any sign of tissue injury during surgery were excluded from further analysis.

Studies with human blood

Blood sampling

Blood was drawn from healthy volunteers who had not ingested drugs affecting platelet function in the previous 10 days. All donors gave their written informed consent to participate in the study. The study was performed in agreement with the Declaration of Helsinki and was approved by the Ethical Committee of the Hospital Clinic (Barcelona). Blood was anticoagulated with 110 mM trisodium citrate (1:10, vol/vol) and incubated with different concentrations of Na_2WO_4 (0 µM, 100 µM, 200 µM, and 400 µM) for 1 hour at 37°C.

Experiments in annular perfusion devices

Aorta segments of enzymatically denuded New Zealand rabbits were mounted inverted on the central plastic rod of the perfusion chamber.[20] Citrated blood samples were recirculated for 10 minutes at shear rates of 800/second and 1,200/second adjusted with a peristaltic pump (Renal Systems). Perfused surfaces were rinsed with 0.15 M PBS, fixed (2.5% glutaraldehyde in 0.15 M PBS, 4°C, 24 hours), dehydrated with a raising ethanol gradient, embedded in JB-4, thin sectioned for light microscopy, and stained with methylene blue. Platelet interactions were evaluated with light microscopy and a software[21] that quantifies platelet coverage – expressed as percentage (%SC) – and calculates interactions higher than 5 µm (%T).

Aggregation studies using turbidimetric techniques

PRP was obtained by centrifugation of whole blood (120 g, 15 minutes). PRP aliquots were incubated with Na_2O_4W by gentle stirring in a conventional aggregometer (Aggrecorder PA 3210 aggregometer; Menarini Diagnostic, Firenze, Italy). Platelets were activated with arachidonic acid (AA, 1.6 mM), ADP (4 µM and 2 µM), collagen (Col, 5 µg/mL), and ristocetin (R, 1 mg/mL) (10 minutes, 37°C) under stirring. Changes in turbidimetric patterns were registered and results expressed as percentage maximal aggregation.[22]

Thromboelastometry studies

Dynamic thrombelastography of whole-blood coagulation was explored using the EXTEM, INTEM, and FIBTEM tests in the rotational thromboelastometry analyzer (PentapharmGmbH, Munich, Germany).[23] EXTEM uses tissue factor as activator and measures changes in the extrinsic pathway of coagulation, fibrinogen and fibrin polymerization, and platelet function. In the FIBTEM test, platelet function is eliminated with cytochalasin D, obtaining a fibrin clot. The INTEM test uses ellagic acid to measure changes in the intrinsic pathway of coagulation.

Clotting time, clot formation time, clot amplitude after 10 minutes (A10), maximum velocity (MaxV), and time to maximum clot formation velocity (MaxV-t) were evaluated. The clotting time and the clot formation time indicate the dynamics of clot formation. The clot amplitude gives information about clot strength and stability, largely dependent on fibrinogen and platelets, and MaxV and MaxV-t express the clot formation velocity.

Statistics

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with raw data using Student's *t*-tests for paired and unpaired samples. The results were considered significant at $P<0.05$.

Results

Effect of sodium tungstate on mouse platelets

Platelet function in the PFA-100

In WT mice (n=8), closure times were 148±17.4 seconds and 211±18 seconds when using the Col–ADP and Col–Epi cartridges, respectively. Closure times in the Col–Epi cartridges were already prolonged in untreated animals and no significant additional effect of Na_2O_4W was detected (227±10 seconds). In Col–ADP cartridges, Na_2O_4W treatment significantly prolonged the closure time up to 272.8±12.4 seconds ($P<0.01$) (Figure 1).

In PTP1B$^{-/-}$ mice (n=8), closure times in untreated animals were 292.9±4.5 seconds and 256±10 seconds when using the Col–ADP and Col–Epi cartridges, respectively. In Na_2O_4W-treated mice, closure times did not significantly differ either in the Col–ADP cartridge (258.6±13.3 seconds) or in the Col–Epi cartridge (196.6±61 seconds) groups (Figure 1).

