High potassium diets greatly reduce intimal and medial thickening in stroke-prone spontaneously hypertensive rats (SHRSP). In vascular smooth muscle cells, transforming growth factor-β (TGF-β) inhibits proliferation. To test whether high potassium diets decrease aortic thickening through TGF-β, we measured TGF-β-like activity in medium bathing aortas from rats fed either normal potassium or high potassium diets. Five-week-old SHRSP were fed 6% high NaCl diets containing either normal (0.5%) potassium (11 rats) or high (2.1%) potassium (14 rats) for 7 weeks. Aortas were freshly excised and perfused for 3 hours with tissue culture medium at ordinary arterial pressures. TGF-β-like activity in the acid-activated perfusing medium was assessed using the growth inhibitory action on mink lung cells. Growth inhibition was assessed by [3H]thymidine incorporation. In the medium perfusing the outside of the aorta, the growth inhibitory rates were 2.5 times higher in high potassium SHRSP than in normal potassium SHRSP (-49% versus -20%, p<0.03). Antibodies to TGF-β, and TGF-β were added to other aliquots and did not alter the results whatsoever. Thus, the difference in growth inhibition was not due to differences in TGF-β. The high potassium aortas released 2.5 times more growth-inhibiting agents than the normal potassium aortas. The same pattern of growth inhibition was also seen using vascular smooth muscle cells rather than mink lung cells (r= +0.818, p<0.001, n = 13). The increased growth inhibition of high potassium aortas was not due to an increased release of heparin. These antiproliferative agents in high potassium aortas may partially explain why the high potassium aortas have so much less intimal and medial thickening.

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KEY WORDS • growth inhibitors • essential hypertension • vascular diseases • potassium • stroke-prone spontaneously hypertensive rats

Hypertension can cause endothelial injury and a thickening of the arterial media and intima associated with vascular smooth muscle cell (VSMC) hypertrophy. We have previously reported that high potassium diets reduce hypertensive endothelial injury and arterial wall thickening in salt-fed hypertensive rats. This can occur without any reduction in intra-arterial blood pressure, as measured at one point during the day. Thus, some mechanism other than a blood pressure-lowering effect must be present to account for the protective effect of high potassium diets against hypertensive arterial damage. Growth factors play key roles in the pathogenesis of atherosclerosis. Growth factors are involved in the hypertrophy of the arterial media and in the migration of cells from the media into the intima. Transforming growth factor-β (TGF-β) stimulates not only proliferation in connective tissue cells but also the production of collagen, fibronectin, and proteoglycans, while it inhibits the growth of some other cells. TGF-β suppresses serum or other mitogen-induced proliferation of cultured VSMCs. However, TGF-β also induces hypertrophy and hyperplasia in cultured VSMCs. In addition, Chobanian et al found an increase of TGF-β and fibronectin messenger RNA in the aorta of deoxycorticosterone acetate-salt hypertensive rats. These findings suggest that TGF-β may be involved in arterial wall thickening in hypertensive animals.

The original purpose of the present study was to explore whether dietary potassium reduces arterial wall thickening through changes of TGF-β in salt-fed stroke-prone spontaneously hypertensive rats (SHRSP). We used a perfusion chamber to perfuse isolated aortas to provide more physiological conditions for examining the release of humoral agents of vascular origin independent of circulating vasoactive substances.

Methods

Materials

Five-week-old male SHRSP from our breeding colony were all fed 6% high NaCl diets containing either 0.5% normal potassium (n=11) or 2.1% high potassium (n=14). In the high potassium diet, half the added potassium was potassium citrate and half was KHCO3. After 7 weeks on the two diets, the blood pressures were measured in the femoral artery under thiobutabarbitral (Inactin) anesthesia (100 mg/kg i.p.). Thereafter, all branches of the aorta from the iliac bifurcation to the
aortic arch were tied off, and the aorta was cannulated at the highest portion of the descending thoracic aorta and just above the iliac bifurcation. Then, the aorta was excised and mounted in a specially designed perfusion chamber.

Both the inside and outside surfaces of the aorta were perfused at 37°C with modified medium 199 (Sigma Chemical Co., St. Louis, Mo.) that contained the electrolyte concentrations of Krebs solution. The medium was aerated with 95% air-5% CO₂ throughout the experiment. The inside of the aorta was perfused at a normal pressure of 105/60 mm Hg. After 3 hours of recirculation, the perfusate bathing the outside surface of the aorta was collected and stored at -70°C.

