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The mechanism of action of angiotensin II is dependent on direct activation of vascular smooth muscle carbonic anhydrase I

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Abstract Our previous studies have shown that angiotensin II increases carbonic anhydrase activity both in vitro and in vivo. In this study we investigated in vitro the effect of angiotensin II on carbonic anhydrase I and II from erythrocytes and on arteriolar vascular smooth muscle carbonic anhydrase I. We also studied in vitro and in vivo the effect of angiotensin II receptor blockers (irbesartan and candesartan) on purified carbonic anhydrase I and II, on vascular smooth muscle carbonic anhydrase I and on arterial blood pressure in humans and in animals. In vitro results showed that angiotensin II is a direct and stronger activator of carbonic anhydrase I than II. Angiotensin II receptor blockers reduced mainly carbonic anhydrase I activity and completely antagonized the activating effect of angiotensin II both on purified and on vascular smooth muscle carbonic anhydrase I. Our in vivo experiments showed that irbesartan and candesartan are powerful inhibitors of carbonic anhydrase I both in erythrocytes (in humans) and in vascular smooth muscles (in animals). In humans, irbesartan and candesartan progressively reduce arterial blood pressure in hypertensive subjects, in parallel with progressive reduction of erythrocyte carbonic anhydrase I activity. We believe that angiotensin II could have a dual mechanism of action: (1) angiotensin interacting with its receptor to form a stimulus-receptor complex; (2) the same stimulus directly acts on the carbonic anhydrase I isozyme (which might be coupled with angiotensin II receptors), ensuring an adequate pH for stimulus-receptor coupling for signal transmission into the cell and hence vasoconstriction.

Key words Angiotensin II · Vascular smooth muscle CA I · Angiotensin II receptor blockers · pH · Mechanism of action

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Introduction

Angiotensin II is an octapeptide which acts via diverse, yet coordinated, mechanisms to increase arterial blood pressure. [1]. Angiotensin II acts directly on vascular smooth muscle and the adrenal cortex, but also within the heart, kidneys, and central and autonomic nervous systems. These actions amplify its volume-retaining and vasoconstrictive effects on the peripheral vascular system. Angiotensin II also induces free radical generation within vessel walls, accompanied by impairment of endothelium-derived nitric oxide [2].

The action of angiotensin II is triggered by its interaction with receptors on the plasma membrane of the tissues responsive to the hormone. Two receptors have been cloned: one (A II type 1 receptor, AT1) mediating virtually all of the known effects of angiotensin II [3] and another (AT 2) apparently involved with cell proliferation and perhaps renal sodium excretion [4]. Detailed information is now available regarding mechanisms by which AT 1 receptors alter cell function [5–7].

Agents that selectively block the AT 1 receptor have been synthetized and marketed for the treatment of hyper-

tension. Losartan was the first, valsartan the second; more compounds are under development, including irbesartan, candesartan, and tasosartan [8]. These agents displace angiotensin II from its specific AT 1 receptor, antagonizing all of its known effects and resulting in a dose-dependent fall in peripheral resistance and little change in heart rate or cardiac output [9].

Carbonic anhydrase (CA) is a zinc enzyme detected in red blood cells and other cells. Its main role is in acid-base balance; its activation leading to a fall in pH and its inhibition leading to an increase in pH [10].

Our previous studies of CA isozymes, have shown that CA I is involved in vascular changes [11], while CA II and CA IV are involved in secretory processes [12]. The same studies showed that CA I and CA II are activated by non-steroidal anti-inflammatory drugs [13] and that vasodilatory prostaglandins and diuretic agents [14, 15] inhibit CA, while vasoconstrictive prostaglandins activate the enzyme [14].

Our previous studies showed that angiotensin II increased CA activity both in vitro and in vivo, CA I activation being significantly higher than CA II activation [16]. These studies suggested that the binding of angiotensin II to its receptor is influenced by changes in pH caused by CA I activation. In this study we investigated the effect of angiotensin II on erythrocyte-purified CA I and CA II and on vascular smooth muscle CA I. We also studied the effect of angiotensin II receptor antagonists on CA I and CA II and on arterial blood pressure.