Platelet interaction in the CPA

In WT animals (n=8), SC (%), AS (μm^2), and OB (n) in the absence of Na_2O_4W were 1.40%±0.39%, 32.9±1.82 μm^2, and 47.9±11.7, respectively. After treatment, both SC and OB increased significantly to 4.5%±1.4% and 783.1±229.4, respectively ($P<0.05$); AS decreased significantly to

22.2±0.8 μm^2 ($P<0.05$) (Figure 2). The increased SC may be due to the higher number of single platelets (OB) that are unable to aggregate among them in the presence of Na_2O_4W but that may adhere onto the plastic surface, probably through unspecific mechanisms.

In untreated PTP1B$^{-/-}$ mice (n=8), SC, AS, and OB were 1.75%±0.79%, 32.1±2.8 μm^2, and 100.1±25.28, respectively. After treatment with Na_2O_4W, SC and AS decreased slightly to 0.64%±0.14% and 26.13±02.1 μm^2 ($P<0.01$), respectively, and OB increased moderately to 146.5±33.87 (Figure 2).

Platelet interaction with collagen under flow conditions

The %SC on collagen-coated surfaces perfused for 5 minutes with blood from WT mice was 44.39%±3.43% (mean ± SEM, n=5). When adhesion studies were performed with the blood of Na_2O_4W-treated animals, %SC was reduced to 30.72%±1.26%, this decrease being statistically significant ($P<0.01$) (Figure 3).

With blood from PTP1B$^{-/-}$ mice, no changes were observed after Na_2O_4W treatment. The %SC was 13.86%±0.35% and 16.23%±0.74% before and after treatment with Na_2O_4W, respectively (Figure 3).

