Chlorthalidone Decreases Platelet Aggregation and Vascular Permeability and Promotes Angiogenesis

Ryan Woodman, Christina Brown, Warren Lockette

Abstract—Variations in diuretic-mediated inhibition of carbonic anhydrase-dependent chloride transport in platelets and vascular smooth muscle could account for the contrasting efficacy of the thiazide and thiazide-like diuretics in reducing cardiovascular morbidity in patients with hypertension. We assessed platelet carbonic anhydrase activity and catecholamine-induced platelet aggregation in the presence of a thiazide and a “thiazide-like” inhibitor of the sodium-chloride cotransporter. Individual variation in platelet carbonic anhydrase activity correlated with contrasting sensitivity to epinephrine-mediated platelet aggregation. Both chlorthalidone, which potently inhibits platelet carbonic anhydrase, and bendroflumethiazide, which has much less effect on this enzyme, increased the amount of epinephrine needed to induce platelet aggregation when compared with the absence of a diuretic. However, chlorthalidone was significantly more effective than bendroflumethiazide in reducing epinephrine-mediated platelet aggregation. Chlorthalidone also induced marked changes in the number of gene transcripts for two proteins that mediate angiogenesis and vascular permeability, vascular endothelial growth factor C and transforming growth factor-β3; chlorthalidone and bendroflumethiazide had contrasting effects on the expression of vascular endothelial growth factor C. Chlorthalidone and bendroflumethiazide reduced vascular permeability to albumin, but only chlorthalidone increased angiogenesis. Thiazides and thiazide-like diuretics can comparably reduce blood pressure, but the drugs in this class are not all alike. It can be suggested from our findings that thiazide and thiazide-like diuretics vary in their pleiotropic effects on platelets and in the vasculature, and these differences could explain the contrasting ability of these drugs to reduce cardiovascular morbidity despite comparable reduction in blood pressure. (Hypertension. 2010;56:463-470.)

Key Words: humans ▪ hypertension ▪ stroke ▪ thiazides ▪ heart failure ▪ VEGF-C ▪ TGF-β3 ▪ carbonic anhydrase ▪ ALLHAT study ▪ ACCOMPLISH study

The Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT) was a randomized, double-blind, multicenter intervention of >33 000 patients who showed the superiority of the “thiazide-like” diuretic chlorthalidone over lisinopril and amlodipine in reducing cardiovascular morbidities such as stroke and congestive heart failure.1 More recently, the Avoiding Cardiovascular Events in Combination Therapy in Patients Living With Systolic Hypertension (ACCOMPLISH) Trial interventions showed that patients receiving benazepril and hydrochlorothiazide had a 20% greater likelihood of having a heart attack or stroke compared with patients taking benazepril and amlodipine.2 On the basis of this last trial, some have advocated overturning the recommendation of the Joint National Committee for the Detection, Evaluation, and Treatment of High Blood Pressure that favored the use of a low-dose diuretic as initial therapy in the treatment of essential hypertension as reported in ALLHAT.3,4

The outcomes over the ALLHAT and ACCOMPLISH Trial have focused the debate on the relative advantages between antihypertensive drug classes, not variations in the benefits of agents within the same class. Alternatively, such discrepant findings from these prospective, population-based studies raise the provocative possibility that, contrary to popular belief, the thiazides and thiazide-like diuretics are not alike. Despite inducing comparable effects on blood pressure reduction, the thiazide-like diuretic chlorthalidone and the thiazides hydrochlorothiazide and bendroflumethiazide may have contrasting effects on platelet and blood vessel function that variably affect cardiovascular morbidity. This hypothesis has not been explored.

