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Catecholamine-induced vasoconstriction is sensitive to carbonic anhydrase I activation

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Abstract

We studied the relationship between alpha- and beta-adrenergic agonists and the activity of carbonic anhydrase I and II in erythrocyte, clinical and vessel studies. Kinetic studies were performed. Adrenergic agonists increased erythrocyte carbonic anhydrase as follows: adrenaline by 75%, noradrenaline by 68%, isoprenaline by 55%, and orciprenaline by 62%. The kinetic data indicated a non-competitive mechanism of action. In clinical studies carbonic anhydrase I from erythrocytes increased by 87% after noradrenaline administration, by 71% after orciprenaline and by 82% after isoprenaline. The increase in carbonic anhydrase I paralleled the increase in blood pressure. Similar results were obtained in vessel studies on piglet vascular smooth muscle. We believe that adrenergic agonists may have a dual mechanism of action: the first one consists of a catecholamine action on its receptor with the formation of a stimulus-receptor complex. The second mechanism proposed completes the first one. By this second component of the mechanism, the same stimulus directly acts on the carbonic anhydrase I isozyme (that might be functionally coupled with adrenergic receptors), so that its activation ensures an adequate pH for stimulus-receptor coupling for signal transduction into the cell, resulting in vasoconstriction.

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- Arterial blood pressure

Introduction

Catecholamines released by the sympathetic nervous system and adrenal medulla are involved in regulating a host of physiological functions, particularly the integration of responses to a range of stresses (1). Norepinephrine is the major neurotransmitter in the peripheral sympathetic nervous system, whereas epinephrine is the primary hormone secreted by the adrenal medulla in mammals (2).

Important factors in the response of any cell or organ to sympathomimetic amines are the density and proportion of alpha- and beta-adrenergic receptors (3,4). Studies on DNA cloning have demonstrated the existence of at least nine types of adrenergic receptors (5). Other studies have proved that binding of agonists to these receptors occurs when the catecholamine molecule is in the protonated state (6), all of these receptors being coupled with G proteins.

Carbonic anhydrase (CA) is a zinc-enzyme discovered by Meldrum and Roughton in 1932 which catalyzes the reversible hydration reac-

tion of CO_2, having a main role in the maintenance of acid-basic equilibrium (7).

$$H_2O + CO_2 \overset{CA}{\Leftrightarrow} H_2CO_3 \overset{CA}{\Leftrightarrow} H^+ + HCO_3^-$$

Eight isozymes have been described so far, located in the membranes, cytoplasm and mitochondria of all organs. CA I is present both in erythrocytes and in vascular walls and its physiologic role has been incompletely elucidated (8). CA II is to be found both in erythrocytes and in the cytoplasm. By its presence in the parietal cells of the gastric mucosa, it has a central role in HCl production, while in the kidney CA II is involved in the maintenance of urinary pH along with CA IV.

Regarding the physiological role of CA isozymes, our studies have shown that CA I is involved in the vascular changes (9) while CA II and CA IV are isozymes involved in the secretory processes (10).

The same studies showed that CA I and CA II are activated by nonsteroidal antiinflammatory drugs (11) and vasodilating prostaglandins and diuretic agents (12,13) inhibit CA while vasoconstrictive prostaglandins activate the enzyme (12). These studies have also demonstrated the involvement of carbonic anhydrase in the regulation of vascular and secretory processes in the organism (14).

Our previous work has shown that alpha- and beta-adrenergic agonists activated purified and red cell CA while adrenergic antagonists inhibited CA and reduced the activating effect of agonists on this enzyme (15,16).

In the present investigation we studied the relationship between alpha- and beta-adrenergic agonists and CA activity in vasoconstriction mechanism.

Material and Methods

Material

Purified human erythrocyte CA I and CA II, adrenaline, noradrenaline, isoprenaline, orciprenaline, HEPES buffer, p-nitrophenol, and Na_2SO_4 were obtained from Sigma Chemical Co. (Deisenhofen, Germany), orciprenaline (Alupent) was purchased from Boehringer (Ingelheim, Germany), and noradrenaline and isoprenaline (vials) were obtained from Sicomed (Bucharest, Romania).

Experimental designs

Erythrocyte studies. We studied the effects of adrenaline, noradrenaline, isoprenaline and orciprenaline on CA I and CA II purified from human erythrocytes. Kinetic determinations were performed at concentrations between 10 nM and 100 µM.

