Inhibition of Carbonic Anhydrase Accounts for the Direct Vascular Effects of Hydrochlorothiazide

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Abstract—Hydrochlorothiazide has been shown to exert direct vasodilator effects by activation of calcium-activated potassium (K\textsubscript{Ca}) channels in human and guinea pig isolated resistance arteries. Since hydrochlorothiazide binds to and inhibits the enzyme carbonic anhydrase and because K\textsubscript{Ca} channel activation is pH sensitive, we investigated the role of intracellular and extracellular carbonic anhydrase in the vascular effects of thiazide diuretics. Small arteries were isolated from guinea pig mesentery and studied by use of a microvascular myograph technique. In some experiments, tone and intracellular pH (pHi) were measured simultaneously with 2',7'-bis(2-carboxyethyl)-5(6)'-carboxyfluorescein (BCECF-AM). Bendroflumethiazide, a thiazide diuretic with minimal inhibitory effects on carbonic anhydrase, had little effect on noradrenaline-induced tone (16±8% relaxation) compared with hydrochlorothiazide (74±12% relaxation). In contrast to hydrochlorothiazide, the action of bendroflumethiazide was unaffected by 100 nmol/L charybdotoxin, a selective blocker of K\textsubscript{Ca} channels. All inhibitors of carbonic anhydrase relaxed noradrenaline-induced tone in a concentration-dependent manner, and this effect was blocked by charybdotoxin. Hydrochlorothiazide and the inhibitors of carbonic anhydrase failed to relax tone induced by a depolarizing potassium solution. Acetazolamide and hydrochlorothiazide increased pHi by 0.27±0.07 and 0.21±0.04, respectively, whereas bendroflumethiazide had a much smaller effect: 0.06±0.03. The rise in pHi induced by any agent was not inhibited by charybdotoxin. The vasorelaxant effect of hydrochlorothiazide is shared by other inhibitors of carbonic anhydrase. Inhibitors of carbonic anhydrase, but not bendroflumethiazide, cause intracellular alkalinization, which is associated with K\textsubscript{Ca} channel opening. These data suggest that the vasodilator effect of thiazide diuretics results primarily from inhibition of vascular smooth muscle cell carbonic anhydrase, which results in a rise in pHi, leading to K\textsubscript{Ca} channel activation and vasorelaxation. (Hypertension. 1999;33:1043-1048.)

Key Words: hydrochlorothiazide ■ carbonic anhydrase inhibition ■ muscle, smooth, vascular ■ pH

Thiazide diuretics were developed in the 1950s by chemical modification of carbonic anhydrase inhibitors. Although their blood pressure-lowering effects have been well documented, their mechanism of action is still not fully resolved. The principal site of action of thiazides is the early segment of the distal nephron, where they inhibit a luminal transmembrane–coupled NaCl transport system. In the long term, thiazides act by reducing peripheral resistance rather than by their diuretic effects, and therefore a direct vascular effect has been proposed.

Previous studies in isolated human and guinea pig resistance arteries have established a direct vasodilator activity of hydrochlorothiazide. This vasorelaxant response to hydrochlorothiazide was abolished by charybdotoxin and iberiotoxin, both selective blockers of large-conductance Ca\textsuperscript{2+}-activated potassium (K\textsubscript{Ca}) channels, but not by inhibitors of other vascular K\textsuperscript{+} channels. On the basis of the fact that thiazide-like drugs such as cicletanine and diazoxide lead to hyperpolarization in vascular smooth muscle cells and the fact that hydrochlorothiazide increases 86 Rb efflux as a marker of K\textsuperscript{+} efflux, it was proposed that hydrochlorothiazide opens K\textsubscript{Ca} channels, thereby leading to K\textsuperscript{+} efflux and membrane hyperpolarization. The resultant closure of voltage-dependent Ca\textsuperscript{2+} channels leads to a fall in [Ca\textsuperscript{2+}], and vasorelaxation.