Thiazides and thiazide-like diuretics bind with nearly equal affinity to the sodium chloride cotransporter in the distal tubule. However, the thiazides and thiazide-like diuretics differ in their ability to inhibit carbonic anhydrase. Reduction in carbonic anhydrase-dependent chloride and bicarbonate exchange decrease catecholamine- and thrombin-mediated platelet aggregation and vascular contractility.5–7 We postulate that the relative effectiveness by which the
various thiazides and thiazide-like diuretics reduce the incidence of stroke in patients with essential hypertension is directly commensurate with their ability to inhibit carbonic anhydrase-dependent chloride/bicarbonate exchange despite inducing comparable reductions in blood pressure. We anticipated that, because chlorthalidone was much more effective than equivalent concentrations of bendroflumethiazide at inhibiting carbonic anhydrase activity, chlorthalidone would more effectively decrease catecholamine-mediated platelet aggregation than a thiazide.

It has also been shown that diuretics do not consistently reduce plasma volume in patients with essential hypertension. Accordingly, other explanations must be sought for the mechanisms by which some diuretics, but not others, are so effective at reducing the prevalence of congestive heart failure in hypertensive patients. Conceivably, diuretics could increase blood flow through the development of collateral blood or lymph vessels, that is, promote angiogenesis or lymphangiogenesis, or directly diminish vascular permeability in the lung. We determined whether these classes of diuretics variably reduce the transcription and expression of candidate genes that mediate angiogenesis or vascular permeability. Finally, we determined whether chlorthalidone and bendroflumethiazide differentially affect angiogenesis and vascular permeability.

Methods

Measurement of Platelet Carbonic Anhydrase Activity

The effects of diuretics on platelet aggregation were assessed using 2 agents reported to have the most widely disparate abilities to inhibit isolated carbonic anhydrase in vitro: chlorthalidone (CTD; inhibition constant: 60 nmol/L) and bendroflumethiazide (BFTZ; inhibition constant: 49 000 nmol/L). To confirm the relative inhibition of carbonic anhydrase activity in platelets by these agents, we measured platelet carbonic anhydrase, as described previously. Briefly, carbonic anhydrase activity was assessed by measuring the increases in platelet size induced by the suspension of platelets in isoosmotic solutions of NH4Cl. When platelets are incubated in an aqueous, impermeant and osmotically active, NH4Cl-dependent platelet swelling is an indicator of carbonic anhydrase activity. Platelet swelling induced by NH4Cl is increased and diminished, by agents that raise or lower carbonic anhydrase activity, respectively. Platelet samples were obtained by venipuncture from healthy volunteers, either college-age students or hospital personnel, following informed consent. Platelets were sized using a Model II Coulter Counter. All of the chemicals were reagent grade (Sigma Chemical Co).

Assessment of Catecholamine-Induced Platelet Aggregation

Next, we compared platelet aggregation in response to increasing concentrations of epinephrine (0.1 to 10.0 μmol/L) in platelet-rich plasma that had been incubated for 20 minutes with CTD or BFTZ by recording changes in the optical density of platelet-rich plasma using standard aggregometry techniques.

Gene Transcription and Protein Expression in Vascular Smooth Muscle Cells

To identify whether CTD or BFTZ differentially affected the transcription of genes that modify vascular permeability or blood vessel formation, we assayed the number of mRNA transcripts in vascular smooth muscle cells (VSMCs) of 82 genes essential for these activities using a commercially prepared RT-PCR assay (RT2 Profiler PCR Array System, SuperArray). Briefly, cultured, nearly confluent a7r5 VSMCs (American Type Tissue Culture) were incubated in DMEM with 20% FBS for 24 hours with vehicle, CTD (100 μmol/L), BFTZ (100 μmol/L), or acetazolamide (ACZ; 100 μmol/L), then mRNA was isolated, and transcript levels were quantified using RT-PCR (Table S1, available in the online Data Supplement at http://hyper.ahajournals.org).

To determine whether diuretic-induced modifications in the number of gene transcripts that we found correlated with changes in the expression of the protein that most affects permeability and angiogenesis in VSMCs, we also assessed the levels of vascular endothelial growth factor (VEGF)-C in monolayers of cultured a7r5 VSMCs. For these experiments, the medium was changed to DMEM and 1% FBS, and the cells were then treated with vehicle, CTD (10 to 100 μmol/L), or BFTZ (10 to 100 μmol/L) for 24 hours. The supernatant was assayed for VEGF-C using a commercially available, isofom specific, ELISA assay (Rodenf VEGF C, Bender MedSystems).