Materials and methods

In vitro studies

We studied the effect of angiotensin II on purified CA I and CA II and on CA I isolated from vascular smooth muscle from piglet arteriole. The dose-response relationship at concentrations between 10^{-8} and 10^{-4} M was assessed. We studied the effect of angiotensin II receptor antagonists, irbesartan and candesartan, on purified CA I and CA II and on vascular smooth muscle CA I. We also investigated the effect of irbesartan and candesartan on the activation of CA I and CA II by angiotensin II.

In vivo studies

The study was conducted according to the Declaration of Helsinki as modified by the 21st World Medical Assembly, Venice, Italy, 1983, and later by the 41st World Medical Assembly, Hong Kong, 1989. All patients gave informed consent for a protocol that had been approved by the ethics committee of the Center for Research and Medical Assistance in Simleu Silvaniei.

Two groups of hypertensive volunteers, aged between 45 and 60 years, weighing 65–80 kg, of both sexes were studied. All subjects were living at home and in good general health apart from their hypertension. Patients were characterized as hypertensive if they had a seated diastolic blood pressure >90 mmHg and a systolic blood pressure of >140 mmHg. Blood pressure was measured with a standard mercury sphygmomanometer. Values were always the mean of three measurements in the classical sitting position. Subjects were screened before participation with a history and physical examination, a complete blood count, fasting serum glucose, and routine chemistries, urinalysis, and electrocardiogram. Patients were excluded from participation if they exceeded 135% of ideal body weight, had a past history of diabetes mellitus, had a fasting serum glucose of >6.7 mM, were taking any medications, had orthostatic hypotension, or had evidence from the screening tests of underlying illness or significant laboratory or electrocardiogram abnormalities. Both groups have been treated orally for 30 days as follows: group 1 (n=19), irbesartan (Aprovel, SANOFI Pharma, Paris, France) a single dose of 150 mg/day; group 2 (n=21), candesartan (Blopress, Takeda Pharma, Vienna, Austria) a single dose of 16 mg/day.

We measured blood pressure values daily; red cell CA I and CA II activity was determined before treatment, after 5 days, after 10 days, and after 30 days of treatment. Each sample was taken 6 h after drug administration.

In animal experiments we selected 24 piglets, weighing between 30 and 35 kg. They were housed in air-conditioned quarters and had free access to tap water and standard food. Animals were divided into three groups of 8 piglets each and received oral treatment for 30 days as follows: group A-control group, placebo; group B-irbesartan (Aprovel), a single dose of 150 mg/day; group C-candesartan (Blopress), a single dose of 16 mg/day.

After 6 h from the last administration we collected blood samples and isolated vascular smooth muscle CA I from femoral arterioles according to the method of Lonnerholm et al. [17]. The CA I and CA II activities were determined and compared with the control group.

Differentiation of red cell CA I from CA II activity was performed by the test with nicotinates [18], which relies on selective inhibition of CA I activity. CA I and CA II activity was assessed using the stopped-flow method [19]. This method involves in measuring the enzymatic activity of CO_2 hydration and relies on the colorimetric method of changing pH. The time in which the pH of the reagent mixture decreases from its initial value of 7.5 to its final value of 6.5 is measured. The reaction is followed spectrophotometrically at 400 nm, using a rapid kinetic spectrophotometer HI-TECH SF-51MX (England), equipped with a mixing unit and a system of two syringes which supply the reagents. The signal transmitted by photomultiplier from the mixing chamber is received and visualized by a computer equipped with a mathematical coprocessor and a kinetic software package RKBIN IS1.

The reagents used were: p-nitrophenol (Sigma, Deisenhofen, Germany) as color indicator at a concentration of 0.2 mM, pH=7.5, temperature 20–25°C; HEPES buffer (Sigma) at a concentration of 20 mM, pH=7.5, at room temperature; CO_2 solution at a concentration of 15 mM (as substrate) obtained by bubbling CO_2 in bidistilled water to saturation; sodium sulfate at a concentration of 0.1 M is used to keep a constant ionic strength. Purified human erythrocyte CA I and CA II and angiotensin II were obtained from Sigma.