Mink Lung Cell Growth Inhibition Assay

We used the MV-LLu mink lung cell growth inhibition assay¹⁴,¹⁵ to detect TGF-β activity in samples of the medium that had bathed the outside of the aorta. Relative rates of DNA synthesis were assessed by measuring the amount of [³H]thymidine incorporated into trichloroacetic acid (TCA)-precipitable material. The effect of this perfused medium was compared with an aliquot of the identical aerated medium that had never been in contact with an aorta. Mink lung cells (CCL-64) were maintained in Dulbecco’s modified Eagle medium (DMEM) (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO₂ incubator. For the growth inhibition assay, subconfluent cells 3 days after previous passage were trypsinized and resuspended in DMEM supplemented with 0.2% FBS and plated at 5 x 10⁴ cells per well in 24 multwell dishes. After 1 hour, the perfused medium samples were added to the cells and incubated for 22 hours. The cells were then pulsed for 2 hours with 0.25 μCi of [³H]thymidine (20 Ci/mmol, New England Nuclear, Boston, Mass.). Then, the cells were fixed with 1 ml ice-cold 10% TCA for 10 minutes and washed once with 10% TCA. The TCA-precipitable materials were dissolved with 0.25 ml of 0.2N NaOH for 30 minutes at room temperature and counted with a liquid scintillation counter. Half-maximal growth inhibition of mink lung cells was achieved at 25 pg/ml porcine TGF-β₁ (R&D Systems, Inc., Minneapolis, Minn.), and the interassay coefficient of variation was 12.4% in four different runs. All samples were analyzed in quadruplicate. All the samples of perfused tissue culture medium were acidified to activate the TGF-β or other agents and were then reneutralized just before the assay.¹⁵,¹⁶

To determine whether the growth inhibitory effect caused by the media perfusing the aortas was due to TGF-β, we applied a neutralizing antibody against TGF-β₁ and TGF-β₂ (R&D Systems). The antibody was added to an aliquot of acid-activated perfused medium (final concentration, 100 μg/ml) and incubated for 1 hour at room temperature just before the growth inhibition assay.

Smooth Muscle Cell Growth Assay

Rat aortic VSMCs from male SHRSP (150-200 g in weight) on a 0.3% NaCl chow were cultured and characterized as previously described.¹⁷ The aortic ex-}

plants were cultured with DMEM supplemented with 20% FBS. The cultures were fed biweekly and trypsinized after 2-3 weeks. The trypsinized VSMCs (defined as passage 1) were maintained in DMEM with 10% FBS. The experiments were at passage 2 to 3. To assay for VSMC growth, the cells were plated at 5 x 10⁴ cells per well in DMEM with 10% FBS (0.5 ml) into a 24 multwell dish and incubated overnight. The cells were washed twice with serum-free DMEM and once with DMEM containing 0.2% FBS; then, DMEM with 0.2% FBS was added to the washed cells (0.5 ml per well). The cells were incubated for 72 hours. Then, the samples of the perfused tissue culture medium were added to the cells after the medium was changed to DMEM containing 10% FBS. After 24 hours of incubation, the cells were pulsed with [³H] thymidine (0.25 μCi per well). Four hours later, the cells were washed twice with 10% ice-cold TCA and were then dissolved in 0.25N NaOH/0.1% sodium dodecyl sulfate and counted.

Results

Blood Pressure

Blood pressure in the high potassium diet group was 211±5 mm Hg, which was exactly the same as in the normal potassium diet group (211±10 mm Hg). There were also no differences in the length of excised aortas between the high potassium (6.0±0.1 cm) and normal potassium (5.9±0.2 cm, p=NS) diet groups.

Transforming Growth Factor-β Activity in the Perfused Medium

The average inhibition of [³H]thymidine incorporation into mink lung cells caused by the media perfusing the aortas of each group is shown in Figure 1. The perfusion media that bathed the high potassium aortas (49±7% of control) averaged 2.5 times more growth inhibition than the media bathing the normal potassium

![Figure 1. Bar graph shows [³H]thymidine incorporation into mink lung cells exposed to tissue culture media that had perfused the outside surfaces of the aortas of stroke-prone spontaneously hypertensive rats. One group of rats was fed a 2.1% high potassium diet and the other group a 0.5% normal potassium diet. Effect of these perfused media was compared with an aliquot of the identical medium that had never been in contact with an aorta.](http://hyper.ahajournals.org/DownloadedFrom/...)

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aortas (−20±11% of control) (p<0.03). Thus it appeared that the high potassium aortas were releasing a potent amount of growth-inhibiting humoral agents, whereas the normal potassium aortas were releasing 59% less of these growth-inhibiting substances. In five runs of the high potassium group of aortas, samples were collected at 1 hour as well as at 3 hours after the initiation of the perfusion. The media perfusing the aortas showed progressively higher growth inhibition as time elapsed (−19% at 1 hour and −58% at 3 hours, p<0.01 compared with control).

**Effect of a Neutralizing Antibody**

The addition of 100 µg/ml of the antibody to 140 pg/ml of porcine TGF-β neutralized the mink lung cell growth inhibitory effect to −7% of control, compared with −86% of control without the antibody. Considering this result, we incubated an aliquot of the perfused media with 100 µg/ml of the antibody. The antibody, however, did not change the mink lung cell growth inhibition caused by the perfused media. Thus, the anti-TGF-β and -β antibody failed to block the growth inhibitory effect of medium samples bathing either high potassium or normal potassium aortas.

