Aortic Responses to Vanadate: Independence from (Na,K)-ATPase and Comparison of Dahl Salt-Sensitive and Salt-Resistant Rats

JOHN P. RAPP, D.V.M., PH.D.

SUMMARY Vanadate at doses from 10^{-4} to 10^{-3} M caused a dose-dependent contraction of the rat aorta in vitro. Aortas of Dahl salt-hypertension sensitive (S) rats responded to vanadate with a greater contraction than Dahl salt-hypertension resistant (R) rats. In contrast, S and R aortic responses to depolarization with potassium were equal, and responses to norepinephrine were less in S than R. The mechanism by which vanadate causes the aortic response was studied in S rats. In aortic smooth muscle sodium-loaded by exposure to low potassium media followed by a norepinephrine-induced contraction, a relaxation induced with 5 mM potassium was not influenced by 10^{-4} M vanadate. Since this potassium-induced relaxation is known to be a reflection of (Na,K)-ATPase activity, these data show that vanadate (up to 10^{-4} M) does not inhibit (Na,K)-ATPase in intact smooth muscle cells although it is a known potent inhibitor of (Na,K)-ATPase in isolated cell membrane preparations. Response to vanadate was not changed by α-blockade with phentolamine or by blocking (Na,K)-ATPase with ouabain. Vanadate contraction was blocked by 4,4'-diisothio cyanido-2,2'-disulfonic acid stilbene, a known inhibitor of anion transport, suggesting that vanadate anions must enter smooth muscle cells to induce contraction. (Hypertension 3 (supp 1): I-168-I-172, 1981)

KEY WORDS • vanadate • vascular smooth muscle • genetic hypertension • salt-sensitive rats

Vanadate has recently been recognized as a potent inhibitor of (Na,K)-ATPase enzyme activity in cell membrane preparations.1 It has been argued that inhibition of (Na,K)-ATPase in vascular smooth muscle could result in contraction of the muscle.2-4 Our primary goal was to determine if vanadate caused a response in vascular smooth muscle in vitro, and, if so, whether this response was related to inhibition of the (Na,K)-ATPase pump. Second, we compared aortic responses to vanadate in rats selectively bred by Dahl et al.5 for sensitivity (S strain) or resistance (R strain) to the hypertensive effect of high salt diet.

Materials and Methods

Salt-sensitive (S) and salt-resistant (R) rats were bred in our laboratory from stock originally obtained from Dahl in 1972. Rats were fed normal laboratory rat chow that contained 1% NaCl. Blood pressure was obtained by the tail microphonic method6 with the rats under ether anesthesia.

Rats were killed by cervical dislocation. The thoracic aorta was removed, placed in ice cold medium (see below), and dissected free of fat. Rings (5 mm long, measured against a steel ruler) were cut from the thoracic aorta just above the diaphragm and mounted on stainless steel hangers in a muscle bath at 37°C under 2 g of tension. Muscles were allowed to equilibrate 1 hour before use. The medium contained 118 mM NaCl, 4.7 mM KCl, 12.5 mM NaHCO3, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 11.1 mM glucose, and was gassed with 95% O2, 5% CO2, pH 7.3. Tension was recorded using a Grass Instrument (Quincy, Massachusetts) FT.03 force displacement transducer and a Grass Model 79 polygraph. At the end of experiments, aortic rings were blotted to remove excess media and weighed on a Cohn microbalance. Injections into the muscle chamber were made using microliter syringes. Sodium orthovanadate (Na3VO4) (Fisher) was added as a 0.1 M solution to yield the concentrations indicated. Ouabain, (-)-norepinephrine and 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS) were obtained from Sigma. Phentolamine was a gift from Ciba.

Results

The addition of vanadate to the muscle chamber caused a pronounced and immediate increase in tension. Figure 1 shows cumulative dose response recordings of responses to vanadate (10^{-4}-10^{-3} M) for in...
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individually S and R aortas. Figure 2 shows that the average dose response obtained to vanadate was greater in S than R rats at all doses. Although the weight of the S rings was greater than the R rings, the strains responded similarly to a depolarizing concentration of K+ (table 1), and S aortas responded less to norepinephrine than did R (fig. 3). The higher response of S aortas to vanadate was, therefore, not a generalized hyperresponsiveness to all stimuli.