Vascular and Endothelial Cell Permeability Studies

Additional cultures of confluent rodent a7r5 VSMCs and human vascular endothelial cells (from umbilical veins, Cascade Biologics) were grown to confluence in DMEM with 20% FBS or Medium 200 with 2% FBS, respectively, and each was supplemented with 10 ng/mL of VEGF-C on a semipermeable membrane (Millicell 96-well insert, Millipore Corporation). The monolayers were then treated with vehicle, CTD, or BFTZ for 8 hours. Albumin (4%) labeled with Evans blue (0.05 mg/mL) was added to the culture medium above the cells, and aliquots of the infranatant were serially sampled from 1 to 3 hours to measure the passage of the labeled albumin across the monolayer as reflected by changes in spectrophotometric absorbance units recorded at 620 nm.

Angiogenesis Studies

To measure changes in angiogenesis, cultures of the human endothelial cells were seeded onto a sarcoma-derived extracellular matrix (BD Matrigel, BD Biosciences) in Medium 200 and 2% FBS with and without vehicle, CTD, or BFTZ. In some experiments, the pH of the medium was adjusted by titration with NaOH. Eighteen to 24 hours after seeding, the number of endothelial tubules formed was analyzed using a light microscope; images were analyzed using the Image Pro Plus software for tubule quantification (Media Cybernetics).

Data Analysis

Data were analyzed using commercially available software (Prism version 3.03, GraphPad). For platelet aggregation studies, dose-response curves were constructed, the ED50 values were compared using a paired t test, and a Bonferroni adjustment was applied for multiple comparisons. Data from the gene transcription and protein expression were compared with unpaired t tests, as were the data for the angiogenesis experiments. Median values were compared with a Mann–Whitney test if data were not normally distributed.

Results

Diuretics and Carbonic Anhydrase Activity

As reflected by reductions in NH4Cl-induced platelet swelling, CTD inhibited maximum carbonic anhydrase activity to a much greater degree than BFTZ. BFTZ (30 μmol/L) inhibited NH4Cl-induced platelet swelling by only 8%, whereas 30 μmol/L of CTD demonstrated a statistically significant 3-fold greater inhibitory capacity of 24% (P<0.01; Figure 1). ACZ, an inhibitor of carbonic anhydrase
that does not bind appreciably to the sodium-chloride transporter, reduced platelet swelling by 50%.

Diuretics and Epinephrine-Induced Platelet Aggregation

Both CTD and BFTZ shifted the dose-response curve for epinephrine-mediated platelet aggregation to the right (Figure 2). Compared with vehicle, CTD increased the concentration of epinephrine required to induce platelet aggregation (EC$_{50}$ values expressed in $\mu$M±SEM: 0.51±0.12, 1.19±0.25, and 2.32±0.46, respectively. The rank order of potency for the 3 diuretics at inhibiting carbonic anhydrase activity was ACZ (30 $\mu$mol/L) > chlorthalidone > BFTZ = vehicle.

Diuretics and Epinephrine-Induced Platelet Aggregation

Both CTD and BFTZ shifted the dose-response curve for epinephrine-mediated platelet aggregation to the right (Figure 2). Compared with vehicle, CTD increased the concentration of epinephrine required to induce platelet aggregation (EC$_{50}$ values expressed in $\mu$M±SEM: 0.51±0.07; $P=0.003$) to a much greater degree than BFTZ (1.19±0.25 versus 0.51±0.07; $P<0.03$; Figure 3). Similarly, the EC$_{50}$ values in the presence of CTD were significantly higher than the EC$_{50}$ value in the presence of BFTZ (2.32±0.46 versus 1.19±0.25; $P=0.005$). Furthermore, the shift in an individual’s EC$_{50}$ for epinephrine-mediated platelet aggregation induced by 30 $\mu$mol/L of CTD was very highly correlated with their platelet carbonic anhydrase activity. Those individuals having the highest platelet carbonic anhydrase activity had the least reduction in epinephrine-mediated platelet aggregation in the presence of CTD (Figure 4). In other words, CTD induced a smaller shift in the EC$_{50}$ for epinephrine-mediated platelet aggregation in subjects whose platelets had higher carbonic anhydrase activity.