In vivo thrombosis model

Because the previous experiments showed that Na_2O_4W decreases the hemostatic capacity of platelets, we wanted to explore the effect of this compound in vivo. Mice treated with Na_2O_4W showed decreased thrombi weights after 3 hours in the IVC stasis model (4.6±0.7 mg in treated mice vs 8.9±0.7 mg in nontreated mice; $P<0.001$;
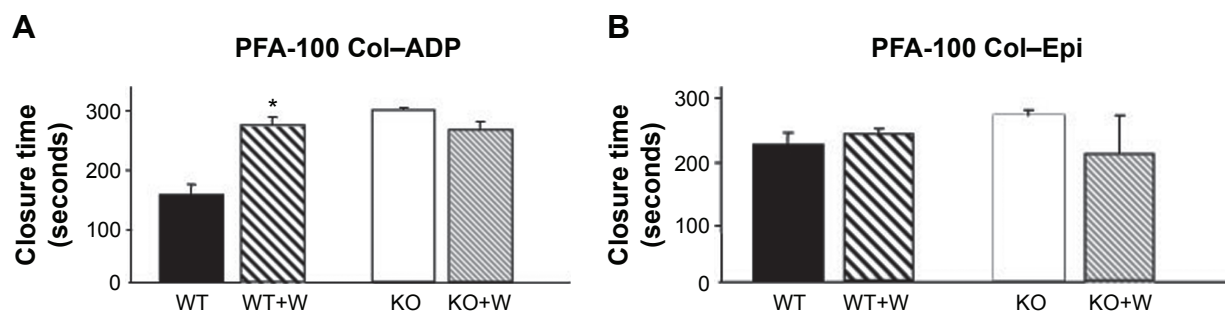
**Figure 1** Mouse platelet function after Na_2O_4W treatment.
Notes: CTs at the PFA-100 were measured using citrated blood samples. Na_2O_4W treatment of WT mice (**A**) prolonged the CT in Col–ADP cartridges, (**B**) being unchanged in Col–Epi cartridges. CT in PTP1B$^{-/-}$ mice were almost identical to those obtained in WT animals treated with W. Data are expressed as mean ± SEM (n=8, *$P<0.01$).
Abbreviations: Col–ADP, collagen–adenosine 5′-diphosphate; Col–Epi, collagen–epinephrine; CT, closure time; KO, knockout mice; PFA-100, platelet function analyzer; PTP1B$^{-/-}$, protein tyrosine phosphatase 1B knockout; SEM, standard error of the mean; W, Na_2O_4W; WT, wild type.
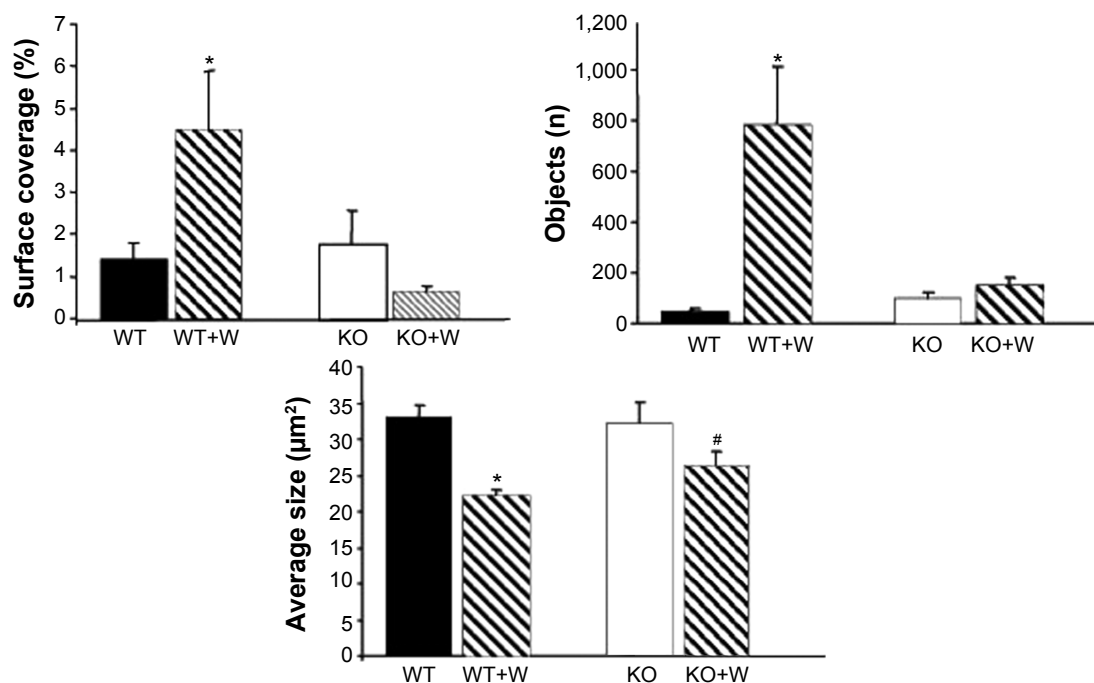
Figure 2 Effect of Na_2O_4W on different parameters measured in a cone and plate analyzer.
