Isosorbide Nitrates, Nitroglycerin, and Sodium Nitroprusside Induce Vasodilation Concomitantly With Inhibition of Carbonic Anhydrase I in Erythrocytes

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This study describes the relationship between nitroglycerin, isosorbide dinitrate, sodium nitroprusside, and carbonic anhydrase I, as well as the involvement of this carbonic anhydrase I in vasodilation. Two groups of coronary patients and a group of rabbits underwent treatment with the above-mentioned vasodilating drugs. The activity of red blood cell carbonic anhydrase was monitored and determined by the stopped-flow method. The results show that these drugs inhibit the activity of the isozyme in parallel to their vasodilating effect. The results of this study lead to the hypothesis that through the pH modifications induced by these vasodilators by nitroprusside, and carbonic anhydrase I, the isozyme may be involved in the regulation of vascular tonus.

KEY WORDS: Organic nitrates, nitric oxide, carbonic anhydrase, vasodilatation, pH, isosorbide dinitrate, sodium nitroprusside, nitroglycerin, rabbit.

Discovered in 1932, carbonic anhydrase (CA) is a zinc-containing enzyme, which catalyzes the reversible reaction of CO₂ hydration, with production of H⁺ and HCO₃⁻, holding a central role in maintaining the acid-base equilibrium in the organism. Eight isozymes have been described so far, located in membranes, cytoplasm, mitochondria, etc. CA I is present both in erythrocytes and in the vascular walls; its physiologic role is incompletely elucidated. CA II is to be found both in erythrocytes and in the cytoplasm; through its presence in the parietal cells of the gastric mucosa, it has a central role in HCl production, while in the kidney it is involved in the maintenance of urinary pH, along with CA IV. Isosorbide nitrates (ISDN), nitroglycerin (NG), and sodium nitroprusside (SNP) are substances with vasodilating effects, known and successfully used in the treatment of coronary disorders and arterial hypertension.

The vasodilating action of these agents has been proved to be initiated by nitric oxide (NO) production. The differences between the vasodilating actions of these drugs consist in the ways in which NO is released, ie, spontaneously in the case of SNP and by bioactivation and metabolic conversion in the case of organic nitrates.

Our studies have shown that vasoconstrictive substances, such as F₂α prostaglandins (PGF₂α), B₄ and C₄ leukotrienes, catecholamines, angiotensin II, vasopressin and l-arginine analogs (N⁶-monomethyl-l-arginine and N⁷-nitro-l-arginine methyl ester), stimulate the activity of red blood cell CA I, while drugs...
possessing vasodilating effects, such as adrenergic antagonists, prostaglandins (PGs) E₂ and I₂, the inhibitors of the conversion enzyme, NO, and calcium channel blockers, reduce the activity of this isozyme. Considering the role of CA in maintaining the acid-base equilibrium, we formulated the hypothesis that the pH modifications induced through the activation or inhibition of CA I by these vasoconstrictive and vasodilating drugs might have a bearing on vascular tone.

Studies carried out so far have shown that there is a relationship between intracellular pH and human and experimental hypertension; other studies have also demonstrated the involvement of intracellular pH in the regulation of the tonus of smooth vascular muscles.

In this paper we are studying the relationship between NG, ISDN, SNP, and CA I, an isozyme present both in erythrocytes and in the vascular endothelium. The modifications of CA I activity in red cells followed by us in this study will reflect the modifications of the same isozyme in the vascular endothelium.

**MATERIALS AND METHODS**

This study was carried out in vivo, in three experimental groups. Group I (N = 59) consisted of patients with coronary affections who were given glycerol trinitrate (Trinitrosan, Merck, Darmstadt, Germany) in single intravenous doses of 5 mg/day on two consecutive days. Red cell CA I and CA II activity was determined every day from blood collected before and 30 min after the administration of glycerol trinitrate. Group II (N = 55) consisted of coronary patients who received ISDN in oral doses of 180 mg/day, administered at 12-h intervals (90 mg at a time), on 3 consecutive days. Red cell CA I and CA II activity was determined every day, before and 2 h after drug administration. Group III (N = 12) consisted of rabbits that were given 60 µg/min SNP by endovenous infusion over 10 min periods. The blood samples for the determination of red cell CA I and CA II activity were collected before treatment, after 5 and 10 min of infusion, and 30 min after discontinuing treatment.

The study was carried out according to international ethical standards.

Methyl nicotinate at a concentration of $5 \times 10^{-4}$ mol/L was added to every blood sample in which CA activity was determined. This was done in order to distinguish red cell CA I from CA II activity and to obtain a separate value for each CA isozyme.

