Corpus Cavernosum from Men with Vasculogenic Impotence Is Partially Resistant to Adenosine Relaxation due to Endothelial A$_{2B}$ Receptor Dysfunction

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ABSTRACT

Although adenosine has been implicated in penile erection in human males, the receptor subtype responsible for adenosine regulation of human corpus cavernosum (HCC) smooth muscle tone is still a matter of debate. Using selective adenosine agonists and antagonists, we aimed at characterizing the adenosine receptors mediating relaxation of precontracted (with 1 μM phenylephrine) HCC strips. HCC specimens were collected from control subjects (organ donors) and from patients with severe vasculogenic erectile dysfunction (ED). In control subjects, adenosine and 5′-N-ethyl-carboxamide adenosine (NECA) fully relaxed HCC. The selective A$_{2A}$ receptor agonist 2-[4-(2-p-carboxy ethyl)phenylamino]-5′-N-ethylcarboxamido adenosine (CGS21680C) produced only a partial relaxation (30–50%) of HCC, which could be further enhanced by simultaneous application of 100 μM NECA. The selective A$_{2B}$ receptor antagonist N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-il)phenoxy] acetamida (MRS1706) (10 nM) attenuated NECA-induced relaxation with-out affecting CGS21680C action. The A$_{2A}$ receptor antagonist 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl}phenol (ZM241385) (50 nM) consistently reduced the actions of both agonists. In contrast to CGS21680C, NECA-induced relaxation was attenuated when endothelial production of NO and prostanoids was reduced by 100 μM N^G-nitro-L-arginine and 10 μM indomethacin, respectively. HCC strips from patients with vasculogenic ED were partially resistant to NECA but kept relaxation to CGS21680C; the remaining effect was sensitive to blockade of A$_{2A}$ receptors with 50 nM ZM241385. Data suggest that adenosine regulates HCC smooth muscle tone through the activation of two receptor populations, CGS21680C-sensitive (A$_{2A}$) and -insensitive (A$_{2B}$) receptors, located on smooth muscle fibers and on endothelial cells, respectively. Endothelial dysfunction may be correlated with a loss of adenosine A$_{2B}$ receptor activity in penile vessels from men with vasculogenic ED.

It is known that purinergic transmission is important for initiation and maintenance of penile erection (Tong et al., 1992). Due to its short half-life, adenosine has been used as an agent for the diagnosis of vasculogenic impotence (Kilic et al., 1994) without interfering with systemic blood pressure (Takahashi et al., 1992). Extracellular adenosine may be released from a variety of cells as result of increased meta-bolic rates or decreased oxygen supply (Tabrizchi and Bedi, 2001) in parallel with the formation of adenosine from the hydrolysis of released ATP through the ectonucleotidase pathway (Zimmermann, 2000). The typical vascular response to adenosine in many vascular beds is vasodilatation, although vasoconstriction may also be seen depending on which type of adenosine receptor is activated (Tabrizchi and Bedi, 2001).

Four subtypes of membrane-bound adenosine receptors (A$_1$, A$_{2A}$, A$_{2B}$, and A$_3$) have been identified by convergent data from molecular, biochemical, and pharmacological studies (Fredholm et al., 2001). Direct effects on blood vessel tone via adenosine actions on A$_1$ receptors are rare and most

ABBREVIATIONS: CGS21680C, 2-[4-(2-p-carboxy ethyl)phenylamino]-5′-N-ethylcarboxamido adenosine; NECA, 5′-N-ethylcarboxamide adenosine; MRS1706, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-il)phenoxy] acetamida; HCC, human corpus cavernosum; ED, erectile dysfunction; PE, phenylephrine; ACh, acetylcholine; ZM241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl}phenol; L-NOARG, N^G-nitro-L-arginine; SIN-1, amino-3-morpholinyl-1,2,3-oxadyazolio.

frequently mediate vasoconstriction. The adenosine A_3 receptors are expressed in human vascular smooth muscle cells mediating rapidly desensitizing focal vasoconstriction. It is becoming increasingly clear that A_3 receptors are not involved in the relaxation of isolated blood vessels (Tabrizchi and Bedi, 2001). The A_2 receptors are coupled to G_s proteins, and their activation results in the stimulation of adenylate cyclase and thus vasorelaxation. A_2 receptors are further subdivided into structurally different high-affinity A_{2A} and low-affinity A_{2B} receptor subtypes. The adenosine analog CGS21680C has only very low affinity at the human A_{2B} receptor and is used extensively to discriminate between A_{2A} (K_i of 27 nM) and A_{2B} (K_i of 88,000 nM) subtypes in humans (Klotz, 2000). No potent and selective A_{2B} receptor agonist has been reported so far; 5'-N-ethylcarboxamide adenosine (NECA) is currently the most potent agonist at A_{2B} receptor, having low micromolar affinity (IC_{50} = 330-3100 nM) (Klotz, 2000). The xanthine amide derivative MRS1706 antagonizes human A_{2B} receptors with a K_i value of 1.39 nM and is 81-fold selective versus human A_{2A} receptors (Kim et al., 2000). This compound has been instrumental to determine A_{2B} receptor contribution in tissues with a mixed population of receptors.