Kinetic studies were carried out in order to identify the mechanism of action of adrenergic agonists on CA. Maximum reaction rate (V_{max}) and the Michaelis constant (K_m) were determined.

Clinical 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 approved by the Ethics Committee of the Center for Research and Medical Assistance in Simleu Silvaniei.

We selected 42 healthy male volunteers aged 30 to 50 years and weighing 60-74 kg, who were randomly divided into three groups. All subjects resided in the community and were in good general health. Subjects were screened before participation by being submitted to physical examination, a complete blood count, fasting serum glucose, and routine chemistry, urinalysis, and electrocardiogram and their medical history was taken. Patients were excluded from participation if they exceeded 135% of ideal body weight, had a past history of hypertension, diabetes mellitus, had a fasting serum glucose of >6.7 mM, were taking any medications, had ortho-

static hypotension, or had evidence from the screening tests of underlying illness or significant laboratory or electrocardiogram abnormalities. In the acute experiment, group I patients (N = 12) received noradrenaline *iv* at the dose of 4 mg/1000 ml isotonic solution (4 µg/min over 30-min periods), group II patients (N = 14) received orciprenaline (Alupent) *iv* at the dose of 0.5 mg, and group III patients (N = 16) received isoprenaline (Isoprenalin) *sc* at the dose of 0.2 mg.

Red cell CA I and CA II activity and arterial blood pressure values were determined before and 30 min after drug administration and the blood count, routine chemistry, urinalysis and electrocardiogram were repeated.

Vessel studies. In the animal experiments, 20 piglets weighing 25-30 kg were housed in air-conditioned quarters and had free access to tap water and standard food. Animals were divided into 4 groups of 5 piglets each and treated as follows in the acute experiment: group 1 - noradrenaline, *iv* doses of 2 µg/min for 30 min; group 2 - orciprenaline (Alupent), *iv* doses of 0.25 mg; group 3 - isoprenaline (Isoprenalin), *sc* doses of 0.1 mg, and group 4 (control group) - placebo.

Arterial blood pressure was determined 30 min after drug administration and all piglets were sacrificed for isolation of vascular smooth muscle CA I. CA I activity was determined and compared to that obtained for the control group.

Experimental procedure

Differentiation of red cell CA I from CA II activity was performed by the nicotinate test (17), which relies on selective inhibition of CA I activity.

Vascular smooth muscle CA I was isolated from the small mesenteric arteries of the animals according to the technique of Lonnerholm et al. (8).

CA I and CA II activity was assessed using the stopped-flow method (18), which consists of measuring the enzymatic activity of CO_2 hydration and is based on a colorimetric method which measures changing pH. The time needed for the pH of the reagent mixture to decrease from its initial value of 7.5 to its final value of 6.5 was measured. The reaction was monitored spectrophotometrically at 400 nm using a rapid kinetic Hi-Tech SF-51MX spectrophotometer (Hi-Tech Scientific Ltd., Salisbury, England) equipped with a mixing unit and a system of two syringes which supply the reagents. The signal transmitted by the photomultiplier from the mixing chamber is received and visualized by a computer equipped with a mathematical coprocessor and the kinetic software package RKBIN IS1.

We used p-nitrophenol (0.2 mM) as color indicator and HEPES (20 mM) as buffer. Na_2SO_4 (0.1 M) was used to keep a constant ionic strength. The CO_2 solution at a concentration of 15 mM (as substrate) was obtained by bubbling twice-distilled water with CO_2 to saturation. All reagents were maintained at pH 7.5 and at room temperature (22-25°C).

Carbonic anhydrase activity was obtained by the formula:

$$A = \frac{T_0 - T}{T} \text{ [enzyme units/ml]}$$

where T_0 represents the uncatalyzed reaction time, and T the catalyzed reaction time (in the presence of CA). Activity is reported as enzyme units (EU) per ml.

In the CO_2 hydration reaction catalyzed by CA one enzyme unit will cause the pH to drop from 7.5 to 6.5 per minute, at 25°C.

In humans, blood pressure was measured with a standard mercury sphygmomanometer in the classical sitting position and is reported as the mean of three measurements.

In piglets, blood pressure was measured under anesthesia by intraperitoneal injection of pentobarbital sodium (35 mg/kg body weight) and catheterization of the femoral artery.