CA activity was obtained from the formula:

$$A = \frac{T_0 - T}{T} \quad [EU/ml]$$

where T_0 represents the uncatalyzed reaction time and T represents the catalyzed reaction time (in the presence of CA I).

The sensitivity of the CA assay was calculated from a least-squares fit of the CA values using RKBIN software.

Statistics

To determine whether the CA I and CA II activity was affected by angiotensin II and by angiotensin II receptor blockers, a repeated measure ANOVA was performed. Comparisons between treatments were made using the Neuman-Keuls multiple comparison test. A paired *t*-test was used to compare initial values with steady-state values for a given treatment. $P<0.05$ was considered significant.

Results

In vitro studies

Angiotensin II increased purified CA I and CA II activity in a dose-dependent manner. The effect starts at 10^{-8} M and reaches a peak at 10^{-4} M. CA I activity increased from 0.435±0.02 to 0.748±0.04 EU/ml ($P<0.001$), and CA II activity from 1.00±0.01 to 1.203±0.02 EU/ml ($P<0.05$) (Table 1).

Angiotensin II also increased vascular smooth muscle CA I activity in a dose-dependent manner. The effect starts at 10^{-8} M and reaches a peak at 10^{-4} M. CA I activity increased from 0.816±0.06 EU/ml to 1.510±0.08 EU/ml ($P<0.001$) (Table 1).

Angiotensin II receptor antagonists studied here inhibited purified CA I and CA II activity and vascular smooth muscle CA I in a dose-dependent manner as follows: irbesartan inhibited purified CA I from 0.435±0.02 EU/ml to 0.183±0.01 EU/ml ($P<0.001$), CA II from 1.00±0.02 EU/ml to 0.815±0.03 EU/ml ($P<0.05$), and vascular smooth muscle CA I from 0.816±0.06 EU/ml to 0.301±0.05 EU/ml ($P<0.001$) (Table 1); candesartan inhibited purified CA I from 0.435±0.02 EU/ml to 0.214±0.01 EU/ml ($P<0.001$), CA II from 1.00±0.02 EU/ml to 0.843±0.03 EU/ml ($P<0.05$), and vascular smooth muscle CA I from 0.816±0.06 EU/ml to 0.350±0.04 EU/ml (P<0.001) (Table 1).

Irbesartan and candesartan completely antagonized the activating effect of angiotensin II both on CA I and CA II (Table 1).

In vivo studies

A 30-day treatment with angiotensin II receptor antagonists reduced the activity of red blood cell CA I and CA II and blood pressure in patients who received oral drugs as follows: group 1 - irbesartan (Aprovel) reduced the activity of CA I from 0.506±0.056 to 0.134±0.011 EU/ml ($P<0.001$) (74%), CA II from 1.328±0.126 EU/ml to 0.821±0.076 EU/ml (38%) (Fig. 1), and systolic blood pressure from 215±15 to 135±10 mmHg ($P<0.001$) (Fig. 2); group 2 - candesartan (Blopress) reduced the activity of CA I from 0.487±0.028 to 0.181±0.010 EU/ml ($P<0.05$) (63%), CA II from 1.415±0.135 EU/ml to 0.952±0.066 EU/ml (33%) (Fig. 1), and systolic blood pressure from 200±10 to 130±10 mmHg ($P<0.001$) (Fig. 2).

Table 1 The effect of angiotensin II and of angiotensin II receptor antagonists on carbonic anhydrase (CA) isozymes. There was a powerfull increase in purified erythrocyte CA I activity and vascular smooth muscle CA I in response to angiotensin II and a small increase in erythrocyte CA II activity. Irbesartan and candesartan reduced erythrocyte CA I activity, vascular smooth muscle CA I, and erythrocyte CA II activity. Angiotensin II receptor antagonists completely antagonized the simulatory effect of angiotensin II on this isozyme. Values are means ± SEM; $n=5$ assessments; * $P<0.05$ compared with isozymes basal activity (paired *t*-test)