Notes: W treatment of WT mice increased the surface covered by platelets (SC, %) and the number of objects (OB, n) adhered, though it decreased the average size of the aggregates (AS, μm^2). In PTP1B$^{-/-}$ mice, both SC and OB were lower and a slight decrease in the AS was also detected in response to W. Data are expressed as mean $\pm$ SEM (n=8, *$P<0.05$ vs nontreated WT, #$P<0.01$ vs nontreated PTP1B$^{-/-}$).
Abbreviations: KO, knockout mice; PTP1B$^{-/-}$, protein tyrosine phosphatase 1B knockout; SEM, standard error of the mean; W, Na_2O_4W; WT, wild type.
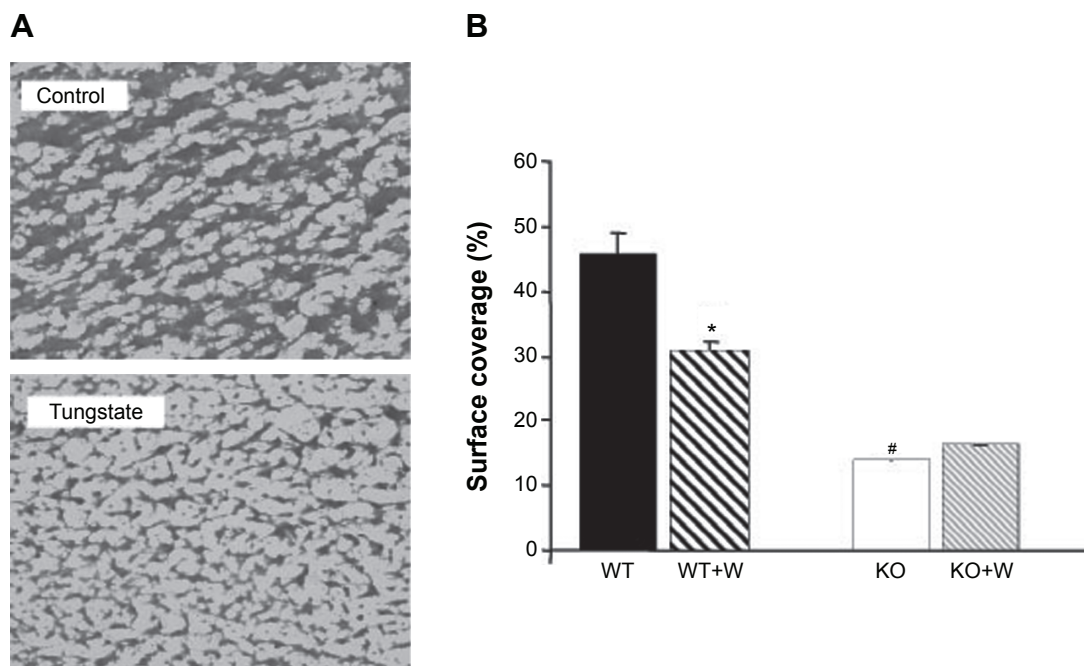
Figure 3 Mouse platelet adhesion on collagen under flow conditions.
Notes: (**A**) Micrographs show groups of platelets adhered onto a collagen-rich surface perfused with citrated blood from untreated WT mice (WT) or treated with Na_2O_4W (WT+W). (**B**) Bar diagram represents the corresponding surface covered by platelets (%) expressed as mean $\pm$ SEM (n=5, *$P<0.01$ vs untreated WT; #$P<0.001$ untreated PTP1B$^{-/-}$ vs untreated WT).
Abbreviations: KO, knockout mice; SEM, standard error of the mean; W, Na_2O_4W; WT, wild type; PTP1B$^{-/-}$, protein tyrosine phosphatase 1B knockout.