CA activity was determined by the stopped-flow method. Determinations were carried out with a rapid kinetics HI-TECH SF-51 MX apparatus (HI-TECH Scientific Ltd., Salisbury, Wiltshire, England), the following reagents being used: 0.2 mmol/L p-nitrophenol as pH indicator, 20 mmol/L HEPES buffer, and 15 mmol/L CO₂ solution. Assays were carried out spectrophotometrically at 400 nm, T = 25°C, and pH = 7.5.

Data were analyzed statistically by Student’s t test.

Glycerol trinitrate (Trinitrosan) was obtained from Merck; isosorbide dinitrate (Isoket) from Schwarz Pharma AG (Monheim, Germany); sodium nitroprusside (Nipride) from Hoffman La Roche (Switzerland); and p-nitrophenol and HEPES buffer from Sigma (Deisenhofen, Germany).

**RESULTS**

These results are summarized in Table 1 and Figure 1.

**Group I** NG reduced total red cell CA activity by 40% on the first day and by 51% after 2 days of treatment. CA I activity decreased by 86% after the first day and was abolished (100% inhibition) after the second day of treatment. CA II activity was reduced by 14% after the first day and by 25% after the second day of treatment.

NG predominantly inhibited CA I, the inhibition being complete after 2 days of treatment. CA I inhibition was six times more powerful than CA II inhibition.

**Group II** ISDN reduced total red cell CA activity by 57% after 3 days of treatment. CA I activity was reduced by 90% after 2 days and by 100% after the third day of treatment. CA II activity decreased by 30% after 3 days of treatment.

Treatment with ISDN progressively reduced CA activity in red blood cells. Inhibition of CA I was five times more powerful than inhibition of CA II. Inhibition of CA I was progressive and became complete after 3 days of treatment with ISDN.

**Group III** SNP reduced total CA activity in the red blood cells of rabbits; the decrease was progressive over the whole period of treatment. After 10 min of infusion, inhibition of total CA reached 60%, CA I being completely inhibited (100%), while CA II activity decreased by 48%; 30 min after discontinuing treatment, CA II was still inhibited by 27%, CA I by 30%.

Acute administration of SNP to rabbits induced complete inhibition of red cell CA I (100%) after 10 min of drug infusion. After discontinuing treatment with SNP, CA I activity recovered sooner than CA II activity.

**DISCUSSION**

The results obtained in this experiment show that NG, ISDN, and SNP induce strong and progressive inhibition of red cell CA I, an isozyme also present in vascular walls. The presence of the same isozyme, CA I, both in erythrocytes and in vascular walls leads to the assumption that the modifications of its activity occur in parallel in both locations, which allows us to use the erythrocyte activity of CA I (which can easily be
TABLE 1. RED BLOOD CELL CA ACTIVITY INHIBITION AFTER TREATMENT WITH NITROGLYCERIN (GROUP I), ISOSORBIDE DINITRATE (GROUP II), AND SODIUM NITROPRUSSIDE (GROUP III)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Determinations</th>
<th>Total CA</th>
<th>CAI</th>
<th>CAII</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Nitroglycerin</td>
<td>After first day</td>
<td>−40%</td>
<td>−86%</td>
<td>−14%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After second day</td>
<td>−51%</td>
<td>−100%</td>
<td>−25%</td>
</tr>
<tr>
<td>II</td>
<td>Isosorbide dinitrate</td>
<td>After first day</td>
<td>−12%</td>
<td>−37%</td>
<td>−10%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After second day</td>
<td>−33%</td>
<td>−90%</td>
<td>−19%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After third day</td>
<td>−57%</td>
<td>−100%</td>
<td>−30%</td>
</tr>
<tr>
<td>III</td>
<td>Sodium nitroprusside</td>
<td>During 5 min</td>
<td>−45%</td>
<td>−75%</td>
<td>−39%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>During 10 min</td>
<td>−60%</td>
<td>−100%</td>
<td>−48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After 30 min</td>
<td>−36%</td>
<td>−30%</td>
<td>−27%</td>
</tr>
</tbody>
</table>

determined) as an indicator for CA I activity changes in vascular walls.

The vasodilating mechanism of organic nitrates, such as NG and ISDN, described so far consists in generating NO by metabolic conversion mediated by free thiols or by an enzymatic mechanism such as is achieved by glutathione-S-transferase or the P-450 cytochrome. Unlike the vasodilating effects of these organic nitrates, that of SNP is very powerful and rapid and is achieved by spontaneous release of NO.

These results show that, along with the vasodilating effects of the three substances studied here, there also occurs a parallel inhibition of CA I. This inhibition is progressive over the duration of the treatment, CA I activity being completely inhibited after 2 or 3 days of treatment, respectively, with NG and ISDN. After administration of SNP, whose effect sets in immediately, CA I activity is abolished within a 10 min infusion, but, after discontinuing treatment, the activity of the enzyme soon and gradually returns to initial values. Other studies performed here show that the rapid return of CA I activity to initial values after discontinuing treatment with SNP is parallel to the return of arterial hypertension to its initial values after the same therapy. Consequently, our results suggest that the NO released from NG, ISDN and SNP is responsible for the inhibition of CA I.