The effect of α-receptor blockade with phenolamine (10^-4 M) on vanadate contractions was studied using aortic rings from S rats. The vanadate (10^-4 M)-induced contraction was expressed as percentage of a K+ (60 mM) contraction to minimize variability between strips. The results were 79.0% ± 4.7% (mean ± SE, n = 6) for vanadate alone, and 73.1% ± 3.9% (n = 6) for vanadate following blockade with phenolamine. These values were not statistically different by a t test (p > 0.25). Vanadate, therefore, must act by some mechanism independent of α-receptors, since it was still effective when α-receptors were blocked. (It was demonstrated that, in fact, α-receptors in S rats were blocked by phenolamine; the response to norepinephrine 10^-7 M without phenolamine was 291.7 ± 37.2 mg, n = 8, and with phenolamine was zero, n = 8.)

The highest concentration of vanadate used (10^-4 M) caused a transient increase in pH from 7.3 to 8.1. The peak increase of pH occurred within 5 seconds of adding 10^-4 M vanadate to the muscle chamber, but returned to pH 7.3 by 3 minutes. The next lower dose, 10^-5 M vanadate, caused no measurable pH change but still caused a muscle response. Changes in pH do not account for the effect of vanadate on the aortic smooth muscle, because adding NaOH at a concentration of 2.5 mM duplicated the maximum pH change but did not cause any change in tension.

Since vanadate is a very potent inhibitor of (Na,K)-ATPase in cell membrane preparations, we wanted to evaluate the effect of vanadate on (Na,K)-ATPase function with intact smooth muscle cells. Webb and Bohr7 have developed a protocol for evaluating (Na,K)-ATPase function as follows. The smooth muscle is exposed to K+ free media, which reduces (Na,K)-ATPase ion transport and allows Na+ to accumulate intracellularly. The muscle is caused to contract with norepinephrine (10^-7 M). When K+ (5 mM) is added to the medium, (Na,K)-ATPase ion transport resumes, resulting in hyperpolarization of the muscle membrane and muscle relaxation. This relaxation is blocked by ouabain (10^-4 M), which is a well-known inhibitor of (Na,K)-ATPase. These phenomena are illustrated in figure 4 (top two recordings). If vanadate were acting to inhibit (Na,K)-ATPase in this system, it should, like ouabain, prevent the K+ stimulated

<table>
<thead>
<tr>
<th>Variable</th>
<th>R</th>
<th>S</th>
<th>S vs R t test probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>206 ± 5.5</td>
<td>236 ± 5.4</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Blood pressure, mm Hg</td>
<td>120 ± 3.8</td>
<td>182 ± 8.7</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Aortic ring weight, mg</td>
<td>3.18 ± 0.14</td>
<td>4.09 ± 0.19</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>Response to 60 mM K+, mg</td>
<td>687 ± 64</td>
<td>635 ± 39</td>
<td>&gt; 0.5</td>
</tr>
<tr>
<td>No. of rats</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE.

Figure 1. Recordings of tension developed by aortic rings from S and R rats in response to cumulative doses of vanadate (VO2). The recordings are redrawn from the original tracings and placed one above the other for comparison. Both recordings actually started from the same baseline.

Figure 2. Mean responses of rings from thoracic aortas of S and R rats to cumulative doses of vanadate (VO2). Error bars are standard errors, n = 6 rats for each strain. S and R responses were statistically different (p < 0.05) from each other by a t test at, and above, 10^-4 M vanadate.
smooth muscle relaxation. Vanadate, however, does not affect the K+ relaxation at all (fig. 4, bottom two recordings, and table 2).