Statistical analysis

When repeated measure ANOVA showed significant differences between groups, the Newman-Keuls multiple comparison test was performed to determine which groups differed significantly. Probabilities of $P<0.05$ were considered significant.

Results

Erythrocyte studies

Adrenaline, noradrenaline, isoprenaline and orciprenaline increased CA I and CA II activity in a dose-dependent manner. The effect started at 10 nM and reached a peak at 100 µM (Table 1).

Adrenaline increased CA I activity from 0.425 ± 0.01 to 0.743 ± 0.02 EU/ml (75%) ($P<0.001$), and CA II activity from 1.00 ± 0.01 to 1.472 ± 0.02 EU/ml (47%) ($P<0.001$).

Noradrenaline increased CA I activity from 0.425 ± 0.01 to 0.714 ± 0.01 EU/ml (68%) ($P<0.001$), and CA II activity from 1.00 ± 0.01 to 1.436 ± 0.02 EU/ml (43%) ($P<0.001$).

Isoprenaline increased CA I activity from 0.425 ± 0.01 to 0.658 ± 0.02 EU/ml (55%) ($P<0.001$), and CA II activity from 1.00 ± 0.01 to 1.342 ± 0.01 EU/ml (34%) ($P<0.001$).

Orciprenaline increased CA I activity from 0.425 ± 0.01 to 0.688 ± 0.02 EU/ml (62%) ($P<0.001$), and CA II activity from 1.00 ± 0.01 to 1.488 ± 0.01 EU/ml (49%) ($P<0.001$).

The kinetic data processed according to the Michaelis-Menten equation showed a non-competitive mechanism of action with an increase in V_{max} and a constant K_m. The kinetic results show that adrenergic agonists were bound to the active site of CA I in a position different from that of the enzyme substrate, CO_2 (Table 2).

Table 1 - Effect of adrenergic agonists on isozyme I and II of carbonic anhydrase (CA).

The table shows the increase of the activity of CA isozymes induced by therapeutic agents such as adrenaline, noradrenaline, isoprenaline and orciprenaline. Values are reported as means ± SEM (N = 5 assessments) *P<0.05 compared with basal activity for each isozyme (Newman-Keuls multiple comparison test).

Substance	Concentration	Purified CA I (basal activity = 0.425 ± 0.01 EU/ml)	Purified CA II (basal activity = 1.00 ± 0.01 EU/ml)
Adrenaline	10 nM	0.519 ± 0.01*	1.194 ± 0.01*
	1 µM	0.638 ± 0.01*	1.341 ± 0.02*
	100 µM	0.743 ± 0.02*	1.472 ± 0.02*
Noradrenaline	10 nM	0.472 ± 0.02*	1.168 ± 0.02*
	1 µM	0.603 ± 0.01*	1.305 ± 0.03*
	100 µM	0.714 ± 0.01*	1.436 ± 0.02*
Isoprenaline	10 nM	0.468 ± 0.01*	1.082 ± 0.01*
	1 µM	0.595 ± 0.02*	1.258 ± 0.02*
	100 µM	0.658 ± 0.02*	1.342 ± 0.01*
Orciprenaline	10 nM	0.476 ± 0.03*	1.203 ± 0.01*
	1 µM	0.611 ± 0.01*	1.376 ± 0.02*
	100 µM	0.688 ± 0.02*	1.488 ± 0.01*

Table 2 - Kinetic data for interaction between alpha- and beta-adrenergic agonists and purified carbonic anhydrase (CA) I.

CA I concentration = 3.68 x 10 nM, pH 7.5, T = 25ºC. Data are reported as means ± SD (N = 5 assessments). *P<0.05 compared to purified CA I (Newman-Keuls multiple comparison test).

System	V_{max} (mM s^{-1})	K_m (mM)
CA I	1.332 ± 0.01	8.99 ± 0.2
CA I + adrenaline (100 µM)	1.796 ± 0.02*	8.87 ± 0.1
CA I + noradrenaline (100 µM)	1.733 ± 0.01*	8.91 ± 0.1
CA I + isoprenaline (100 µM)	1.710 ± 0.02*	8.95 ± 0.2
CA I + orciprenaline (100 µM)	1.742 ± 0.02*	8.89 ± 0.1

Clinical studies

In group 1, noradrenaline increased red cell CA I activity from 0.268 ± 0.026 to 0.501 ± 0.042 EU/ml (87%) ($P<0.001$) and CA II activity from 1.081 ± 0.116 to 1.598 ± 0.134 EU/ml (48%) ($P<0.05$) (Figure 1). Arterial blood pressure rose from 130 ± 10 to 175 ± 15 mmHg ($P<0.05$) (Figure 2).