The receptor subtype responsible for adenosine regulation of human cavernosal smooth muscle tone is still a matter of debate. For example, A_{2A} receptors might contribute to adenosine-induced relaxation of human and rabbit corpus cavernosum (Filippi et al., 2000), but these authors failed to induce erection by intracavernous injection of adenosine in human volunteers. In this study, we aimed at characterizing the receptor subtype underlying adenosine actions on human corpus cavernosum (HCC) in control subjects (organ donors) and in patients with severe vasculogenic erectile dysfunction (ED), because this condition is the most common cause of failure to achieve erections with usual vasodilators and of patients referred for surgical treatment.

Endothelial dysfunction is a milestone in the pathophysiology of both erectile dysfunction and cardiovascular disease, the two entities sharing a high degree of interdependence concerning severity (Thompson et al., 2005). Adenosine and endothelium-derived NO and prostacyclin are important local mediators of vasodilatation. In several human vascular beds, adenosine A_2 receptors mediate vasodilatation in part through endothelium, possibly by releasing NO (Sobrevia et al., 1997; Li et al., 1998) and prostacyclin (Chiang et al., 1994; Donoso et al., 2005). Other studies, however, failed to demonstrate endothelium-dependent responses in the presence of adenosine (Sabouni et al., 1990; Tsai et al., 1996; Kemp and Cocks, 1999). Controversy also exists on the receptor subtype (A_{2A} or A_{2B}) predominating on endothelial cells (Chiang et al., 1994; Iwamoto et al., 1994; Sobrevia et al., 1997; Li et al., 1998; Donoso et al., 2005). Since little is known about the adenosine receptor present on endothelial cells of HCC and the impairment of endothelium function may be one of the factors for the attenuation of adenosine receptor-mediated responses, we thought it would be interesting to explore adenosine-induced relaxation of HCC smooth muscle in control subjects after inhibiting endothelial production of NO and prostacyclin compared with patients with vasculogenic impotence exhibiting multiple risk factors for endothelial lesion (e.g., type II diabetes, hypercholesterolemia, hypertension, heavy smoking habits). Another impor-

tant feature of adenosine signaling is its extensive inactivation by cellular uptake and extracellular deamination in most vascular beds, particularly by endothelial cells. Therefore, we also investigated the kinetics of adenosine inactivation in HCC strips to probe its relevance for erectile dysfunction in patients with diffuse vascular disease.

Materials and Methods

HCC Tissues. HCC specimens were obtained from control subjects (bodies donated for harvesting organs, 18–50 years) and from patients suffering from severe vasculogenic ED (48–58 years) at the time of penile prosthesis insertion. All the patients were informed of procedures and signed their written consent. The protocol was approved by the Ethics Committee of Hospital Geral de Santo Antó-nio–SA (University Hospital) and of Instituto de Ciências Biomédi-cas de Abel Salazar (Medical School) of the University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki. Tissues were maintained at 4–6°C in M-400 transplantation solution not supplemented with ATP or adenosine (4.190 g/100 ml mannitol, 0.205 g/100 ml KH_2PO_4, 0.970 g/100 ml $K_2HPO_4 \cdot 3H_2O$, 0.112 g/100 ml KCl, and 0.084 g/100 ml $NaHCO_3$, pH 7.4) until used, which was between 2 and 16 h after extraction (Simonsen et al., 1997).

Relaxation of HCC Strips. Longitudinal strips of corpus cavernosum tissue ($3 \times 3 \times 7$ mm) were mounted in 12-ml organ chambers containing oxygenated (95% O_2, 5% CO_2, pH 7.4) Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 11.2 mM glucose, and 100 mM ascorbic acid) at 37°C. HCC strips were connected to isometric force transducers (Hugo-Sachs, Hugstetten, Germany), and changes in tension were recorded continuously by using either a polygraph linearcorder (Hugo-Sachs) or a PowerLab data acquisition system (Chart 5, version 4.2; AD Instruments, Colorado Springs, CO). Tissues were preloaded with 2 g of tension and allowed to equilibrate for 90 min in Tyrode's solution. Each strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1 μM phenylephrine (PE) (Azadzoi et al., 1992). After standardization, relaxation to 1 to 10 μM acetylcholine (ACh) was taken as a measure of endothelium integrity. To evaluate tissue relaxant responsiveness, HCC strips were contracted with 1 μM PE, and once a stable contraction was achieved (15–20 min) the strips were challenged with cumulative additions of adenosine and its stable analogs NECA or CGS21680C to the chambers by transferring the inlet tube of the peristaltic pump from one flask to another. In some of the experiments, HCC strips were pretreated with adenosine receptor antagonists (MRS1706 and ZM241385) or with inhibitors of NO synthase (L-NOARG) and inhibitors of cyclooxygenase (indomethacin). These compounds were tested directly on basal tone and on 1 μM PE-contracted strips to assess any contractile/relaxation response, and they were added to the organ bath at least 15 min before adenosine receptor agonists. Adenosine receptor antagonists did not change constriction of HCC strips to 1 μM PE, implying that endogenous adenosine production was irrelevant under these experimental conditions. Thus, adenosine deaminase was not required to eliminate the effects of endogenous adenosine when testing the action of the stable adenosine analogs.