Figure 1. Platelet carbonic anhydrase activity was measured by suspending human platelets in 145 mmol/L of NH$_4$Cl. To maximally stimulate carbonic anhydrase activity, 15 mmol/L of NH$_4$HCO$_3$ was added (with a concomitant reduction of NH$_4$Cl to 130 mmol/L). BFTZ (30 $\mu$mol/L) did not inhibit carbonic anhydrase-dependent platelet swelling; CTD (30 $\mu$mol/L) reduced maximum carbonic anhydrase activity. The rank order of potency for the 3 diuretics at inhibiting carbonic anhydrase activity was ACZ (30 $\mu$mol/L) > chlorthalidone > BFTZ = vehicle.

Figure 2. Chlorthalidone inhibited platelet aggregation induced by increasing concentrations of epinephrine in platelet-rich plasma (PRP) to a much more significant degree than BFTZ. Although the sensitivity to epinephrine differed in the presence of CTD or BFTZ, the maximum responses to epinephrine-induced platelet aggregation did not differ between these 2 groups. Hydrochlorothiazide (not shown) inhibited epinephrine-mediated platelet aggregation with a potency in between that of BFTZ and chlorthalidone.

Figure 3. The concentrations of epinephrine required to induce 50% of the maximal aggregation response of the platelets (ie, EC$_{50}$ dose) were greatly increased by the diuretics. The EC$_{50}$ values for epinephrine in the presence of the vehicle, BFTZ, and chlorthalidone were (values in $\mu$M±SEM) 0.51±0.12, 1.19±0.25, and 2.32±0.46, respectively.

Figure 4. Chlorthalidone induced the greatest shift in the EC$_{50}$ for epinephrine-mediated platelet aggregation in those platelets that had the least levels of carbonic anhydrase activity. Data points represent values from subjects that had both measurements performed.
Diuretics and Steady-State mRNA Levels in VSMCs

CTD did not inhibit VSMC growth at any of the concentrations studied (data not shown). We found that incubation of VSMCs with 100 µmol/L of CTD for 24 hours significantly reduced the expression of 2 of the 82 genes that we screened that are known to play a major role in controlling angiogenesis and vascular permeability: VEGF-C (P<0.001; Figure 5, left) and transforming growth factor (TGF)-β3 (P<0.001; Figure 5, right); 2 other genes had moderately significant reductions in expression: fibroblast growth factor receptor 3 (P=0.001; data not shown) and neuropilin 2 (NRP2; P=0.001; data not shown). However, these CTD-induced changes in gene transcription were not dependent on CTD-dependent inhibition of carbonic anhydrase or inhibition of the sodium-chloride cotransporter; ACZ and BFTZ had no significant effect on the transcription of these genes (data not shown).

Diuretics, VEGF-C Levels, and Vascular Permeability

To determine whether the diuretic-induced changes in VEGF-C steady-state mRNA levels translated into differences in protein expression, we assayed the supernatant of cultured a7r5 blood vessels for VEGF-C after exposure to vehicle or 10 to 100 µmol/L of CTD or BFTZ. As depicted in Figure 6, there were contrasting dose-dependent effects of CTD and BFTZ on VEGF-C concentrations. CTD (10 µmol/L) greatly reduced VEGF-C, where a higher concentration of CTD slightly increased VEGF-C. Bendroflumethiazide decreased VEGF-C levels, but not to the degree induced by equivalent concentrations of CTD.

Although only CTD reduced the steady-state levels of VEGF-C gene transcripts, CTD and BFTZ both diminished the permeability of VSMC monolayers to labeled albumin (Figure 7). Unlike the findings in VSMCs, the CTD-induced reductions in vascular permeability were not seen across monolayers of endothelial cells (data not shown).