In addition to [Ca\textsuperscript{2+}], the open state probability of the K\textsubscript{Ca}, channel is also modulated by intracellular pH (pHi). Channel opening is inhibited by intracellular acidosis in carotid body cells, while in isolated blood vessels intracellular alkalinization leads to relaxation associated with hyperpolarization of the cell membrane and a consequent fall of [Ca\textsuperscript{2+}]. At present, how hydrochlorothiazide opens the K\textsubscript{Ca} channel is unknown; it could act by direct interaction with the channel or involve an intermediate intracellular biochemical effect.
Since it is known that most thiazide diuretics bind to and inhibit carbonic anhydrase, we hypothesized that a rise in pH by inhibition of carbonic anhydrase could represent the mechanism of action by which thiazide diuretics open vascular K<sub>Ca</sub> channels and relax resistance arteries. We were able to examine the influence of carbonic anhydrase inhibitor activity since different thiazide compounds exert different degrees of carbonic anhydrase inhibitor activity. It was also possible to distinguish between an effect on intracellular or extracellular membrane–bound forms of carbonic anhydrase by the use of lipophilic and hydrophilic carbonic anhydrase inhibitors.

The vasodilator effects of thiazides may contribute to their antihypertensive properties, and opening of K<sub>Ca</sub> channels by these agents may represent a novel mode of action of these drugs in the vasculature.

**Methods**

**Tissue and Myograph Procedure**

After approval of our ethics committee, guinea pig mesentery was removed from male animals (250 to 300 g) killed by cervical dislocation. Mesenteric resistance arteries (n = 44 in total; ID, 320±1.9 μm) were dissected free of surrounding tissue and mounted as ring segments in an isometric microvascular myograph. The myograph contained 10 mL physiological saline solution (PSS) (in mmol/L: NaCl 118, KCl 4.7, CaCl<sub>2</sub> \cdot 2H<sub>2</sub>O 2.5, MgSO<sub>4</sub> \cdot 7H<sub>2</sub>O 1.17, NaHCO<sub>3</sub> 25.0, Na<sub>2</sub>PO<sub>4</sub> \cdot 7H<sub>2</sub>O 1.0, Na<sub>2</sub>EDTA 0.03, glucose 5.5) maintained at 37°C and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The vessels were allowed to equilibrate for 1 hour and then were set at a “normalized” internal circumference of 0.9L100, estimated to be 90% of the circumference they would maintain if relaxed and exposed to 100 mm Hg transmural pressure. This was calculated for each individual artery on the basis of the passive length-tension characteristics of the artery and the LaPlace relationship. At this setting near-maximal force development can be obtained, and the internal diameters referred to were derived from this calculation.

Before start of the studies, vessels were tested for viability with the use of a depolarizing potassium solution (KPSS; PSS with equimolar substitution of 118 mmol/L KCl for NaCl) and noradrenaline (10 μmol/L) exert direct effects of the K<sub>Ca</sub> channel. To confirm viability of arteries, a concentration-response curve (n = 4 for each agent; 10<sup>−9</sup> to 10<sup>−5</sup> mol/L) was constructed for acetylcholine (10 μmol/L). Those vessels failing to produce a tension equivalent to 90 mm Hg to these stimulants were discarded.

**Effects of Carbonic Anhydrase Inhibitors on Vascular Tone**

Vessels were precontracted with noradrenaline (10 μmol/L), and once stable tone was attained, concentration-response curves (n = 4 for each agent; 10<sup>−9</sup> to 10<sup>−5</sup> mol/L) were constructed for acetazolamide, benzolamide (hydrophilic), or ethoxzolamide (lipophilic). Because benzolamide has a lower ether/water partition coefficient and a markedly lower pK<sub>a</sub> value (3.2) than acetazolamide (7.4), it is generally assumed that it permeates into cells very slowly and therefore more or less specifically inhibits the activation of extracellular carbonic anhydrase.