Adenosine Inactivation in HCC Strips. To study the kinetics of adenosine inactivation in HCC taken from control subjects and from patients with vasculogenic ED, longitudinal strips ($3 \times 3 \times 7$ mm) were mounted in 1.5-ml organ baths containing oxygenated (95% O_2, 5% CO_2; pH 7.4) Tyrode's solution at 37°C. After a 30-min equilibration period, the preparations were incubated with 30 μM adenosine (zero time). Samples of 75 μl were collected from the organ bath at different times up to 45 min for high-performance liquid chromatography (L-6200 Intelligent pump with an L-4000 UV detector; Hitachi, Sachen, Germany) analysis of the variation of substrate

disappearance and product formation (Magalhães-Cardoso et al., 2003). Concentrations of the substrate and products were plotted as a function of time (progress curves). The spontaneous degradation of adenosine at 37°C in the absence of the preparation was negligible (0–5%) over 45 min. At the end of experiments, the remaining incubation medium was collected and used to quantify the lactate dehydrogenase (EC 1.1.1.27) activity. The negligible (0.56 ± 0.04 U/ml; n = 7) activity of lactate dehydrogenase in bath samples collected at the end of the experiments is an indication of the integrity of the cells during the experimental procedure.

Materials and Solutions. Adenosine, ADP, AMP, ATP, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid (indomethacin), L-PE hydrochloride, hypoxanthine, inosine, NECA, and L-NO-ARG were from (Sigma-Aldrich, St. Louis, MO). CGS21680C was from Sigma/RBI (Natick, MA). Amino-3-morpholinyl-1,2,3-oxadyazolio, MRS1706, and ZM241385 were from Tocris Cookson Inc. (Bristol, UK). MRS1706 and ZM241385 were made up in 5 mM stock solutions in dimethyl sulfoxide, NECA was made up in a 10 mM stock solution in dimethyl sulfoxide, and indomethacin was made up in a 10 mM stock solution in ethanol. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily, and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used [0.5% (v/v)], were observed. The pH of the superfusion solution did not change by the addition of the drugs in the maximum concentrations applied to the preparations.

Presentation of Data and Statistical Analysis. The data are expressed as mean ± S.E.M. from an n number of individuals. At least four strips were used for each experiment. The responses are expressed as percentage of 1 μM phenylephrine contractions. For multiple comparisons, results were analyzed by analysis of variance followed by the Bonferroni's post test. For comparison between two values, the unpaired Student's t test was used. A value of $P < 0.05$ was considered significant.

Results

Pharmacological Characterization of Adenosine Receptors in HCC. Cumulative application of 1 to 1000 μM adenosine concentration dependently relaxed precontracted (with 1 μM PE) HCC strips from control subjects (Fig. 1, a and b); a maximal relaxation effect of about 80% was obtained with 300 μM adenosine. Although maximal relaxation of HCC strips achieved with 0.1 to 300 μM NECA was similar to that caused by adenosine (IC$_{50}$ of ~30 μM), the nonselective A$_2$ receptor agonist exhibited a highest potency (IC$_{50}$ of ~3 μM) (Fig. 1a). Surprisingly, the selective A$_{2A}$ receptor agonist CGS 21680C (0.001–10 μM) caused only a partial relaxation of HCC strips (IC$_{50}$ of ~0.03 μM), which was not greater than 30 to 50% of the maximal isometric contraction produced by 1 μM PE (Fig. 1, a and c). As illustrated in Fig. 1c, 100 μM NECA was still able to cause relaxation of HCC strips in the presence of CGS 21680C, when this drug was used in a 10 μM concentration high enough to saturate adenosine A$_{2A}$ receptors (Fig. 1a).

Preincubation with the selective adenosine A$_{2B}$ receptor antagonist MRS1706 (10 nM) (Kim et al., 2000) shifted the NECA (0.1–300 μM) concentration-response curve to the right, i.e., the IC$_{50}$ values for relaxation of HCC strips calculated for NECA increased from 3 to 300 μM in the presence of 10 nM MRS1706 (Fig. 2a). Preincubation with 10 nM MRS1706 did not significantly ($P > 0.05$) modify relaxation of HCC strips by 0.001 to 10 μM CGS21680C (Fig. 2b). This contrasted with that detected in the presence of the preferential A$_{2A}$ receptor antagonist ZM241385 (50 nM) (Poucher et al., 1995) (Fig. 2d). Preincubation of HCC strips with 50 nM ZM241385 shifted the concentration-response curves for both agonists to the right, NECA (0.1–300 μM) and CGS21680C (0.001–10 μM); under these circumstances, the IC$_{50}$ values for the two agonists increased to 80 and 3 μM, respectively. None of the adenosine receptor antagonists significantly changed ($P > 0.05$) contractile responses of HCC strips to 1 μM PE, implying that endogenous adenosine was not importantly produced. These results suggest that human penile vessels possess two populations of adenosine receptors, A$_{2A}$ and A$_{2B}$, acting complementarily to cause smooth muscle relaxation.