Figure 4). Moreover, we also studied thrombus formation in the PTP1B$^{-/-}$ mice and analyzed whether these differences could be potentiated by Na$_2$O$_4$W. PTP1B$^{-/-}$ mice showed smaller thrombi than WT mice (5.0±0.3 mg in nontreated PTP1B$^{-/-}$ mice vs 8.9±0.7 mg in nontreated WT mice; $P<0.001$), reflecting a defect in platelet function in these mice, as previously described.[14] Na$_2$O$_4$W reduced thrombi weight to a significantly greater extent in these animals (2.8±0.4 mg in treated PTP1B$^{-/-}$ mice vs 5.0±0.3 mg in nontreated PTP1B$^{-/-}$ mice; $P<0.01$), indicating that the mechanisms by which Na$_2$O$_4$W affects hemostasis would be not only through PTP1B. Therefore, other potential targets should not be disregarded.

Effect of sodium tungstate on human platelets

Platelet function in the PFA-100

Closure times in the absence of Na$_2$O$_4$W were 149.1±6.1 seconds (mean ± SEM, n=10) and 99.9±4.3 seconds in Col–Epi and Col–ADP cartridges, respectively (Figure 5).

In the presence of 100 µM, 200 µM, and 400 µM of Na$_2$O$_4$W, closure times were prolonged in a concentration-dependent manner to 182.3±5.5 seconds ($P<0.01$), 203.6±17.9 seconds ($P<0.01$), and 198.3±7.8 seconds ($P<0.001$), respectively, in the Col–Epi cartridges (Figure 5). In the Col–ADP cartridges, only the highest concentration of Na$_2$O$_4$W used (400 µM) resulted in a significantly delayed closure time (175.7±36 seconds, $P<0.005$) (Figure 5).
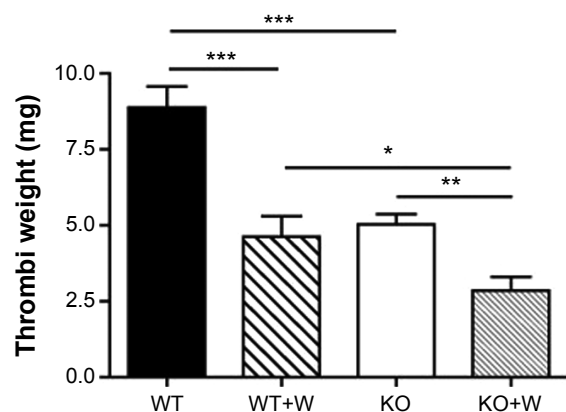
Figure 4 IVC ligation model.
Notes: Bars represent weights (milligrams) of harvested IVC wet thrombi 3 hours after DVT induction, obtained from untreated WT mice (WT), WT mice treated with Na$_2$O$_4$W (WT+W), untreated PTP1B$^{-/-}$ mice (KO), or PTP1B$^{-/-}$ mice treated with Na$_2$O$_4$W (KO+W). Eight mice for each group were assayed (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; Student's t-test).
Abbreviations: DVT, deep vein thrombosis; IVC, inferior vein cava; KO, knockout mice; PTP1B$^{-/-}$, protein tyrosine phosphatase 1B knockout; SEM, standard error of the mean; W, Na$_2$O$_4$W; WT, wild type.

Platelet interaction with subendothelial surfaces under flow conditions

Perfusion assays were carried out with denuded vessel segments at moderate shear rates. In the absence of Na$_2$O$_4$W, morphometric analysis of the surfaces perfused at 800/second showed that %SC in control samples after 10 minutes of perfusion was 33.0%±1.0% (mean ± SEM, n=9). In the presence of 100 µM (n=4), 200 µM (n=9), and 400 µM (n=4) Na$_2$O$_4$W, %SC was significantly reduced to 30.7%±3.9%, 18.0%±2.4% ($P<0.001$), and 27.0%±2.7% ($P<0.05$). Aggregates of >5 µm (%T) were found covering 56.3%±4.4% of the SC in the absence of Na$_2$O$_4$W, while on those surfaces perfused with blood samples preincubated with 100 µM, 200 µM, and 400 µM of this compound, there was a significant decrease in the percentage of the surface covered by large thrombi, to 38.1%±12.7%, 32.1%±4.0% ($P<0.005$), and 38.2%±9.4%, respectively (Figure 5B). Representative micrographs are shown in Figure 6.

The concentration of 200 µM of Na$_2$O$_4$W was used to perform the remaining experiments with human blood. Using this concentration, similar results were obtained at a shear rate of 1,200/second (reductions of 63.8%±4.8% and 67.4%±3.6% in the %SC and %T, both $P<0.05$, respectively, were observed in the presence of Na$_2$O$_4$W).

### Platelet aggregation using turbidimetric techniques

Samples of PRP before and after being incubated with 200 µM Na$_2$O$_4$W for 1 hour were activated with AA, ADP 4, ADP 2, Col, and R for 5 minutes. Results expressed as percentage of maximal aggregation (mean ± SEM, n=10) were 82.5%±3.3%, 86.4%±3.6%, 78.0%±8.1%, 91.5%±2.8%, and 71.1%±6.8%, respectively. In the presence of Na$_2$O$_4$W, percentage of maximal aggregation was 81.2%±7.0%, 82.8%±5.4%, 54.3%±9.0%, 84.7%±6.4%, and 72.7%±6.2%, respectively. Although results did not differ significantly, a decrease in the aggregating response to the lower concentration of ADP assayed was detected ($P=0.06$).