Additional evidence that the vanadate-induced contraction does not involve (Na,K)-ATPase is the fact that rat aortic rings in complete medium, which were blocked by ouabain (10⁻³ M), did not show an altered response to vanadate 10⁻³ M. Vanadate responses were expressed as a percentage of the contraction due to 60 mM K+ and were: 102.5% ± 3.89% for unblocked

### Table 2. Effect of Ouabain or Vanadate on the K⁺-Induced Relaxation of Rat Aortic Rings Contracted by Norepinephrine in K⁺ Free Media

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>K⁺ relaxation, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 24)</td>
<td>299 ± 28.4</td>
</tr>
<tr>
<td>Ouabain, 10⁻³ M (n = 16)</td>
<td>30.3 ± 10.0*</td>
</tr>
<tr>
<td>Vanadate, 10⁻³ M (n = 16)</td>
<td>281 ± 16.0</td>
</tr>
</tbody>
</table>

Mean ± SE are given. Conditions are given in legend to figure 4. Results of a one-way analysis of variance showed significant differences (p < 0.001) between the three conditions (control, ouabain, and vanadate). Results of multiple comparisons by the S-method of Scheffé (see ref. 33) were: control vs ouabain p < 0.001; control vs vanadate p > 0.5; ouabain vs vanadate p < 0.001.

*The K⁺ relaxation recorded for ouabain treatment was read at the time of maximal K⁺ relaxation in concomitantly recorded control rings without ouabain and is due to a slow downward drift of the norepinephrine contraction rather than a discrete relaxation (see fig. 4).
tissue, and 107.6% ± 5.90% for ouabain-blocked tissue (mean ± se, n = 8 for each group, p > 0.25 by t test).

In contrast to the ability of ouabain and phenetolamine to alter vanadate-induced aortic responses, we found that 10^{-3} M DIDS completely blocked the response to 10^{-3} M vanadate. DIDS had no effect on contractions induced by 60 mM K+ or 10^{-3} M norepinephrine (table 3).

**Table 3. Effect of DIDS on Contractions of Rat Aortic Rings Induced by Potassium, Norepinephrine, and Vanadate**

<table>
<thead>
<tr>
<th>Agonist Concentration</th>
<th>Without DIDS (n = 8) (%)</th>
<th>With DIDS (n = 8) (%)</th>
<th>Results of (n = 8) (%)</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium, 60 mM</td>
<td>101.7 ± 3.7</td>
<td>113.9 ± 5.2</td>
<td>0.05-0.1</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine, 10^{-3} M</td>
<td>102.6 ± 8.4</td>
<td>113.3 ± 6.5</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Vanadate, 10^{-3} M</td>
<td>125.2 ± 5.7</td>
<td>91.1 ± 3.7*</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Two thoracic aortic rings from each of eight female rats were set up in paired muscle chambers in complete media (see Materials and Methods). Responses to 60 mM K+ were recorded from both rings. One chamber received 10^{-3} M of 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS) for 1 hour, and both chambers were flushed. Responses to 60 mM K+, 10^{-3} M norepinephrine, and 10^{-3} M vanadate in that order were recorded. The blocking effect of DIDS is not removed by repeated flushing of media. In the present work, the aorta was stained yellow by DIDS, and this color did not wash out. The effect of DIDS in blocking vanadate contraction remained complete up to 4 hours after removal of DIDS. The data are expressed as a percentage of the initial 60 mM K+ contraction to remove variability between preparations, mean ± se are given. ns = not significant.

*A negative number represents a relaxation.

**Discussion**

Vanadate apparently penetrates red blood cells and cultured heart cells. Vanadate uptake in red blood cells is blocked by a disulfonic stilbene derivative, and such compounds are known to inhibit anionic transport (Cl−, SO₄²⁻) in red cells and organic anion transport (para-amino hippurate) in kidney tissue. We found that 4,4'-diisothiocyanato-2,2'-disulfonic acid stilbene (DIDS) at 10^{-3} M completely blocked the anion response to 10^{-3} M vanadate but had no effect on contractions induced by 60 mM K+ or 10^{-3} M norepinephrine. Thus, it appears that the vanadate anion, VO₄³⁻, must be taken into smooth muscle cells by the anion transport system prior to causing contraction, although we have no direct studies of radioactive vanadate uptake in aortic smooth muscle cells. In contrast, ouabain binds to the external cell surface.