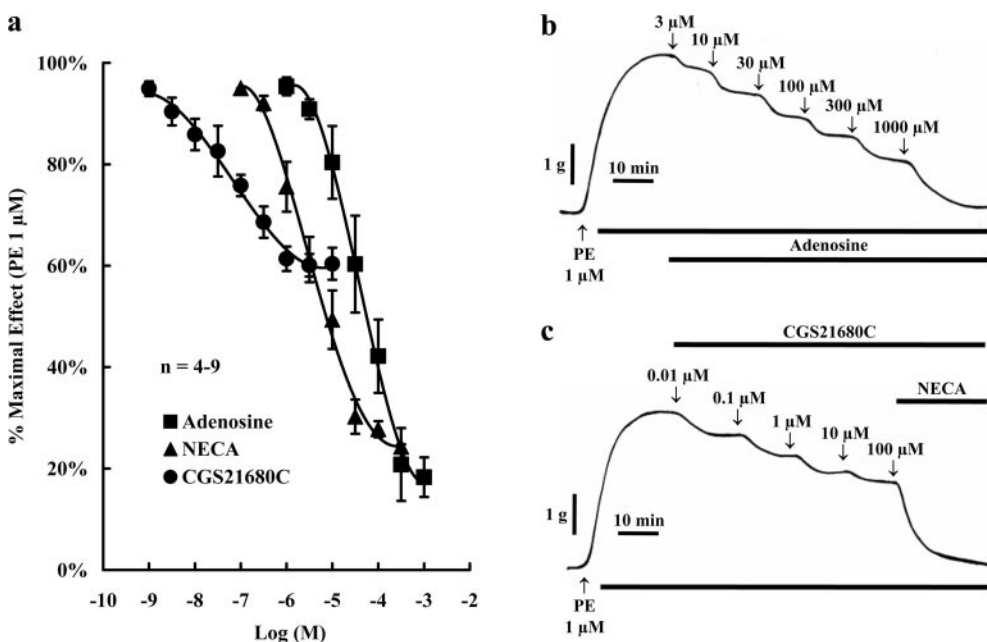**Role of the Endothelium in Adenosine-Induced Relaxation of HCC.** At the beginning of each experiment,