Thromboelastometric properties of blood clot

In the presence of 200 µM Na$_2$O$_4$W, no significant differences were observed in the parameters measured in the three different tests applied (Table 1).

Discussion

The majority of drugs developed as antiplatelet agents basically affect platelet membrane receptors and metabolic pathways and have a major impact on platelet function. Although most of them have shown clinical efficacy in reducing
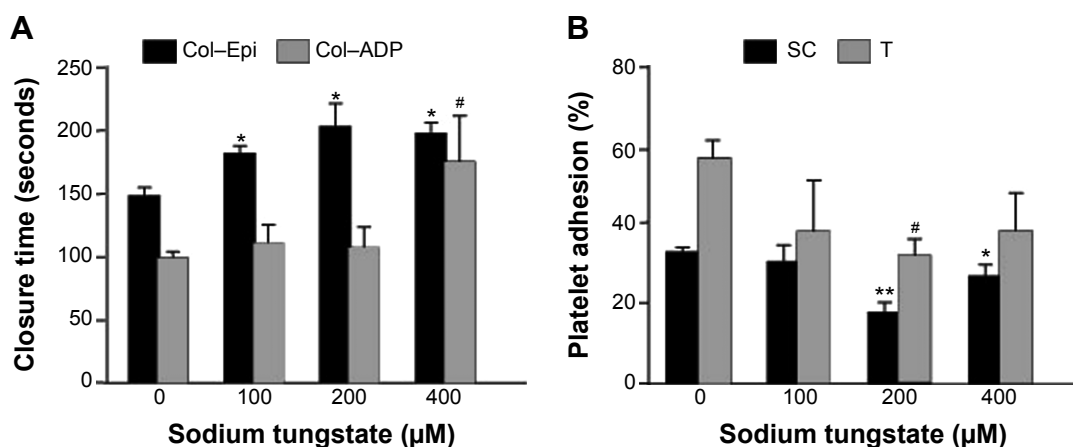
Figure 5 Platelet interaction with subendothelial surfaces under flow conditions.
Notes: Bar diagrams show the effect of incubating citrated blood samples with different concentrations of Na_2O_4W on (**A**) the closure times (seconds) in the PFA-100 device when using Col–Epi (black bars) and Col–ADP (gray bars) cartridges and (**B**) the platelet adhesion on denuded vascular segments (perfused at 800/second for 10 minutes). Surface coverage (SC; black bars); surface covered by thrombi ([T] with respect to the total SC; gray bars). Data are expressed as mean ± SEM (*$P<0.01$, **$P<0.001$, #$P<0.005$ vs the corresponding nontreated sample's value).
**Abbreviations:** Col–ADP, collagen–adenosine 5′-diphosphate; Col–Epi, collagen–epinephrine; PFA-100, platelet function analyzer; SEM, standard error of the mean; W, Na_2O_4W; WT, wild type.

morbidity and mortality in patients with atherothrombotic disease, these agents are associated with a residual risk of thrombotic events, risk of bleeding, and high variability in the patients' responses. The use of a simple molecule targeting signaling proteins such as PTPs, which regulate partial aspects of platelet function, could lead to a more manageable effect on platelet function. Our present in vivo and in vitro studies demonstrate that Na_2O_4W significantly inhibits platelet interaction with subendothelial surfaces under flow conditions, decreasing the adhesive and cohesive properties of platelets without interfering with the thromboelastometric properties of blood clot. These differences resulted in smaller thrombi in a deep vein thrombosis mouse model induced by blood stasis.