Vanadate is an analog of phosphate and as such interferes with a wide variety of enzymes involving phosphorylation and/or dephosphorylation reactions. Vanadate is a potent inhibitor of (Na,K)-ATPase in isolated membrane preparations. It has been postulated that inhibition of the (Na,K)-ATPase electrogenic pump in vascular smooth muscle would result in vasoconstriction either by: 1) depolarizing the membrane and opening voltage-dependent Ca²⁺ channels with subsequent increase in intracellular Ca²⁺; or 2) allowing an increase in intracellular Na⁺ and consequently increased Na⁺-Ca²⁺ exchange, with Ca²⁺ moving into smooth muscle cells. By either of these two possible mechanisms, inhibition of (Na,K)-ATPase would increase cell Ca²⁺ and initiate muscle contraction.

In our present experiments, vanadate was used on intact smooth muscle cells at concentrations 1000-fold greater than those known to inhibit (Na,K)-ATPase in isolated membrane preparations. Although these concentrations caused smooth muscle to contract, they failed to inhibit the K+ relaxation phenomenon which is known to result from (Na,K)-ATPase pump activity. (Na,K)-ATPase was therefore not inhibited. This is compatible with the fact that vanadate also fails to inhibit (Na,K)-ATPase pump activity, as measured by rubidium uptake in dog saphenous vein or in adipocytes. Vanadate does, however, inhibit rubidium uptake in red blood cells, suggesting important differences between cell types. Inside cells vanadate is reduced by glutathione and binds to cytoplasmic proteins. Vanadate could also be reduced by an NADH-vanadate-oxidoreductase described in cardiac cell membranes. Such phenomena may result in a different accessibility of vanadate anion to (Na,K)-ATPase between cell types and account for the lower sensitivity to vanadate of intact cells compared to cell membrane preparations.

Although vanadate obviously does not inhibit (Na,K)-ATPase in intact smooth muscle cells, it still does cause a pronounced increase in tension. What are the possible mechanisms? For example, vanadate at low concentrations also inhibits Ca-ATPase activity in isolated cell membrane preparations. This would be expected to result in increased intracellular calcium and therefore muscle contraction. Although there are no data on which to reject this hypothesis, it seems unlikely that vanadate would be able to reach an intracellular site and inhibit plasma membrane Ca-ATPase and yet fail to inhibit plasma membrane (Na,K)-ATPase. Vanadate also stimulates adenylate cyclase and increases cellular cyclic AMP levels. Such an action of vanadate on smooth muscle cyclic AMP, however, would be expected to cause relaxation, not a contraction, as was observed.

Protein phosphorylation may be involved in regulating contractile proteins in both smooth muscle and non-smooth muscle cells. Since vanadate alters phosphorylation and/or dephosphorylation reactions, it might interfere with the phosphorylation of proteins involved in smooth muscle contraction. Solaro et al. have shown that troponin I from rabbit hearts treated with vanadate had a reduced phosphate content.

It has been suggested that tissue vanadate may function as a physiologic regulator of (Na,K)-ATPase. Although vanadate infused into intact animals causes vasoconstriction and feeding of vanadate to rats increases blood pressure, we view
vanadate in the context of our own work not as a physiologic regulator but as an interesting pharmacological probe. The difference in aortic response to vanadate in vitro between S and R rats could be a reflection of mutant enzymes or proteins involved in smooth muscle contraction. Obviously, this can only be established by demonstrating directly the strain-specific differences in such molecules. Although it appears that the increased sensitivity to vanadate in the S rat was not shared with other agonists, it is impossible to know from the data available so far whether such a strain difference reflects a causative mechanism for hypertension or arises as a result of hypertension. Clearly, data in young S and R rats with similar blood pressures will be required. The data also showed that 5 mm rings cut from hypertensive S rats were heavier (wet weight) than those from R rats. It is unknown at this point what component of the aorta causes the increased weight (smooth muscle, connective tissue, water, etc.). It is also emphasized that changes observed in the aorta do not necessarily reflect changes in the much smaller, more muscular, more highly innervated resistance vessels.

It is concluded that: 1) vanadate causes a dose-related contraction of rat aortic smooth muscle in vitro; 2) vanadate-induced responses with intact cells are not mediated by inhibition of (Na,K)-ATPase, although vanadate is a known potent inhibitor of (Na,K)-ATPase in cell membrane preparations; 3) vanadate apparently enters smooth muscle cells via the anion transport mechanism prior to inducing a contraction; 4) aortas from hypertensive S rats show a greater response to vanadate than aortas from R rats.

References


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