Substance	Concentration (M)	Purified CA I (basal activity = 0.435±0.01 EU/ml)	Purified CA II (basal activity = 1.00±0.01 EU/ml)	Vascular smooth muscle CA I (0.816±0.06 EU/ml)
Angiotensin II	10^{-8}	0.566±0.01 *	1.085±0.01	1.173±0.01 *
	10^{-6}	0.672±0.02 *	1.162±0.02 *	1.382±0.02 *
	10^{-4}	0.748±0.04 *	1.203±0.02 *	1.510±0.08 *
Irbesartan	10^{-8}	0.368±0.02 *	0.961±0.02	0.624±0.03 *
	10^{-6}	0.295±0.01 *	0.904±0.03	0.487±0.02 *
	10^{-4}	0.183±0.01 *	0.815±0.02 *	0.301±0.05 *
Candesartan	10^{-8}	0.395±0.01 *	0.983±0.01	0.708±0.03 *
	10^{-6}	0.307±0.02 *	0.921±0.02	0.536±0.02 *
	10^{-4}	0.214±0.02 *	0.843±0.01 *	0.350±0.04 *
Angiotensin II+ irbesartan	10^{-8}	0.428±0.02	1.012±0.01	0.819±0.03
	10^{-6}	0.434±0.01	1.023±0.03	0.825±0.06
	10^{-4}	0.441±0.01	1.039±0.02	0.835±0.05
Angiotensin II+ candesartan	10^{-8}	0.425±0.01	1.015±0.03	0.824±0.04
	10^{-6}	0.437±0.02	1.031±0.01	0.838±0.06
	10^{-4}	0.452±0.01	1.042±0.02	0.847±0.02

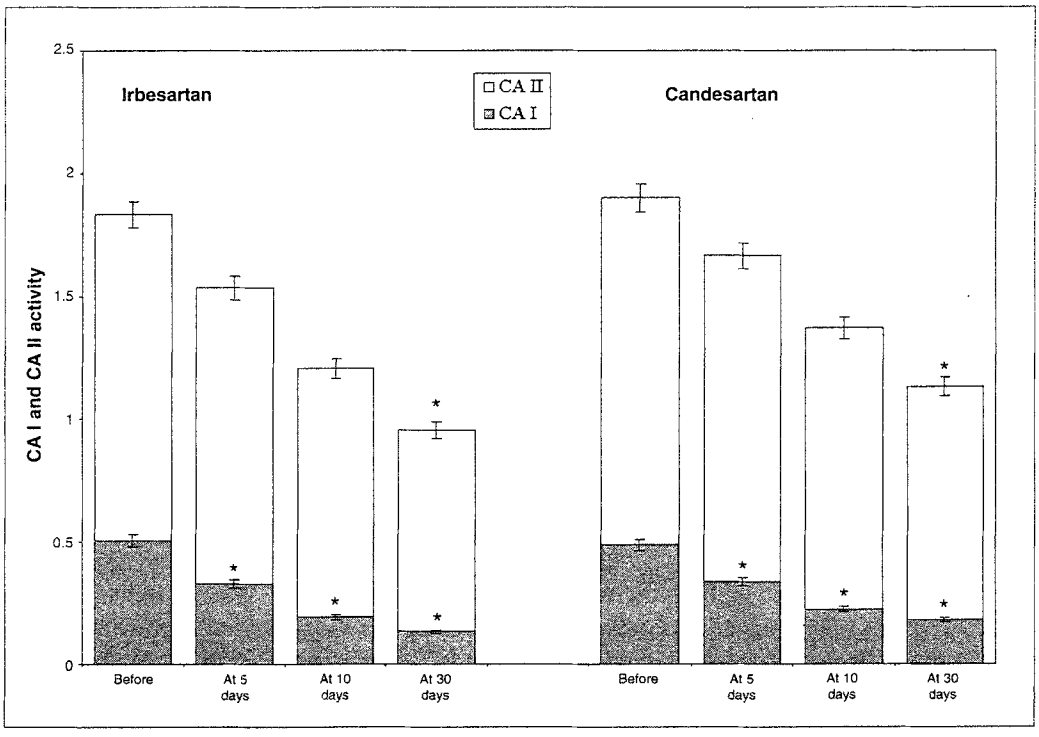

Fig. 1 The inhibition of erythrocyte carbonic anhydrase (*CA*) I and CA II activity after 5 days, after 10 days, and after 30 days of treatment with irbesartan (Aprovel) (group 1) and candesartan (Blopress) (group 2). Values are means±SEM; n=19–21 patients; *P<0.05 compared with values before treatment (paired t-test)