**Interaction Between Carbonic Anhydrase Inhibitors and Vascular Potassium Channels**

It was previously demonstrated<sup>10,11</sup> that the vascular action of hydrochlorothiazide was absent in vessels precontracted with a depolarizing high-potassium solution and also was inhibited by charybdotoxin, but not by antagonists of other potassium channels such as glibenclamide (ATP-dependent K<sup>+</sup> channel) and apamin (small-conductance K<sub>Ca</sub> channel).<sup>2</sup> To demonstrate that carbonic anhydrase inhibitors (n = 4 for each agent; 30 μmol/L) exert direct vasorelaxation, we precontracted some vessels with noradrenaline in the presence of KPSS. Under these depolarized conditions, potassium channel activation will have a negligible effect on membrane potential and therefore should not reduce calcium entry and vascular tone. If the vasodilation induced by carbonic anhydrase inhibitors is evoked by hyperpolarization of vascular smooth muscle due to increased K<sup>+</sup> conductance, its action should be inhibited by depolarized conditions. Additionally, the vasorelaxant properties of the carbonic anhydrase inhibitors (30 μmol/L) were compared before and after incubation with charybdotoxin (n = 5; 20 minutes; 100 μmol/L) or glibenclamide (n = 5; 20 minutes; 100 μmol/L) in noradrenaline-contracted vessels. Charybdotoxin is a selective inhibitor of K<sub>Ca</sub> channels<sup>12</sup> while glibenclamide is a selective blocker of K<sub>ATP</sub> channels.<sup>13</sup>

**Interaction Between Carbonic Anhydrase Inhibitors, the Eicosanoid System, and the Endothelium**

The vasorelaxant effect of acetazolamide (30 μmol/L) was determined with or without 30 minutes of preincubation with 20 μmol/L indomethacin. Indomethacin is a potent NSAID, and it has been well established that NSAIDs inhibit prostaglandin synthesis by blocking the enzyme cyclooxygenase, which is involved in the generation of prostaglandin from arachidonic acid.<sup>14</sup> In addition, the effect of endothelial removal was examined in 4 vessels. Endothelium was removed from vessels mounted in the myograph by passing a hair through the lumen of the vessel.<sup>15</sup> The efficacy of this procedure was confirmed by abolition of relaxation to endothelium-dependent vasodilators acetylcholine (10 μmol/L) or substance P (100 nmol/L).

**Effects of Thiazide Diuretics on Vascular Tone and Their Interaction With K<sub>Ca</sub> Channels**

If the ability of hydrochlorothiazide to activate K<sub>Ca</sub> channels and relax resistance arteries is dependent on its carbonic anhydrase–inhibiting activity, any vascular effects of bendroflumethiazide, a thiazide that practically lacks carbonic anhydrase–inhibiting activity,<sup>9</sup> should not be associated with K<sub>Ca</sub> channel activation. To test this hypothesis, we compared the vascular effects of each drug (n = 8 to 12; 30 μmol/L) and determined whether these effects were inhibited by charybdotoxin (20 minutes; 100 μmol/L). Because it was previously established that the vasorelaxant effect of hydrochlorothiazide is dose dependent,<sup>2,11</sup> we used the concentration that elicited the maximal effect.

**Measurements of pH<sub>i</sub>**

In some vessels, measurements of pH<sub>i</sub> were obtained as described previously.<sup>2,16</sup> In brief, vessel segments were set up in a single-channel myograph dedicated to fluorescence measurements, were incubated with 10 μmol/L of the acetoxymethyl ester of the pH-sensitive dye 2',7'-bis(carboxyethyl)5(6)-carboxyfluorescein (BCECF-AM). Fluorescence was measured with a Deltascan spectrophotometer (Photon Technology International) connected to an inverted Axiovert 35 fluorescence microscope (Carl Zeiss) using only quartz objectives (Ultrafluor ×10). pH<sub>i</sub> was assessed on the basis of the ratio of fluorescence emission measured at 510 nm, which was evoked by excitation at 450- and 495-nm light. Emission signals and vascular tone were measured simultaneously at 1 Hz and acquired online with an analog/digital interface (Photon Technology International) connected to an IBM computer. Data were stored on an optical disk and later analyzed offline with commercially available software (Photon Technology International). At the end of each experiment, the ratio was calibrated with 4 solutions (K<sup>+</sup>/HEPES, in mmol/L: KCl 140, MgCl<sub>2</sub> 1.0, CaCl<sub>2</sub> 1.6, EDTA 0.026, glucose 10, HEPES 10.0) in the pH range of 6.8 to 7.4 containing nigericin (10 μmol/L), as described previously.<sup>16</sup> Nigericin is a K<sup>+</sup>/H<sup>+</sup> ionophore that will equilibrate intracellular and extracellular pH in high-potassium buffers. The first solution was applied to the myograph for 7 minutes, and the subsequent solutions were added for 5 minutes each. With the use of this technique, a linear regression line could be calculated and the other intensity ratios could be evaluated to give true pH readings.
The vasorelaxant effect was probably not due to inhibition of intracellular carbonic anhydrase, since the hydrophilic benzolamide exerted a similar response to the lipophilic ethoxzolamide.