Diuretics and Angiogenesis

We next used an assay that relies on the ability of endothelial cells to form distinct blood vessel–like tubules from endothelial cells when they are seeded onto an extracellular matrix. Twenty-four hours after seeding, CTD, but not BFTZ, potentiated angiogenesis, as measured by sprouting and tubule

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**Figure 5.** Incubation of VSMCs in DMEM with 20% FBS and chlorthalidone or vehicle for 48 hours significantly reduced VEGF-C and TGF-β3 gene transcription. Cell viability, as measured by cell count and measurement of apoptosis (not shown), was not reduced by diuretic treatment. Bendroflumethiazide had no effect on the number of mRNA transcripts of these genes (not shown).

**Figure 6.** Chlorthalidone, in low concentrations (10 µmol/L), decreased VEGF-C, but at high concentrations (100 µmol/L) raised VEGF-C in the media of the VSMCs. In contrast, VEGF-C is reduced by both concentrations of BFTZ. Chlorthalidone and BFTZ had contrasting concentration-dependent effects on the TGF-β1.
formation when visualized (Figure S1, available in the online Data Supplement) and quantified (Figure 8) with light microscopy. Although sprouts formed, we did not ascertain whether these tubules developed a lumen. It is also possible that the high level of baseline angiogenesis that we observed in the control conditions for cells cultured in the presence of BFTZ might have masked BFTZ-induced angiogenesis. We also found that increasing the extracellular pH from 7.0 to 8.0 promoted angiogenesis (Figure S2).

Discussion
The traditional view has been that thiazides and thiazide-like diuretics bind with equal avidity to the sodium chloride cotransporter in the distal tubule, and they lower blood pressure by similarly reducing plasma volume. However, it has been known since 1960 that the reduction in plasma volume brought about by thiazide diuretics is not maintained with the chronic use of these agents.16 Accordingly, the mechanisms by which these agents lower blood pressure and reduce cardiovascular morbidity over time remain to be determined. It is suggested from our findings that CTD has some unique properties, not shared by the thiazides, which could prove it to be more salutary at reducing cardiovascular morbidity independent of the effect of this drug on blood pressure. Our findings could help explain the contrasting outcomes between the ALLHAT and ACCOMPLISH studies.

This hypothesis should not be unexpected. During the course of the Multiple Risk Factor Intervention Trial for the treatment of hypertension, patients were prescribed 50 or 100 mg of hydrochlorothiazide or 50 or 100 mg of CTD. In this study, subjects were randomized to “usual care” or “special intervention.” In the special intervention group, patients receiving hydrochlorothiazide had a 46% increase in cardiovascular mortality compared with the usual care group. Patients receiving CTD in the special intervention group had a 58% decrease in cardiovascular mortality compared with the usual care group. After changing from hydrochlorothiazide to CTD, those patients in the special intervention group had a 28% decrease in cardiovascular mortality compared with the usual care group. More recently, it has been shown in the Hypertension in the Very Elderly Trial that the primary use of the diuretic indapamide showed a striking 39% reduction in death from stroke and a 64% reduction in the rate of heart failure. Indapamide is one of the few diuretics that inhibits carbonic anhydrase as potently as CTD.17

The contrasting efficacy between CTD and most of the thiazides could be because of differences in pharmacokinetics and the varying ability of these agents to lower blood pressure. Ernst et al18 conducted a randomized, single-blinded, 8-week active treatment, crossover study comparing 12.5 mg/d of CTD with forced titration to 25.0 mg/d, and hydrochlorothiazide 25.0 mg/d with forced titration to 50.0 mg/d in untreated hypertensive patients. The authors reported that CTD was more effective in lowering systolic blood pressure than hydrochlorothiazide, as evidenced by 24-hour ambulatory blood pressure recordings; however, these differences were not apparent with measurements made in the clinic. We contend that differences in pharmacokinetics alone may not be the only explanation as to why prospective, population-based studies with CTD favor diuretics over other
classes of drugs, whereas prospective, population-based trials with thiazides have been much less successful in supporting that contention.