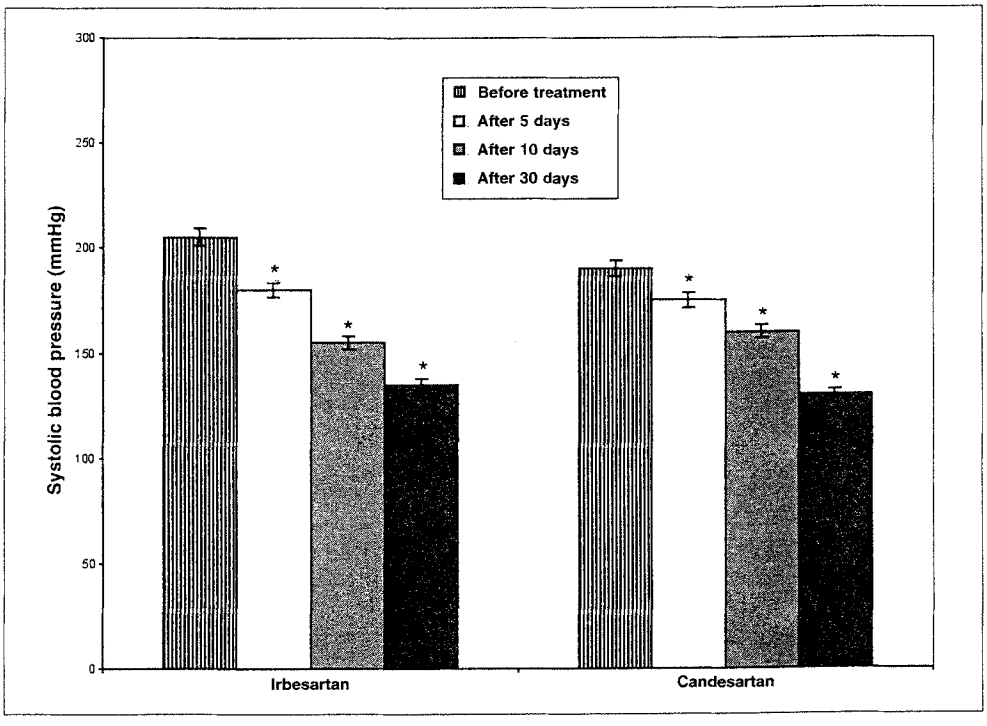

Fig. 2 The reduction of systolic blood pressure after 5 days, after 10 days, and after 30 days of treatment with irbesartan (Aprovel) (group 1) and candesartan (Blopress) group 2). Values are means±SEM; n=19–21 patients; *P<0.05 compared with values before treatment (paired t-test)

Animal studies (Fig. 3)

In the control group (A), the activity of red cell CA I was 0.314±0.026 EU/ml, red cell CA II was 1.216±0.108 EU/ml, and vascular smooth muscle CA I was 0.796±0.058 EU/ml. In group B (irbesartan treated), the erythrocyte CA I activity was 0.116±0.007 EU/ml (P<0.001), erythrocyte CA II activity was 0.873±0.092 EU/ml (P<0.05), and vascular smooth muscle CA I was 0.198±0.143 EU/ml (P<0.001), which was significantly reduced compared with the control group. In group C (candesartan treated), the erythrocyte CA I activity was 0.124±0.008 EU/ml (P<0.001), erythrocyte CA II activity was 0.956±0.085 EU/ml (P<0.05), and vascular smooth muscle CA I was 0.219±0.152 EU/ml (P<0.001), which was significantly reduced compared with the control group.