**Interaction Between Carbonic Anhydrase Inhibitors and Potassium Channels**

All carbonic anhydrase inhibitors failed to relax KPSS-induced tone (\(n=4\)). The effect of incubation with charybdotoxin, an inhibitor of \(K_{ATP}\) channels, on the relaxation of the carbonic anhydrase inhibitors is shown in Figure 2. Incubation with charybdotoxin (100 nmol/L) had no effect on the subsequent contraction to noradrenaline. The vasorelaxant effect of all 3 carbonic anhydrase inhibitors was significantly inhibited by charybdotoxin. Substance P (100 nmol/L) was used as a control, and its vasorelaxant effect was not inhibited by charybdotoxin. Acetazolamide-induced relaxation was unaffected by the \(K_{ATP}\)-selective antagonist glibenclamide; relaxation to acetazolamide was 73.8±8.2\% and 77.5±7.7\% in the absence and presence of glibenclamide, respectively (\(P=NS\)).

**Effects of Thiazide Diuretics on Vascular Tone and Their Interaction With \(K_{Ca}\) Channels**

In agreement with previous reports, \(2,5,11\), hydrochlorothiazide (30 μmol/L) relaxed guinea pig vessels (74±12\%; \(P<0.001\)), and this effect was almost totally abolished by charybdotoxin (\(P<0.001\)). In contrast, bendroflumethiazide had little effect on vascular tone (relaxation 16±8\%; \(n=12\)). The small relaxation seen in response to bendroflumethiazide was not significantly inhibited by charybdotoxin (Figure 3).

**Effects of Carbonic Anhydrase Inhibitors and Thiazides on \(pH_i\)**

Resting \(pH_i\) in isolated guinea pig mesenteric arteries was 7.18±0.19 (\(n=15\)). As shown in Figure 4, the vasorelaxant effect of acetazolamide and hydrochlorothiazide was associated with a rise in \(pH_i\). Bendroflumethiazide caused a small rise in \(pH_i\), but this was not statistically significant. In vessels incubated in charybdotoxin, the acetazolamide- and hydrochlorothiazide-induced rises in \(pH_i\) were not significantly affected (Figure 5).
Discussion

Our experiments were designed to determine to what extent inhibition of carbonic anhydrase by thiazide diuretics accounts for their direct vasodilator effects. We have shown that at clinically relevant concentrations, the vasorelaxant effect of hydrochlorothiazide attributable to K\textsubscript{Ca} channel opening is shared by other agents that inhibit carbonic anhydrase. In contrast, even a high concentration of bendroflumethiazide, a thiazide that practically lacks carbonic anhydrase-inhibiting activity, only minimally affected vascular tone. Furthermore, both hydrochlorothiazide and acetazolamide increased pH\texttextsubscript{i} in association with opening the K\textsubscript{Ca} channel, while bendroflumethiazide had minimal effects on either pH\textsubscript{i} or tone. Since the effect of hydrochlorothiazide and acetazolamide on pH\textsubscript{i} was also present in vessels preincubated with charybdotoxin, a blocker of the K\textsubscript{Ca} channel, we conclude that the rise in pH\textsubscript{i} is likely to be a consequence of K\textsubscript{Ca} channel activation and/or vasorelaxation.

Since we have previously reported that charybdotoxin also inhibits the fall in intracellular calcium induced by hydrochlorothiazide,5 it is also unlikely that changes in intracellular calcium account for the rise in pH\textsubscript{i}. Therefore, we conclude that inhibition of carbonic anhydrase and the associated rise in pH\textsubscript{i} may play a primary role in the vasodilator effect of hydrochlorothiazide. Although our findings concentrate on the carbonic anhydrase-inhibiting properties and not on inhibition of the NaCl cotransporter by thiazide diuretics, these mechanisms may be interrelated, since studies in rat distal colon demonstrated that thiazide-induced inhibition of NaCl absorption is directly due to inhibition of mucosal carbonic anhydrase.17 However, to our knowledge NaCl cotransport has not been demonstrated in vascular smooth muscle cells.