Fig. 1. Relaxation of HCC strips by 1 to 1000 μM adenosine and its stable analogs NECA (0.1–300 μM) and CGS 21680C (0.001–10 μM). Tissue samples were collected from control subjects. a, ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent ± S.E.M. from an n number of separated human specimens (four strips for each experiment). b and c, typical recording traces of relaxation of HCC strips precontracted with 1 μM PE by cumulative application of adenosine (b) and its stable analogs (c) to the incubation fluid.
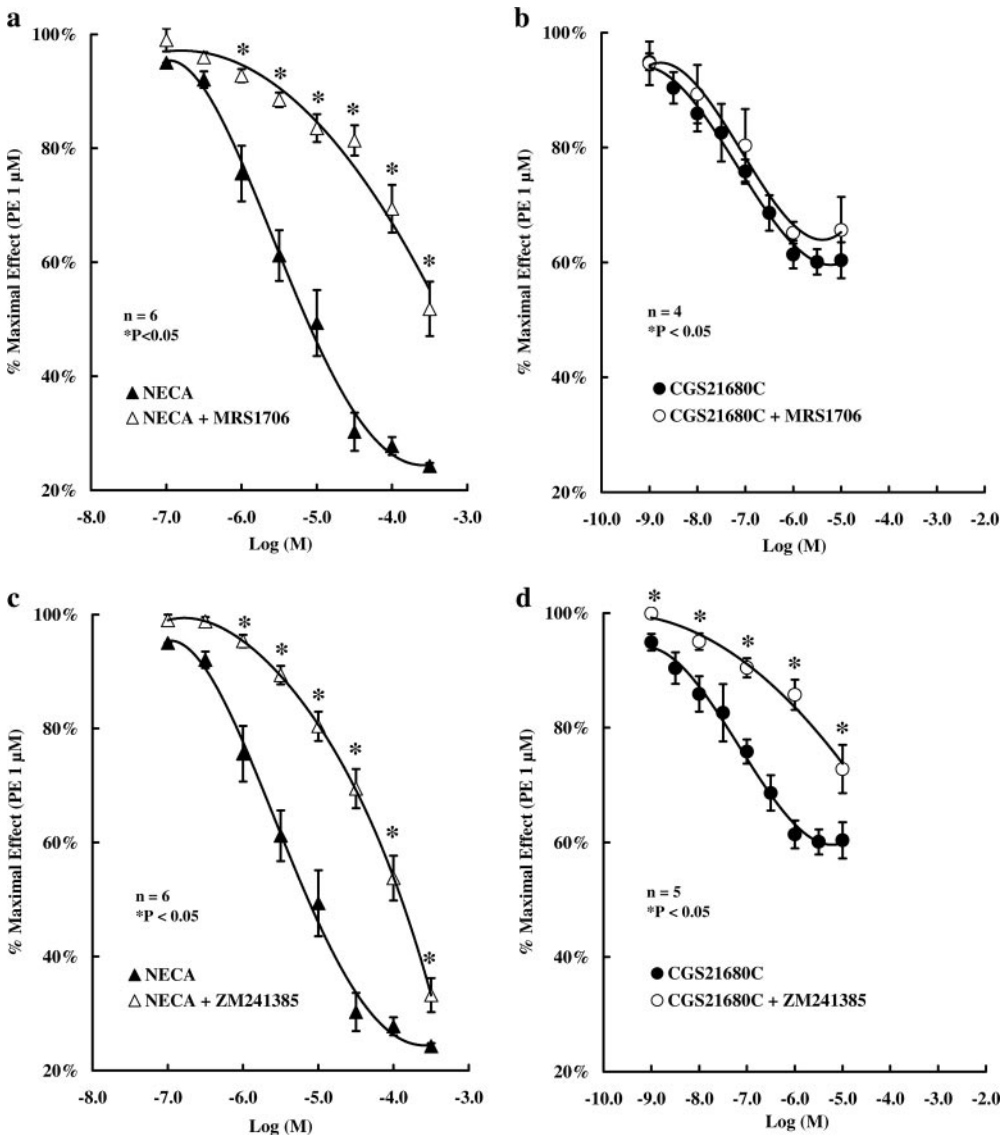
Fig. 2. Concentration-response curves of 0.1 to 300 μM NECA and 0.001 to 10 μM CGS21680C in HCC strips in the absence and in the presence of selective adenosine A_{2A} (50 nM ZM241385) and A_{2B} (10 nM MRS1706) receptor antagonists. Tissue samples were collected from control individuals. Adenosine receptor antagonists were added to the incubation fluid 15 min before application of NECA or CGS21680C. The ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent $\pm$ S.E.M. from an n number of separated human specimens (at least four strips for each experiment). *, $P < 0.05$ compared with the effect of NECA (a and c) and CGS21680C (b and d) in the absence of adenosine receptor antagonists MRS1706 and ZM241385.

endothelium integrity was evaluated by testing relaxation of precontracted HCC strips to ACh. Table 1 shows that HCC strips from control individuals relaxed to 1 to 10 μM ACh in a concentration-dependent manner. Inhibition of NO syn-

TABLE 1
Relaxation of precontracted (with 1 μM PE) HCC strips from control subjects and from patients with vasculogenic ED to 1 to 10 μM ACh: the role of NO production

The NO synthase inhibitor L-NOARG (100 μM) was added to the incubation media 15 min before application of ACh. Values are expressed as percentage $\pm$ S.E.M. of maximal contraction caused by 1 μM PE. The number of separated human specimens (four strips for each experiment) is shown in parentheses.

	Control Individuals	Patients with Vasculogenic ED
	%	
No L-NOARG (100 μM)		
+ ACh (1 μM)	53 $\pm$ 8 (14)	81 $\pm$ 6 (8)*
+ ACh (10 μM)	43 $\pm$ 6 (13)	67 $\pm$ 5 (8)*
With L-NOARG (100 μM)		
+ ACh (1 μM)	92 $\pm$ 2 (5)*	N.T.
+ ACh (10 μM)	77 $\pm$ 6 (5)*	N.T.

N.T., not tested.
* $P < 0.05$ compared with the effect of ACh in control subjects in the absence of L-NOARG.