Carbonic anhydrase-dependent chloride/bicarbonate exchange plays an important role in mediating catecholamine-induced platelet aggregation and increases in vascular tone.5-7,19,20 We would expect that the greater the carbonic anhydrase activity, the more difficult it is to inhibit cellular responses that are carbonic anhydrase dependent (eg, platelet aggregation). It is suggested from our data that CTD inhibits carbonic anhydrase-dependent platelet aggregation more effectively than the thiazides hydrochlorothiazide (data not shown) and BFTZ. Accordingly, greater inhibition of catecholamine sensitive, carbonic anhydrase-dependent platelet aggregation and vascular contractility by CTD may help further reduce stroke in patients with essential hypertension when compared with therapy with thiazides. From our data, we can also suggest that individual variation in platelet (and perhaps vascular) carbonic anhydrase activity may help predict those patients who would most benefit from CTD therapy.

In addition to reducing stroke, CTD protects against the development of congestive heart failure. However, reductions in plasma volume are not maintained with chronic diuretic use. Accordingly, other mechanisms must come into play to explain the diminished incidence of congestive heart failure with CTD use. It is possible that CTD improves cardiac contractility. Alternatively, we postulated that CTD could diminish pulmonary vascular permeability, decrease pulmonary vascular resistance by abetting the development of collateral blood vessels, or increase lymph flow.

VEGF-C gene transcription was strikingly downregulated by CTD. This is an important observation, because overexpression of VEGF in rodents leads to the development of pulmonary edema, the hallmark of congestive heart failure. VEGF has a number of isoforms encoded by different genes. VEGF, also known as VEGF-A, occurs as 1 of 4 isoforms: VEGF121, VEGF165, VEGF189, and VEGF206; VEGF-B, VEGF-C, and VEGF-D are encoded by genes distinct from VEGF-A.21 Most circulating VEGF is stored in platelets, and plasma levels of this cytokine increase after platelet aggregation.22 A number of investigators have found that VEGF levels are increased in patients with essential hypertension and to even a greater degree when hypertension is accompanied by target organ damage.23-25 As already noted, VEGF has been reported to be increased during the development of pulmonary edema.26 We found that CTD specifically diminished the expression of VEGF-C, and both CTD and BFTZ reduced the levels of VEGF-C secreted by cultured VSMCs. Enhanced platelet aggregation in patients with hypertension may contribute to higher levels of VEGF in hypertension and, subsequently, VEGF-dependent increases in vascular permeability and the development of congestive heart failure in some patients.

CTD and BFTZ comparably reduced the permeability of monolayers of VSMCs in the presence of exogenously added VEGF-C. This effect was not observed in cultured endothelial cells. In our assays, we included VEGF to ensure that any diuretic-induced changes in permeability would not be attributed to diuretic-induced reductions in VEGF concentrations. Diuretics could have a direct effect on the integrity of the VSMCs in the intima and medial layers of blood vessels where myointimal plaques erode. Interestingly, indapamide, a thiazide that strongly inhibits carbonic anhydrase, has also been shown to inhibit vascular permeability induced by ischemia in brain capillary cells.27 In those studies, the cells were not cultured with the addition of exogenous VEGF. The baseline permeability of Evans blue-labeled albumin across our cultured endothelial cells was 250% greater (data not shown) than baseline permeability of the cultured VSMCs. Likely, any effect of CTD in reducing endothelial permeability could not be demonstrated in the presence of the high baseline permeability of the endothelial cells.