Thrombotic complications attributed to platelets require prior formation of a mural thrombus. The thrombus or embolized portions can be responsible for downstream ischemic complications. Several factors are known to participate in the regulation of thrombus formation. Platelet adhesion to the vessel wall increases with shear stress.[24] Glycoprotein IIb-IIIa is expressed in an active conformation after platelets become exposed to damaged arterial surfaces under flow conditions.[25,26] Thrombin generated through the activation of the coagulation system, and thromboxane A2 (TXA2) generated through arachidonic acid metabolism, are powerful activating agents, facilitating platelet deposition and growth of aggregates.[27–29] In addition, platelets possess several receptors for ADP on their membrane surface and contain ADP and other vasoactive substances in their storage granules, which are released during platelet secretion,[29] a step of critical importance in the regulation of platelet responses.[30]

Inhibition of platelet aggregation can be achieved by either the blockade of membrane receptors or by interfering with intracellular pathways. Inhibition of arachidonic acid metabolism by aspirin and blockade of the ADP receptors by thienopyridines are currently the more widely used antiplatelet strategies. In fact, current standard treatment for
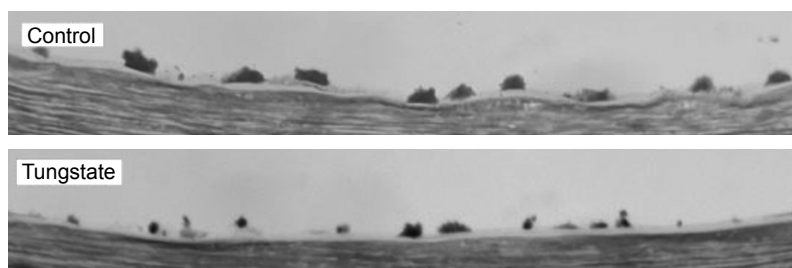
Figure 6 Micrographs showing platelet adhesion on subendothelial surfaces in the presence of Na_2O_4W.
Notes: Citrated blood samples, before (Control) and after being incubated with 200 μM Na_2O_4W (Tungstate), were perfused on denuded rabbit aorta segments at 800/second, for 10 minutes. Images are representative of nine different experiments.

Table 1 Effect of tungstate on the viscoelastic properties of clots

	ClotT	CFT	A10 (mm)	MaxV (mm/min)	MaxV-t (seconds)
EXTEM					
Control	55.5±10.5	95.5±10.5	53.0±2.0	13.5±1.5	113.0±4.0
Tungstate	60.0±5.0	84.5±3.5	54.0±1.0	14.5±0.5	115.0±18.0
INTEM					
Control	168.0±23.0	79.0±11.0	52.0±3.0	16.0±2.0	189.0±29.0
Tungstate	166.0±15.0	75.0±2.0	54.5±1.5	17.0±0.0	191.5±29.0
FIBTEM					
Control	54.5±6.5	NA	11.0±1.0	10.5±2.5	58.0±6.0
Tungstate	56.5±0.5	NA	12.0±0.0	12.5±1.5	58.5±0.5

Notes: ClotT, CFT, A10, MaxV, and MaxV-t were evaluated. Data are expressed as mean ± SEM (n=4).
Abbreviations: ClotT, clotting time; CFT, clot formation time; A10, clot amplitude after 10 minutes; MaxV, maximum velocity; MaxV-t, time to maximum clot formation velocity; NA, not applicable; SEM, standard error of the mean.

the prevention of thrombosis after percutaneous coronary intervention in acute coronary syndrome patients is dual antiplatelet therapy with aspirin and the thienopyridine clopidogrel.[31–33] However, high platelet reactivity and genetic polymorphisms affect patient response to antiplatelet treatment.[34–36] Although newly developed thienopyridine compounds, such as prasugrel, seem to be effective antiplatelet agents, they exhibit increased associated risk of bleeding and higher acquisition cost compared with clopidogrel. Thus, there is continuous search for newer agents with less variable antiplatelet activity and lower associated bleeding risk.