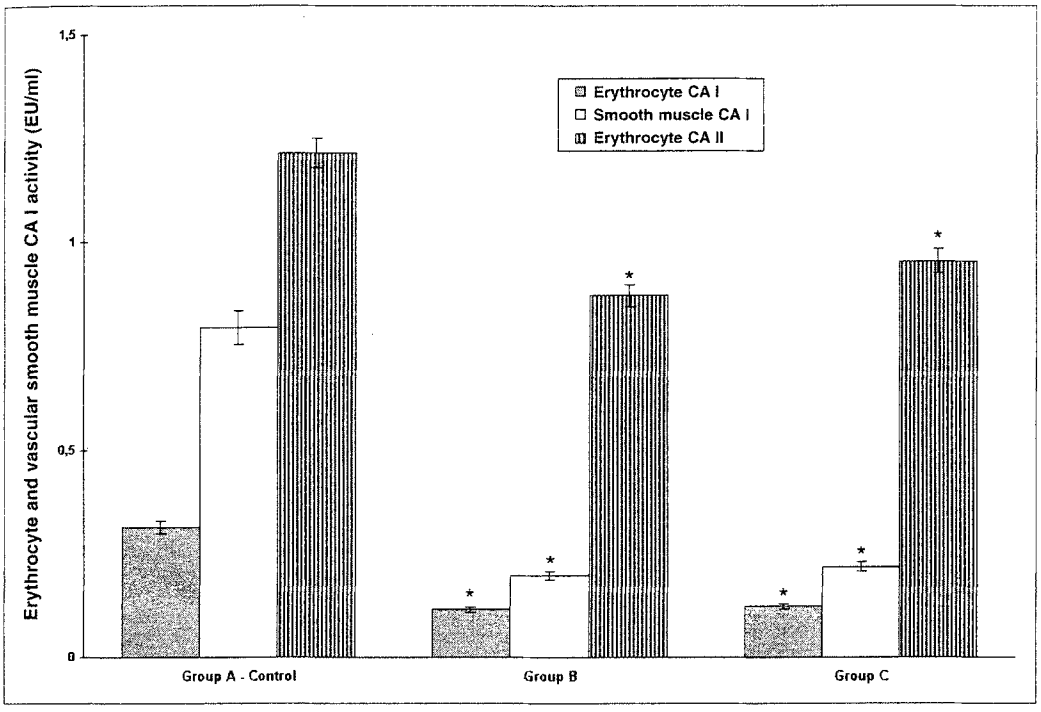

Fig. 3 The reduction of erythrocyte CA I and CA II activity and of vascular smooth muscle CA I activity in piglets after treatment with irbesartan (Aprovel) (group B) and candesartan (Blopress) (group C) compared with the control group (group A). Values are means±SEM; n=8 piglets; $^*P<0.05$ compared with control group

Discussion

The relationship between angiotensin II and CA I and CA II isozymes has been studied for the first time by our group [16] and we have shown that CA I is mainly activated by a direct mechanism of action. The in vitro results of this study showed that angiotensin II is a direct and strong CA I activator, but has less effect on CA II. This activating effect of angiotensin II applies to both erythrocyte-purified CA I and CA I from arteriolar vascular smooth muscle. Angiotensin II receptor blockers reduced mainly CA I activity by a direct mechanism of action.

The specific blockers of angiotensin II receptors, irbersartan and candesartan, completely antagonized the activating effect of angiotensin II both on purified CA I and on vascular smooth muscle CA I.

Our in vivo experiments showed that irbesartan and candesartan are powerful CA I inhibitors both in erythrocytes and vascular smooth muscle. In animals, chronic treatment with angiotensin II receptor blockers such as irbesartan and candesartan significantly reduced CA activity, mainly CA I, both in erythrocytes and in arteriolar smooth muscle compared with controls.

In humans, irbesartan and candesartan progressively reduced arterial blood pressure in hypertensive subjects (Fig. 2), in parallel with a progressive reduction of erythrocyte CA I activity (Fig. 1). A parallel reduction of arterial blood pressure and erythocyte CA I inhibition was noted in all groups of hypertensive patients studied by us.