Carbonic Anhydrase Inhibition and pH\textsubscript{i}

Relatively few studies have focused on the effects of inhibition of carbonic anhydrase on pH\textsubscript{i} and tone in vascular smooth muscle cells. In agreement with our findings, acetazolamide has also been reported to increase pH\textsubscript{i} in turtle bladder cells,18 kidney cells,19 choroid plexus epithelial cells,20 and the mandibular gland.21 Under different conditions and in different cells, acetazolamide has also been reported to decrease22 or have no effect23 on pH\textsubscript{i}. The mechanism of action of the acetazolamide-induced rise in pH\textsubscript{i} is not completely understood, but most reports focus on an intracellular accumulation of HCO\textsubscript{3} due to inhibition of CI/HCO\textsubscript{3} exchange. In addition, an acetazolamide-sensitive inward chloride pump, different from the CI/HCO\textsubscript{3} exchange and NaKCl cotransporter, has been reported in rat arterial vascular smooth muscle cells.26 Others found the same acetazolamide-induced inhibition of renal CI/HCO\textsubscript{3} exchange in vivo and suggested that in the presence of acetazolamide, H\textsuperscript{+} extrusion continues, but the rate of reaction of OH\textsuperscript{-} with CO\textsubscript{2} is diminished as a result of carbonic anhydrase inhibition.19,27 In our experiments the hydrophilic benzolamide was approximately as effective as acetazolamide and ethoxzolamide, indicating that inhibition of the extracellular membrane-bound form of carbonic anhydrase is responsible for the intracellular alkalinization, K\textsubscript{Ca} channel activation, and vasorelaxation. It is unclear how inhibition of this enzyme can mediate changes in pH\textsubscript{i}, but it seems possible that this extracellular enzyme might modulate CI/HCO\textsubscript{3} exchange, resulting in an attenuated HCO\textsubscript{3} extrusion and increase in pH\textsubscript{i}. The present study does not allow definite conclusions on the mechanism of the acetazolamide-induced increase in pH\textsubscript{i}, but our observation that the vascular action and the pH\textsubscript{i} effect are...
shared by a thiazide diuretic that also exerts carbonic anhydrase–inhibiting activity and not by a thiazide that lacks this effect suggests that both effects may be due to inhibition of carbonic anhydrase.

**pH$_i$ and Vascular Tone**

One of the various ways (for review, see Reference 28) in which changes of pH$_i$ could alter the force development in smooth muscle cells is through potassium channel modulation, since marked effects of pH$_i$ on the K$_{Ca}$ channel have been reported in various tissues.\textsuperscript{29–31} Since the vasorelaxant action of hydrochlorothiazide is inhibited by charybdotoxin, we hypothesized that a pH$_i$ change, due to the carbonic anhydrase–inhibiting activity of the drug, was the trigger for K$_{Ca}$ channel activation. In isolated type I cells of the neonatal rat carotid body, the K$^+$ current that was inhibited by intracellular acidosis was also inhibited by charybdotoxin and not by apamin,\textsuperscript{6} suggesting that the K$^+$ current is carried through large-conductance K$_{Ca}$ channels. In accordance, the vascular effects of hydrochlorothiazide have also been reported to be inhibited by charybdotoxin and iberiotoxin\textsuperscript{2,5,11} but not by apamin.\textsuperscript{2,11}

**Direct Vasoactivity of Carbonic Anhydrase Inhibitors**

Although we used the carbonic anhydrase inhibitors in the present study as a tool to elucidate the mechanism of action of thiazide diuretics, the observation that acetazolamide is a direct vasodilator at clinically relevant concentrations in isolated resistance arteries is an interesting finding in itself. The vascular effects of acetazolamide have been well studied, especially on the cerebral vasculature,\textsuperscript{32} but our finding that the vasorelaxant effect of acetazolamide is associated with a rise in pH$_i$ and K$_{Ca}$ channel activation is completely novel. We found that the dose-dependent vasorelaxant effect of acetazolamide is caused by opening of K$_{Ca}$ channels and not mediated by other K$^+$ channels, the eicosanoid system, or the endothelium.