In group 2, orciprenaline increased red cell CA I activity from 0.251 ± 0.030 to 0.429 ± 0.027 EU/ml (71%) ($P<0.001$) and CA II activity from 1.135 ± 0.110 to 1.288 ± 0.194 EU/ml (27%) ($P<0.05$) (Figure 1). Arterial blood pressure rose from 120 ± 10 to 155 ± 5 mmHg ($P<0.05$) (Figure 2).

In group 3, isoprenaline increased red cell CA I activity from 0.247 ± 0.015 to 0.450 ± 0.020 EU/ml (82%) (P<0.001) and CA II activity from 0.983 ± 0.105 to 1.474 ± 0.208 EU/ml (50%) (P<0.05) (Figure 1). Arterial blood pressure rose from 120 ± 10 to 160 ± 5 mmHg (P<0.05) (Figure 2).

Vessel studies

In animals, after the acute experiment the activity of vascular smooth muscle CA I was 0.812 ± 0.062 EU/ml and arterial blood pressure was 120 ± 10 mmHg in the control group. In all adrenergic-treated groups, vascular smooth muscle CA I activity and arterial blood pressure increased significantly (P<0.05) compared to controls, as follows: group 1 - CA I was 1.696 ± 0.124 EU/ml and arterial blood pressure was 180 ± 15 mmHg; group 2 - CA I was 1.408 ± 0.136 EU/ml and arterial blood pressure was 165 ± 10 mmHg; group 3 - CA I was 1.519 ± 0.143 EU/ml and arterial blood pressure was 170 ± 10 mmHg (Figure 3).

Discussion

Our group has studied the relationship between adrenergic agonists and CA (15,16), showing that CA was activated by a direct mechanism. The erythrocyte studies proved that alpha- and beta-adrenergic agonists are direct and strong CA I activators which have less effect on CA II.

Clinical and vessel studies have shown that adrenergic agonists are powerful CA I activators both in erythrocytes and in vascular smooth muscles. In humans, noradrenaline, orciprenaline and isoprenaline increased arterial blood pressure in volunteer subjects, in parallel with an increase of erythrocyte CA I activity. Parallelism between the increase in arterial blood pressure and erythrocyte CA I activation was observed in all groups. The most potent activating effect on CA was induced by noradrenaline which also produced the main increase in blood pressure. None of these patients presented any major side effects during the experiments.

In animals, administration of adrenergic agonists significantly increased CA activity, mainly of CA I in arteriolar smooth muscle as compared to controls, in parallel with an increase in arterial blood pressure.

These results agree with previous studies by our group which showed that CA I is involved in the modulation of vascular pro-

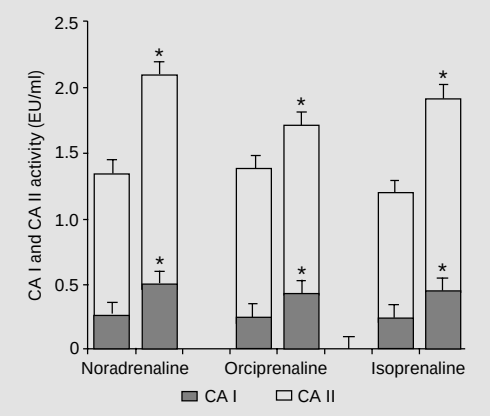

Figure 1 - Effect of noradrenaline (4 µg/min, over 30 min, *iv*), orciprenaline (0.5 mg, *iv*) and isoprenaline (0.2 mg, *sc*) on red blood cell CA I and CA II activity. Values are reported as means ± SEM; N = 12-16 patients. *P<0.05 compared with values before treatment (paired *t*-test).