These results are in accordance with other studies carried out by us which showed that CA I is involved in the modulation of vascular processes. We hypothesize that the pH increase induced by CA I inhibition might influence the binding of hypotensive stimuli to their specific receptors followed by signal transmission into the cytoplasm of smooth muscle cells with subsequent vasodilating effects [11, 20]. In the same way, the reduction in pH induced by vascular smooth muscle CA I activation through hypertensive agents would influence the membrane-specific receptor, signal transmission in the vascular smooth muscle cytosol, and subsequent vasoconstrictive effects [21].

Our results are supported by recent studies which show that primary hypertension may be associated with perturbations of acid-base status, specifically of intracelluar pH [22, 23]. Furthermore, the same studies showed a decrease in intracellular pH in hypertensive animal models compared with normotensive animals [22, 24]. An evaluation of steady-state intracellular pH in erythrocytes, using a nuclear magnetic resonance technique, has indicated that intracellular pH is reduced in erythrocytes from untreated patients with essential hypertension compared with treated patients and normotensive controls [25].

Other studies have reported that the blood pressure-lowering effects of calcium channel blockade were inversely related to intracellular pH - i.e., the lower the initial pH, the greater the antihypertensive effect. Furthermore, nifedipine consistently elevated intracellular pH values [26].

An enhanced activity of H^+-Na^+-antiporter (as a major mechanism of cell defense against cellular acidification) has been reported in lymphocytes [27], as well as in the renal brush border membrane in spontaneous hypertensive rats

[28] and in hypertensive rats [29]. Other authors have shown enhanced responsiveness of the renal proximal Na^+-H^+-antiporter of hypertensive rats to certain hormonal stimulation [30]. It has been assumed that overactivity of this antiporter is not a primary process but rather reflects intracellular acidosis in the spontaneous hypertension of rat models [23, 24].

Angiotensin II-induced CA I activation and its inhibition by specific receptor blockers have suggested the involvement of pH changes (induced by activation/inhibiton of CA I) in vasoconstriction and vasodilation. In keeping with this concept, angiotensin II has a dual mechanism of action: (1) action on its specific receptor, with subsequent formation of a stimulus-receptor complex, coupling of G proteins, and information transmission within the cell; (2) direct action of CA I (which might be coupled to angiotensin II receptors), with a fall in pH, which would facilitate angiotensin II binding to its specific receptor.

However, in vascular smooth cell intracellular pH is lowered by angiotensin II-induced CA I activation, and this leads to an intracellular transmission mechanism, as follows: (1) C-β phospholipase generates inositol triphosphate with release of intracellular calcium, followed by vasoconstriction; (2) phospholipase A_2 stimulation with arachidonic acid formation and subsequent prostaglandin, leukotriene and thromboxan synthesis; (3) adenylyl cyclase inhibiton with reduction of cAMP formation followed by vasoconstriction.

In keeping with this concept, angiotensin II receptor blockers have the same dual mechanism of action: (1) the coupling with angiotensin II specific receptor (the known mechanism) and (2) direct CA I inhibition in vascular smooth muscle cell with a subsequent increase in pH. This alkalinization might contribute to the inhibition of angiotensin-receptor coupling as well as to the inhibition of intracellular transmission of activating stimulus to the effector.

This hypothesis is in accordance with a more-elaborate theory proposed by our group in which CA I modulates vascular tone by means of pH changes [21]. This pH theory is supported by our research which is based on: (1) the known role of CA I in acid-base balance; (2) the effect of vasoconstrictive substances that activate erythrocyte CA I and vascular smooth muscle CA I by a direct mechanism of action; (3) the effect of vasodilatory substances and drugs used in the treatment of hypertension, which inhibit CA I by a direct mechanism both in erythrocytes and in vascular smooth muscle.

Our results suggest that CA I activation induces a decrease in intracellular pH, while its inhibition leads to an increase. These changes in intracellular pH might influence ion channel activity, symport and antiport pump activity, and ATPase activity, all of which are involved in the modulation of vascular processes.

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