VEGF-C is a ligand for VEGF-R2 (Flk-1/KDR), VEGF-R3 (Flt-4), and NRP2, of which the expression was also strongly reduced in the presence of CTD.21 VEGF-C and NRP2 accumulate in regions rich in lymphatic vessels; they are responsible for the sprouting of lymphatic vessels from embryonic veins, and they play a role in renal podocyte survival.28 The role of VEGF-C in lymphangiogenesis is directly supported by the observation that mice lacking the VEGF-C alleles exhibit failure to develop lymphatic vessels, and VEGF-C overexpression causes hyperplasia of lymphatic vessels.29 NRP2, which was downregulated by CTD, is also required for angiogenesis; gene knockouts of NRP2 resulted in less dense capillary networks during embryogenesis.30 VEGF-C could have an indirect effect on angiogenesis through an action on NRP2. We cannot say with certainty whether the effect of CTD on angiogenesis or lymphangiogenesis is protective or not. The actions of VEGF-C are complex; in addition to promoting vasculogenesis, VEGF-C has a role as a negative regulator of pericyte function and vessel maturation.31 Machnik et al32 found that reductions in monocYTE VEGF-C signaling augmented interstitial hypertonic volume retention, diminished lymphangiogenesis, decreased endothelial NO synthase expression, and elevated blood pressure in response to a high-salt diet in rodents. Further studies are clearly needed to help elucidate the exact role that CTD-induced reductions in VEGF-C–mediated gene transcription may have on lymphangiogenesis and angiogenesis in the pathogenesis of end organ damage in hypertension.

We next measured the effect of CTD on angiogenesis. Within 24 hours of culture, we found that CTD, but not BFTZ, promoted the sprouting of blood vessels and new tubule formation from endothelial cells. We could not characterize cell markers to determine whether the vascular formations were of lymphatics or blood vessels. Because inhibition of intracellular carbonic anhydrase is associated with alkalization of the intracellular milieu, we determined the effect of pH on angiogenesis and found that vasculogenesis from cultured endothelial cells was markedly enhanced with rising pH (Figure S2). Interestingly, alkali loading by increasing bicarbonate intake also upregulated the expression of the thiazide receptor in the kidney in rodents.33 Diuretic-induced changes in intracellular pH may be a common link by which these agents influence so many physiological processes.

We also found that transcription of TGF-β3 was downregulated by CTD but not BFTZ. We did not have an assay...
specific for rodent TGF-β3 protein; however, we found a striking reduction in the concentration of TGF-β1 in the supernatants of the VSMCs grown in the presence of CTD (Figure 6 and Supplementary Discussion). A role for TGF-β1 is implicated in the deposition of collagen that accompanies the progression of renal disease in rodent models. It has also been reported that TGF-β1 levels are particularly high in pediatric patients with renal insufficiency and in black hypertensives with target organ damage.2,37 We can find no reports describing an assessment of TGF-β3 levels in patients with hypertension. However, using specific antibodies, others have reported that TGF-β3, rather than TGF-β1, localized in the renal juxtaglomerular apparatus of an infant with severe hypertension and also in the pulmonary vasculature of patients with pulmonary hypertension. Furthermore, TGF-β3 gene expression is upregulated in collateral blood vessels in rodents with genetic hypertension. NO has been shown to diminish TGF-β3 expression in cardiac fibroblasts, but it was not determined whether this cytokine plays a role in promoting cardiac fibrosis. TGF-β3 may play as important a role as TGF-β1 in the development of target organ damage in hypertension. It would also be fruitful to assess levels of VEGF-C and TGF-β3 in hypertensive patients before and after being treated with CTD or a thiazide and with comparable degrees of blood pressure control.