Na$_2$O$_4$W is a phosphatase inhibitor having antidiabetic properties,[37,38] with an excellent therapeutic profile in both long- and short-term treatments.[39,40] Administered orally, Na$_2$O$_4$W normalizes glycemia in mouse models of diabetes.[40,41] It also increases the total amount and translocation of GLUT4 transporter in muscle[42] and restores the glucose hepatic metabolism in streptozotocin-induced diabetic rats.[11] In streptozotocin-treated neonatal rats, Na$_2$O$_4$W administration stimulates insulin secretion[43] and regenerates β-cell population.[41] Na$_2$O$_4$W also reduces weight gain and adiposity by increasing energy dissipation and fatty acid oxidation rate in an obese rat model.[8] In a phase II study[10] in obese patients treated with Na$_2$O$_4$W for 6 weeks, the antiobesity effect was positive although low, probably due to the short treatment duration and the lack of a hypocaloric diet used synergistically, among other limitations. Despite all the evidence generated, the precise molecular mechanisms of Na$_2$O$_4$W action are not yet defined. In the context of diabetes, a recent study in an animal model[44] points to the effect of Na$_2$O$_4$W on different phosphatases, such as PTP1B and PP1, and also on G-proteins, all these being key elements in platelet function.

In our studies with Na$_2$O$_4$W-treated WT mice, hemostasis was significantly delayed, as evidenced by prolonged closure

times at the PFA-100, using the COL–ADP cartridge, and lower rates of surface covered by platelets on perfused collagen surfaces. The PFA-100 assay was designed for the screening of primary hemostasis in humans, and not in other animal species, and some differences between the results with mice and human blood could be expected. The in vivo model of thrombosis in mice showed a consistent inhibitory effect of Na$_2$O$_4$W on thrombi formation. Interestingly, when blood samples were from PTP1B$^{-/-}$ mice, results were similar to those obtained with blood from WT mice in the presence of Na$_2$O$_4$W and no additional effect was observed when PTP1B$^{-/-}$ mice were treated. In experiments with human blood, a moderate and consistent effect decreasing both the adhesive and cohesive properties of platelets, when interacting with denuded vascular segments under flow conditions, was observed in the presence of Na$_2$O$_4$W. Patterns of interaction were more similar to those reported with the ADP receptor inhibitors than with aspirin. It is interesting to mention that the effect of Na$_2$O$_4$W on hemostasis seems to be directed toward platelet function because no action on the coagulation system could be observed. The effect of Na$_2$O$_4$W on the hemostatic properties of platelets was detected only when applying flow techniques using whole blood. Although the effect seems restricted to platelets, an in vivo model showed a marked decrease in venous thrombus formation, indicating that Na$_2$O$_4$W acted as an antithrombotic drug. Indeed, previous studies[45,46] have shown that the IVC stasis model requires a proper platelet function through interaction with endothelial cell-derived von Willebrand factor.

The effects of Na$_2$O$_4$W on the adhesive and cohesive properties of platelets could be related to PTP1B, as derived from results in the mouse model. In fact, results with blood from the PTP1B$^{-/-}$ mice are quite coincident with the results with blood from WT animals treated with Na$_2$O$_4$W, and

treatment of PTP1B$^{-/-}$ mice with Na_2O_4W did not modify this response. PTP1B is a tyrosine phosphatase that regulates platelet functions that are dependent on outside–in $\alpha IIb\beta 3$ signaling,[14] such as platelet spreading on fibrinogen and thrombus formation under flow conditions, by activation of c-Src, although it seems that it is not required for the agonist-induced activation of $\alpha IIb\beta 3$.[14] Despite this evidence, Na_2O_4W may be acting on other signaling mechanisms, which should be further explored.

Na_2O_4W is a compound with a reasonably delimited pharmacotoxicologic profile. It has satisfactorily passed three phase I clinical trials involving healthy volunteers without clinical, laboratory, or electrographic findings suggesting toxicity.[10] Na_2O_4W has been shown to be effective in both normalizing blood glucose levels and decreasing the body weight gain and adiposity. Through the present study, we have generated evidence for the first time demonstrating the effect of Na_2O_4W modulating the adhesive and cohesive functions of platelets. Considering that the socioeconomic burden of atherothrombosis is still increasing in our society and that the current antiplatelet treatments are associated with thrombotic and/or bleeding risks, Na_2O_4W offers a potential antiplatelet strategy that should be further explored.

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Disclosure

The authors report no conflicts of interest in this work.

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