thase with 100 μM L-NOARG attenuated 1 to 10 μM ACh-induced relaxation of HCC strips (Table 1), without significantly ($P > 0.05$) affecting relaxation caused by the NO donor amino-3-morpholinyl-1,2,3-oxadyazolio (SIN-1; 10–30 μM; data not shown). Preincubation with 100 μM L-NOARG shifted the concentration-response curve of NECA to the right (0.3–300 μM; IC_{50} of ~20 μM) (Fig. 3a), with this effect being more evident as the NECA concentration increased above the micromolar range. In contrast, 100 μM L-NOARG was without effect on the ability of 0.001 to 10 μM CGS21680C to relax precontracted HCC strips (Fig. 3b). It is worth noting that 100 μM L-NOARG, like the cyclooxygenase inhibitor indomethacin (10 μM), increased contractile responses to 1 μM PE by 30 $\pm$ 4 ($n = 4$) and 25 $\pm$ 4% ($n = 4$), respectively, and their effects were additive (66 $\pm$ 8%; $n = 6$). Nevertheless, attenuation of the relaxing effect of 0.3 to 300 μM NECA in the presence of 100 μM L-NOARG plus 10 μM indomethacin was not higher than that observed when HCC strips were pretreated with each compound alone (Figs. 3a and 4). The results suggest that endothelium-derived NO and prostacyclin are important mediators of the CGS 21680C-insensitive aden-
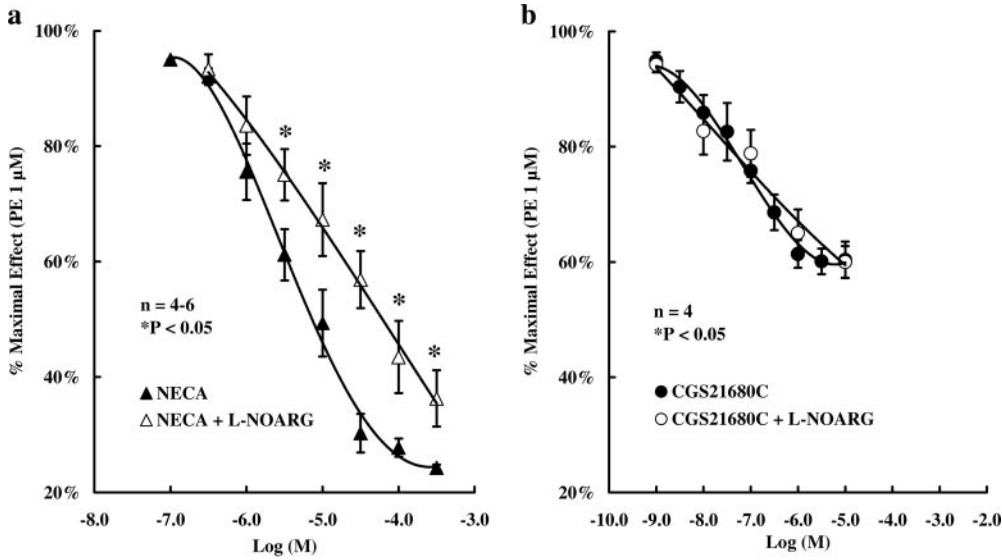
Fig. 3. Concentration-response curves of 0.1 to 300 μM NECA and 0.001 to 10 μM CGS21680C in the absence and in the presence of the NO synthase inhibitor L-NOARG (100 μM). HCC strips were collected from control subjects. L-NOARG was added to the incubation fluid 15 min before application of NECA or CGS21680C. The ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent $\pm$ S.E.M. from an n number of separated human specimens (at least four strips for each experiment). *, $P < 0.05$ compared with the effects of NECA (a) and CGS21680C (b) in the absence of L-NOARG.

osine relaxation of HCC smooth muscle, probably due to activation of the low-affinity A$_{2B}$ receptors on endothelial cells.

Relaxation of Cavernosal Tissue from Men with Vasculogenic ED to Adenosine Receptor Agonists. Patients included in this study possess the most severe forms of ED, requiring surgical penile implantation, because they did not respond to common vasodilators (e.g., sildenafil and alprostadil) and exhibited multiple risk factors for endothelial lesion (e.g., type II diabetes, hypercholesterolemia, hypertension, and heavy smoking habits). Their clinical status was assessed by hemodynamic studies. Endothelial dysfunction was confirmed in vitro at the beginning of each experiment by a significant ($P < 0.05$) reduction to relaxation induced by

ACh, even when this drug was used in high concentrations (10 μM) (Table 1). In some experiments, 10 μM ACh was also tested after application of adenosine receptor agonists for proving its inability to increase relaxation of HCC strips beyond that previously caused by activating adenosine receptors (Fig. 5).

In contrast with the findings obtained in control individuals, 0.1 to 300 μM NECA-induced relaxations of HCC strips from patients with vasculogenic ED were incomplete, i.e., maximal relaxations produced by NECA in these patients did not surpass 30 to 50% of full contractions caused by 1 μM PE (Fig. 6a). Moreover, the effect of 0.1 to 300 μM NECA was not modified after preincubation of HCC strips from patients with vasculogenic ED with the selective A$_{2B}$ receptor antagonist MRS1706 (10 nM) (Fig. 7a). There were no significant ($P > 0.05$) differences between the concentration-response curves for the effect of 0.001 to 10 μM CGS21680C in HCC strips from control subjects and from patients with vasculogenic ED (Fig. 6b), but the selective A$_{2A}$ receptor antagonist ZM241385 (50 nM) was still able to attenuate 0.001 to 10 μM
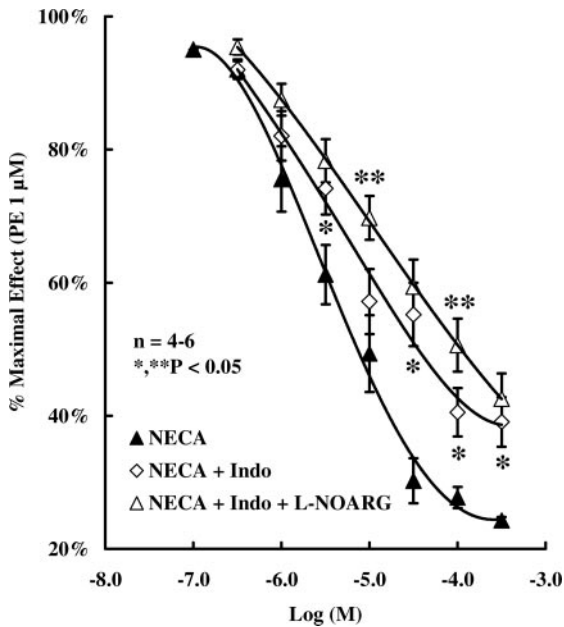