The steady-state concentration in the blood of men after daily oral administration of 50 mg of CTD for 2 weeks is 21.2 μmol/L. Our studies in human platelets used a physiologically relevant concentration of 30 μmol/L. It is not unusual for slightly larger dosages of drugs to be required for in vitro studies when compared with in vivo investigations. However, for the cells in which we used cultured rodent vascular cells, we increased the concentration of the diuretics from 30 to 100 μmol/L. Compared with humans, rodents are less sensitive to thiazides and CTD. For example, to induce comparable levels of blood pressure reduction between rodents and humans, Reungjui et al. had to administer 10 mg/kg per day of hydrochlorothiazide. This is equivalent to 700 mg/d for an average man. In their studies, Komatsu et al. used 80 mg/kg per day in spontaneously hypertensive rats to induce blood pressure reductions comparable to the degree suggested from our findings that much remains to be discovered about the mechanisms by which CTD contributes to reductions in the morbidity and mortality associated with essential hypertension. It is premature to discard the results of the ALLHAT study or the recommendations of the National Heart, Lung, and Blood Institute Joint Committee for the Detection, Evaluation, and Treatment of Hypertension. Special consideration should be given to the selection of the specific diuretic recommended for first-line therapy: all diuretics are not alike. The diuretic CTD should continue to be the first-line treatment for essential hypertension. Also, because CTD differs from thiazides in many important respects, the use of the vague term “thiazide-like diuretic” should be abandoned.

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Disclosures
None.

References


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CHLORTHALIDONE DECREASES PLATELET AGGREGATION
AND VASCULAR PERMEABILITY AND PROMOTES ANGIOGENESIS

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Running head: All diuretics are not alike

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Leukocyte cell derived chemotaxin 1  NM_030854
Leptin  NM_013076
Mitogen activated protein kinase 14  NM_031020
Midkine  NM_030859
Matrix metalloproteinase 19 (predicted)  XM_222317
Matrix metallopeptidase 2  NM_031054
Matrix metallopeptidase 3  NM_133523
Matrix metallopeptidase 9  NM_031055
Natriuretic peptide receptor 1  NM_012613
Neuropilin 1  NM_145098
Neuropilin 2  NM_030869
Platelet derived growth factor, alpha  NM_012801
Platelet derived growth factor, B polypeptide  XM_343293
Platelet/endothelial cell adhesion molecule  NM_031591
Placental growth factor  NM_053595
Plasminogen activator, urokinase  NM_013085
Plasminogen  XM_574314
Prostaglandin-endoperoxide synthase 1  NM_017043
Serine (or cysteine) proteinase inhibitor, clade B, member 5  NM_057108
Serine (or cysteine) proteinase inhibitor, clade F), member 1  NM_177927
Sphingosine kinase 1  NM_133386
T-box 4 (predicted)  XM_220811
Endothelial-specific receptor tyrosine kinase  XM_342863
Transforming growth factor alpha  NM_012671
Transforming growth factor, beta 1  NM_021578
Transforming growth factor, beta 2  NM_031131
Transforming growth factor, beta 3  NM_013174
Transforming growth factor, beta receptor 1  NM_012775
Thrombospondin 4  XM_342172
Tissue inhibitor of metalloproteinase 1  NM_053819
Tissue inhibitor of metalloproteinase 2  NM_021989
Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)  NM_012886
Tumor necrosis factor superfamily, member 2  NM_012675
Vascular endothelial growth factor A  NM_031863
Vascular endothelial growth factor B  NM_053549
Vascular endothelial growth factor C  NM_053653

A7r5 +/- bendroflumethiazide gene expression experiments used custom made to order plates with these genes, below (GenBank accession numbers as above):

Fibroblast growth factor receptor 3
FMS-like tyrosine kinase 1
Neuropilin 2
Transforming growth factor, beta 1
Transforming growth factor, beta 2
Transforming growth factor, beta 3
Vascular endothelial growth factor A
Vascular endothelial growth factor B
Vascular endothelial growth factor C
Supplementary Materials for Result Section

Control

Chlorthalidone (30μM)

Figure S1. Representative images of human endothelial vein cells grown in an extracellular matrix to induce tubule formation with, or without, chlorthalidone.
Figure S2. Changes in the pH of growth media greatly altered tubule formation. The culture supernatant was titrated with NaOH to increase pH from 7.0 to 8.0, and angiogenesis was subsequently quantified.

Supplementary Material for Discussion Section

*Vascular smooth muscle cells TGF-β1 protein expression.* Because a specific rodent TGF-β3 assay was commercially unavailable, we measured TGF-β1 levels using the Quantikine® Rodent TGF-β1 assay (R&D Systems, Minneapolis, MN).