Our previous studies have in fact shown that NO is an inhibitor of CA I by a direct mechanism of action, and that other vasodilators, substances such as PGE₂, PG₁₂, adrenergic receptor antagonists, nicotinates, calcium channel blockers, thiazidic diuretics, amiloride, and triamterene, also inhibit CA I along with vasodilation.

At the same time, vasoconstrictors, such as L-arginine analogs, PG₂, thromboxane A₂, leukotrienes B₄ and C₄, catecholamines, angiotensin II, and vasopressin, will activate CA I; the vasoconstrictive effects again occur in parallel with CA I activation.

Since the decrease of CA I activity accompanies the vasodilating effects of substances with various chemical structures, it leads to the hypothesis that CA I is involved in the modulation of vascular tonus. This hypothesis is supported by the increase of CA I activity induced by vasoconstrictive substances with various chemical structures that activate the same isozyme in parallel with vasoconstriction.

Our hypothesis is strengthened by other studies that show that acetazolamide, known as a specific inhibitor of CA, doubtlessly possesses cerebral vasodilating effects.
properties, as well as the hypotensive properties described by us.\(^5\)

As far as the mechanism of action of CA is concerned, its involvement in the modulation of the intracellular pH in vascular walls has been demonstrated.\(^3\) The stimulation of CA activity by vasoconstrictive substances induces a decrease of pH by excess of H\(^+</\); the opposite phenomenon induced by vasodilating drugs has also been described.\(^5\)

This hypothesis is also supported by recent studies carried out by a highly prestigious team who reported that the blood pressure lowering effects of calcium channel blockade were inversely related to intracellular pH—ie, the lower the initial pH, the greater the antihypertensive effect. Furthermore, nifedipine consistently elevated intracellular pH values.\(^3\)

The literature data prove more and more the role of intracellular pH in the regulation of vascular smooth muscle. An evaluation of steady-state intracellular pH in erythrocytes, using a nuclear magnetic resonance technique, has indicated that intracellular pH is reduced (by about 0.1 pH unit) in erythrocytes from untreated patients with essential hypertension compared to treated patients and normotensive controls.\(^6\) The same authors\(^6\) also found that the intracellular pH of erythrocytes from rats with different experimental forms of hypertension was reduced as related to normotensive control rats.

Other studies show that the intracellular control of pH in vascular smooth muscle cells is also modified. This might be important for the pathogenesis of hypertension by affecting either vascular smooth muscle tone or vascular smooth muscle growth.\(^7\) It was also proved that there is a relationship between hypertension and abnormalities in transport and intracellular concentrations of a variety of ions. The hydrogen ion attracts interest in hypertension because plasma pH affects peripheral resistance (vascular smooth muscle tone).\(^5\)

In vascular smooth muscles, intracellular pH is rapidly modified, within the first 35 sec approximately, under the impact of extracellular pH changes.\(^3\) Previous studies showed that modification of extracellular pH by one unit induces modifications of intracellular pH by 0.2 to 0.4 units. Consequently a slight modification of plasmatic pH will affect intracellular pH and, as a consequence, vascular tonus.

Studies concerning intracellular pH show that the action of primary extracellular messengers on secondary messengers (cyclic nucleotide and calcium) is a pH-dependent process. Thus a rise of intracellular pH is accompanied by activation of adenylyl cyclase and guanylate cyclase, with production of cAMP and cGMP. The decrease of intracellular pH was considered responsible for the reduction of adenylyl and guanylate cyclase activity, and, consequently, for the reduction of cAMP and cGMP production.\(^6\) Intracellular Ca modifications are also influenced by intracellular pH. A summary of the data in the medical literature thus shows that arterial hypertension can be associated with anomalies in the regulation of intracellular pH.

According to our hypothesis, vasodilating substances induce inhibition of CA I, which, through the modifications of intracellular pH CA I causes by reducing H\(^+</\)> concentration, could influence the vasodilation process. This hypothesis maintains that vasodilating stimuli and NO, respectively, possess dual and direct mechanism of action both on soluble guanylate cyclase\(^3\) and on CA I, the latter being followed by a rise of intracellular pH with repercussions on the ligand-receptor complex, the G proteins, and the effector system. Consequently CA is not a mere catalyst of CO\(_2\) hydration, but, through the pH changes induced by its inhibition by means of various endogenous and exogenous vasodilating agents, might modulate the physiologic and pathologic vascular processes in the organism. The activation of CA I by vasoconstrictors, as described by us,\(^5\) supports the hypothesis concerning the involvement of this isozyme in the regulation of vascular tonus.

Further research work is required to confirm this hypothesis and to assess the quantitative relations between CA I inhibition, intracellular pH modifications, and the production of cGMP.

REFERENCES

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