Fig. 4. Effect of cyclooxygenase inhibition with 10 μM indomethacin on the concentration-response curve of 0.1 to 300 μM NECA. HCC strips were collected from control subjects. Indomethacin (with or without L-NOARG) was added to the incubation fluid 15 min before application of NECA. The ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent $\pm$ S.E.M. from an n number of separated human specimens (at least four strips for each experiment). *, $P < 0.05$ compared with the effect of NECA in control conditions.
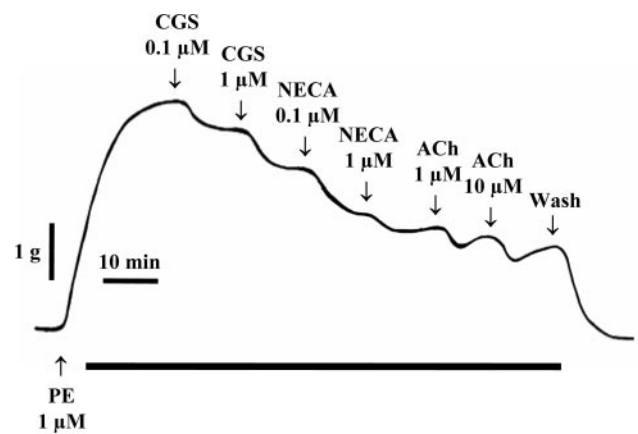
Fig. 5. Representative recordings of the relaxation of HCC strips precontracted with 1 μM PE by cumulative application of 0.1 and 1 μM CGS21680C, 0.1 and 1 μM NECA, and 1 and 10 μM ACh. The tissue sample was collected during surgical implantation of penile prosthesis from a 49-year-old patient (RMJ) with vasculogenic ED. RMJ was a heavy smoker ($\pm$80 cigarettes/day) and had a clinical history of noncontrolled chronic hypertension, type II diabetes (with 3 years of evolution with metformin), hypercholesterolemia (medicated with lovastatin), and severe iliac artery disease.