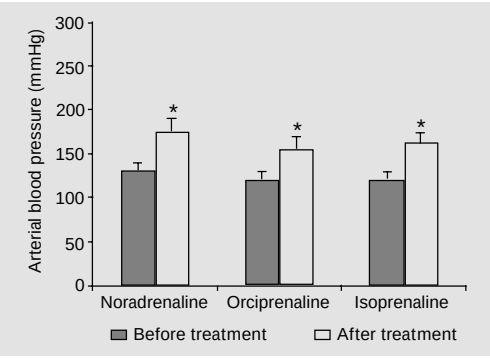

Figure 2 - Effect of noradrenaline (4 µg/min, over 30 min, *iv*), orciprenaline (0.5 mg, *iv*) and isoprenaline (0.2 mg, *sc*) on arterial blood pressure. Values are reported as means ± SEM; N = 12-16 patients. *P<0.05 compared with values before treatment (paired *t*-test).

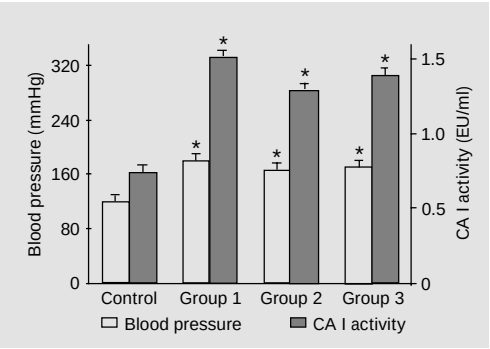

Figure 3 - Increase of vascular smooth muscle CA I activity and of arterial blood pressure values in piglets after treatment with noradrenaline (group 1), orciprenaline (group 2) and isoprenaline (group 3) compared to control. Values are reported as means ± SEM; N = 5 piglets. *P<0.05 compared with control (paired *t*-test).

cesses in the organism. In our conception, the pH increase induced by CA I inhibition might influence the binding of hypotensive stimuli to their specific receptors, followed by signal transduction to the cytoplasm of smooth muscle cells with subsequent vasodilating effects (9,14). Similarly, the reduction in pH induced by vascular smooth muscle CA I activation with hypertensive agents may influence the membrane specific receptor and signal transduction in the vascular smooth muscle cytosol, with subsequent vasoconstrictive effects (14).

Regarding the role of pH changes in hypertension, our results support recent studies which have shown that primary hypertension may be associated with perturbations of acid-base status or intracellular pH, respectively (19,20). Furthermore, the same studies demonstrated a decrease of intracellular pH in hypertensive animal models as compared to normotensive animals (19,21). An evaluation of steady-state intracellular pH in erythrocytes using a nuclear magnetic resonance technique indicated that intracellular pH is reduced in erythrocytes from untreated patients with essential hypertension compared to treated patients and normotensive controls (22).

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 (23).

An enhanced activity of the H^+-Na^+ antiporter (as a major mechanism of cell defense against cellular acidification) has been reported in lymphocytes (24) as well as in the renal brush border membrane of spontaneously hypertensive rats (25) and in hypertensive rats (26). Other authors have shown enhanced responsiveness of the renal proximal Na^+-H^+ antiporter of hypertensive rats to stimulation with some hormones (27). It has been assumed that an overactivity of this antiporter is not a primary process but rather reflects intracellular acidosis in hypertension, as studied in spontaneously hypertensive rat models (20,21).

Catecholamine-induced CA I activation has suggested a concept concerning the involvement of pH changes (induced by activation) in vasoconstrictive processes due to adrenergic agonists. In keeping with this concept, catecholamines have a dual mechanism of action: the first consists of adrenergic agonists acting on their specific receptors with subsequent formation of a stimulus-receptor complex, followed by the coupling of G proteins and information transmission within the cell. The second mechanism suggests that catecholamines directly act on CA I (an isozyme that might be functionally coupled with adrenergic receptors), which by its activation accompanied by a fall in pH may be favorable to catecholamine-binding to its specific receptor and by G proteins to facilitate information transmission into the cell.

Our results suggest that CA I modulates vascular tone by means of pH changes (14). This role for pH in the vascular bed is supported by our data as follows: a) the already known role of CA I in acid-base balance; b) the effect of vasoconstrictive substances that activate erythrocyte CA I and vascular smooth muscle CA I by a direct mechanism of action, and c) the effect of vasodilatory substances along with drugs used in the treatment of hypertension which inhibit CA I by a direct mechanism of action both in erythrocytes and in vascular smooth muscle.

Our results suggest that CA I activation decreases intracellular pH while its inhibition increases it. These changes in intracellular pH might influence ion channel activity, as well as symport and antiport pump and ATPase activity, all being involved in the modulation of vascular processes.

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