In vivo, systemic administration of acetazolamide can produce pronounced hypercapnia. Because hypercapnia is a potent dilator of cerebral blood vessels,\textsuperscript{33} it is possible that the direct vasodilator mechanism that we describe does not account for the cerebral vasodilation in response to acetazolamide. In our experiments in isolated arteries, the maximal response to acetazolamide is reached within minutes, whereas the maximal vasodilator response in the carotid vascular bed after systemic administration of acetazolamide takes up to 1 hour.\textsuperscript{32} Furthermore, the acetazolamide-induced vasodilation of cerebral vessels appears to be dependent on prostaglandin synthesis but not on nitric oxide release, since it was found to be inhibited by indomethacin\textsuperscript{44} but not by N$^\omega$-nitro-L-arginine,\textsuperscript{35} an inhibitor of nitric oxide synthase. In contrast, our results indicate that the direct vasorelaxant effect of acetazolamide is independent of both local prostaglandin synthesis and the endothelium. It is assumed that cerebral vessels are sensitive to a fall in extracellular pH,\textsuperscript{36} whereas our data concentrate on the rise in pH$_i$.

In contrast to the well-studied effects of acetazolamide on the cerebral vasculature, not much is known about its direct vascular effects in other vascular beds. Since vascular effects appear to depend on inhibition of carbonic anhydrase activity, this could account for contradictory reports regarding the vasoactivity of acetazolamide. Vascular carbonic anhydrase activity varies from organ to organ and also between species.\textsuperscript{37} It has been reported that rabbit aorta does not contain carbonic anhydrase activity,\textsuperscript{38} and, in agreement, thiazide diuretics that possess carbonic anhydrase–inhibiting activity do not relax isolated rabbit arteries (A.D.H., unpublished data, 1996). It was reported that the direct vasorelaxant effects of hydrochlorothiazide are present in human and guinea pig vessels but not in rat resistance arteries,\textsuperscript{39} and it is of interest that acetazolamide fails to change pH$_i$ in rat mesenteric resistance arteries (Aalkjær C., written communication, 1997).

It is difficult to speculate whether the aforementioned properties of acetazolamide and hydrochlorothiazide play a role during long-term administration of the drugs in humans. In the present study we used rather high concentrations of the thiazide diuretics and carbonic anhydrase inhibitors; however, the concentration-response curves show that direct vascular effects are seen at clinically relevant concentrations.\textsuperscript{39} Furthermore, the antihypertensive effects of thiazide diuretics take several weeks to reach their maximum and also wear off slowly after termination of therapy. This may suggest that slow accumulation in the target organ takes place, especially since the thiazide-like agent indapamide was found at a 9-fold higher concentration in vascular smooth muscle cells than in the plasma.\textsuperscript{40} Consequently, despite the high concentrations used in this study, it is conceivable that the mechanism described may be relevant to the actions of these agents in vivo after long-term administration.

**Conclusion**

We have previously shown that hydrochlorothiazide relaxes human\textsuperscript{39} and guinea pig\textsuperscript{2,5,11,39} isolated arteries by opening K$_{Ca}$ channels. This effect of hydrochlorothiazide is not shared by bendroflumethiazide and seems related to its activity as an inhibitor of carbonic anhydrase. At clinically relevant concentrations, other inhibitors of carbonic anhydrase also relax vascular smooth muscle by activation of K$_{Ca}$ channels associated with an increase in pH$_i$. In view of the efficacy of the hydrophilic inhibitor benzolamide, this effect probably does not involve an effect on intracellular carbonic anhydrase. As a result of our studies, we propose that acetazolamide and thiazide diuretics that inhibit carbonic anhydrase activity produce intracellular alkalosis of vascular smooth muscle cells. The rise in pH$_i$, as a consequence of inhibition of carbonic anhydrase appears to activate the K$_{Ca}$ channel, resulting in hyperpolarization of the vascular smooth muscle cell, reduction of voltage-dependent calcium channel activity, fall in [Ca$^{2+}$], and vasorelaxation. It is possible that this novel mechanism of vasodilation contributes to the antihypertensive action of thiazides in vivo.

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References


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