**Effect on Smooth Muscle Cell Growth**

Rat aortic VSMCs were incubated with DMEM containing 0.2% FBS for 72 hours to arrest cell growth. Then, aliquots of the perfused media were added to the cells immediately after the cells were exposed to 10% FBS. The samples of the perfused media suppressed the serum-induced DNA synthesis in cultured rat aortic VSMCs (high potassium group, −40±13% [n=7]; normal potassium group, −11±8% [n=6]). There was a strong positive correlation of effects on DNA synthesis between mink lung cells and rat aortic VSMCs (r=+0.818, p=0.0006).

To determine whether the VSMC growth inhibitory effect induced by the perfused fluids was due to a heparin-like substance, aliquots of the samples were incubated with 10 units/ml Flavobacterium heparinase (Sigma) for 120 minutes at 37°C. The samples were then boiled at 90°C for 5 minutes to kill the enzyme activity and were added to VSMCs. This treatment resulted in no change of inhibitory effect on VSMC growth (−32±14% versus −30±13%, n=5, p=NS).

**Discussion**

The media perfusing the aortas of salt-fed SHRSP suppressed the growth of mink lung cells. The growth inhibitory effect in these cells was 2.5 times higher in the high potassium aortas than in the normal potassium group. A similar growth inhibitory pattern was observed in cultured rat aortic VSMCs. The results presented here raise two interrelated possibilities: 1) high potassium diets may cause arteries to release large amounts of growth-inhibiting agents, and 2) high potassium diets may reduce hypertensive arterial wall thickening through the effect of these growth-inhibiting agents.

The initial objective of this study was to investigate the involvement of TGF-β in the reduction of arterial wall thickening during high potassium diets. To examine the release of TGF-β from the aorta, we perfused isolated aortas in a perfusion chamber. This isolated aorta perfusion model permits one to investigate the release of humoral agents from the aorta independent of various circulating vasoactive substances. The aorta can be studied under more physiological conditions than aortic strip incubation or tissue culture.

We measured TGF-β-like activity in the samples by using the mink lung cell growth inhibition assay. This assay has good sensitivity and specificity for TGF-β. Other growth factors such as epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor have only minor effects on the growth of mink lung cells. However, some other nonspecific factors may affect the growth of mink lung cells. Thus, to show the specificity for TGF-β, it is necessary to demonstrate the neutralization of the growth inhibition with anti-TGF-β antibody. The antibody against porcine TGF-β, and TGF-β used in this study failed to block the growth inhibitory effect of the perfusion samples. To explain this failure, it is possible that subtypes of TGF-β other than TGF-β and -β might be responsible for the growth inhibitory effect observed in this study. This failure might be due to species differences of TGF-βs. However, cultured human and bovine vascular endothelial cells secrete mainly TGF-βs, and elevation of blood pressure increases gene expression of TGF-β, in deoxyxocorticosterone acetate–salt hypertensive rats. Therefore, there is a high degree of conservation of amino acid sequences in the TGF-βs of different species. Thus, it does not appear likely that the antibody failed to block the growth inhibitory effect of the samples because of differences of subtypes or species of TGF-βs.

Because we used an isolated aorta perfusion model in the present study, we should consider the possibility that substances of vascular origin inhibit the growth of mink lung cells or VSMCs. Because the agents that suppressed the growth of mink lung cells are not likely to be TGF-β, we should consider some possible substances other than TGF-β. The treatment with heparinase did not change the effect of the perfusion media on VSMC growth. This result suggests that the growth-inhibiting agents are not heparin-like substances. Endothelium-derived relaxing factor and prostacyclin also have growth-inhibiting activity on VSMCs in culture. They are mainly produced by vascular endothelial cells. High potassium diets protect vascular endothelium from hypertensive injury. Taken together, we could speculate that healthier endothelium may produce and release larger amounts of growth-inhibiting substances than injured endothelium does. The growth-inhibiting agents we observed in this study could be such a substance. However, both endothelium-derived relaxing factor and prostacyclin have too brief of a half-life to register in our assay. Further studies will be required to identify the growth-inhibiting substances and to determine the mechanism by which dietary potassium augments the release of these substances from aortas of hypertensive rats.

In conclusion, the perfused aortas released growth-inhibiting humoral agents that apparently were not TGF-β or a heparin-like substance. The high potassium aortas released the growth-inhibiting agents 2.5 times more abundantly than the normal potassium aortas did. Diets high in potassium can markedly reduce the intimal and medial thickening of arteries in hypertensive rats. If
the high potassium diets cause arteries to release large amounts of growth-inhibiting agents, this could partially explain why the high potassium diets can greatly reduce hypertensive arterial thickening.

References


High potassium diets greatly increase growth-inhibiting agents in aortas of hypertensive rats.

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