Fig. 6. Concentration-response curves of 0.1 to 300 μM NECA and 0.001 to 10 μM CGS21680C in HCC strips obtained from control subjects and from vasculogenic ED patients. The ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent ± S.E.M. from an n number of separated human specimens (at least four strips for each experiment). ∗, $P < 0.05$ compared with the effects of NECA (a) and CGS21680C (b) in control subjects.
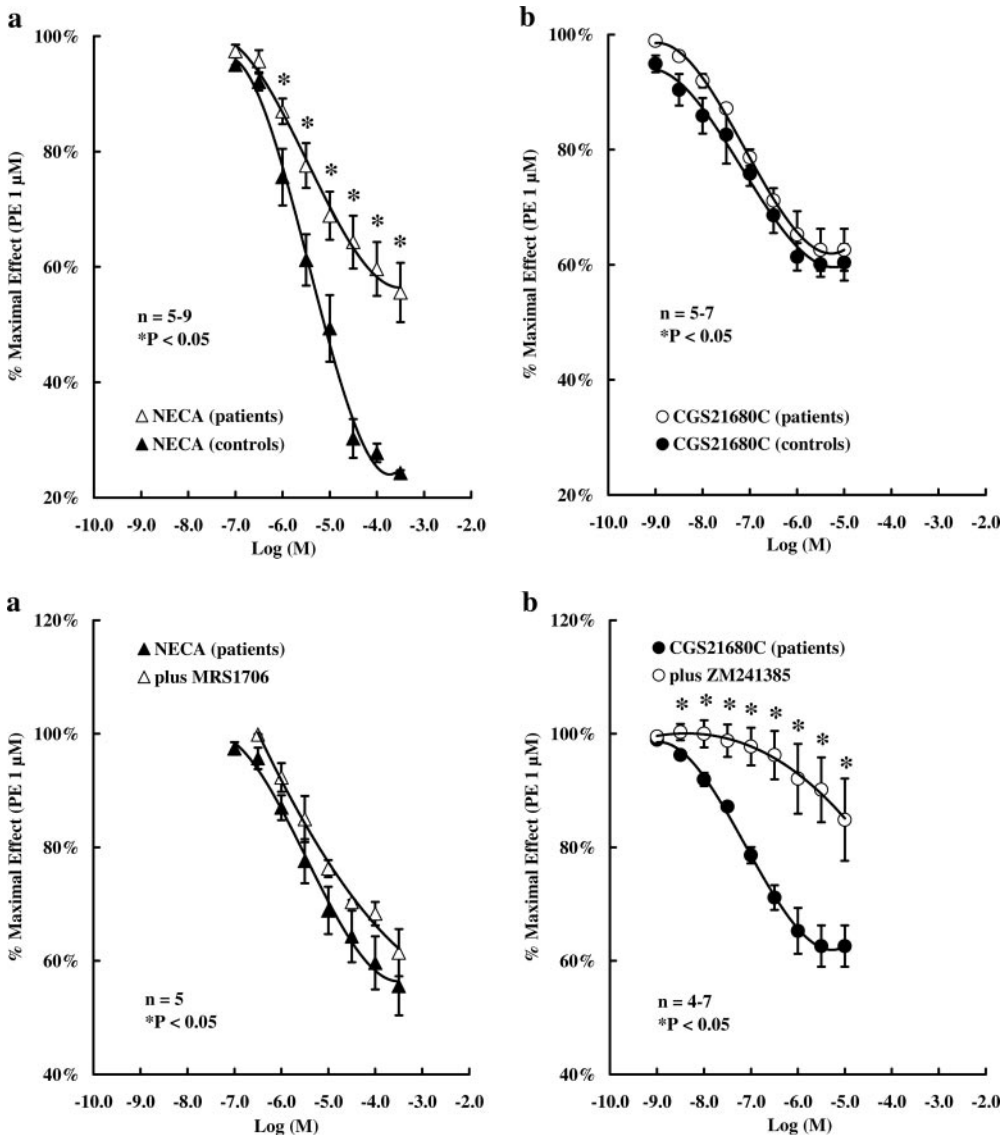
Fig. 7. Adenosine-induced relaxation of precontracted HCC strips from patients with vasculogenic ED is attenuated by blocking adenosine A_{2A} receptors but not the A_{2B} receptors. ZM241385 (50 nM; A_{2A} receptor antagonist) and MRS1706 (10 nM; A_{2B} receptor antagonist) were added to the incubation fluid 15 min before application of 0.1 to 300 μM NECA (a) and 0.001 and 10 μM CGS21680C (b), respectively. The ordinates are percentage of maximal contraction produced by 1 μM PE. The vertical bars represent ± S.E.M. from an n number of separated human specimens (at least four strips for each experiment). ∗, $P < 0.05$ compared with the effect of NECA (a) and CGS21680C (b) in the absence of adenosine receptor antagonists.

CGS21680C-induced relaxation of HCC strips from patients with vasculogenic ED (Fig. 7b). It is also worth noting that maximal relaxations produced by 0.1 to 300 μM NECA and 0.001 to 10 μM CGS21680C were of a similar magnitude if one considers the samples from patients with vasculogenic ED. This contrasts with that observed in control subjects where NECA had about twice the efficacy of CGS21680C (Fig. 6).

For comparison, we also tested the effects of adenosine and its stable analogs NECA and CGS 21680C on HCC strips from two patients with nonvasculogenic ED. Viable cavernosal tissue samples were obtained either during partial amputation of the penis due to in situ carcinoma (JPF, 52 years, history of traumatic paraplegia with 15 years of evolution) or during penile prosthesis insertion using the same surgical procedure as in the other patients with vasculogenic ED in a patient (MJSG, 58 years) submitted to surgical ablation of the prostate gland 4 years before. The two patients responded to local application of vasodilators, and endothelial integrity was confirmed in vitro by a positive relaxation to 1 to 10 μM ACh. The efficacy profile of the adenosine receptor agonists was similar to that observed in HCC strips from control subjects (data not shown).

Kinetics of Adenosine Inactivation in HCC Tissue. The progress curves of 30 μM adenosine disappearance in HCC strips from control subjects and from vasculogenic ED patients are represented in Fig. 8. The results show that extracellular adenosine is slowly inactivated in HCC compared with other tissues (e.g., human urinary bladder) (Faria et al., 2005). There were no significant ($P > 0.05$) differences between the rate of adenosine inactivation and metabolites (inosine and hypoxanthine) formation in the two groups of samples (Fig. 8, a and b). Forty-five minutes after 30 μM adenosine application, the concentration of the nucleoside in the incubation fluid was 26.76 ± 1.42 and 27.78 ± 2.13 μM, respectively, in samples from control subjects and from vasculogenic ED patients, whereas inosine concentrations in the two groups were 3.49 ± 0.94 and 3.50 ± 1.00 μM, respectively. The absence of AMP formation from adenosine suggests that no extracellular adenosine kinase (EC 2.7.1.20) activity is present in HCC.

Discussion

This study is pioneering by the use of selective adenosine agonists and antagonists for the characterization of adeno-
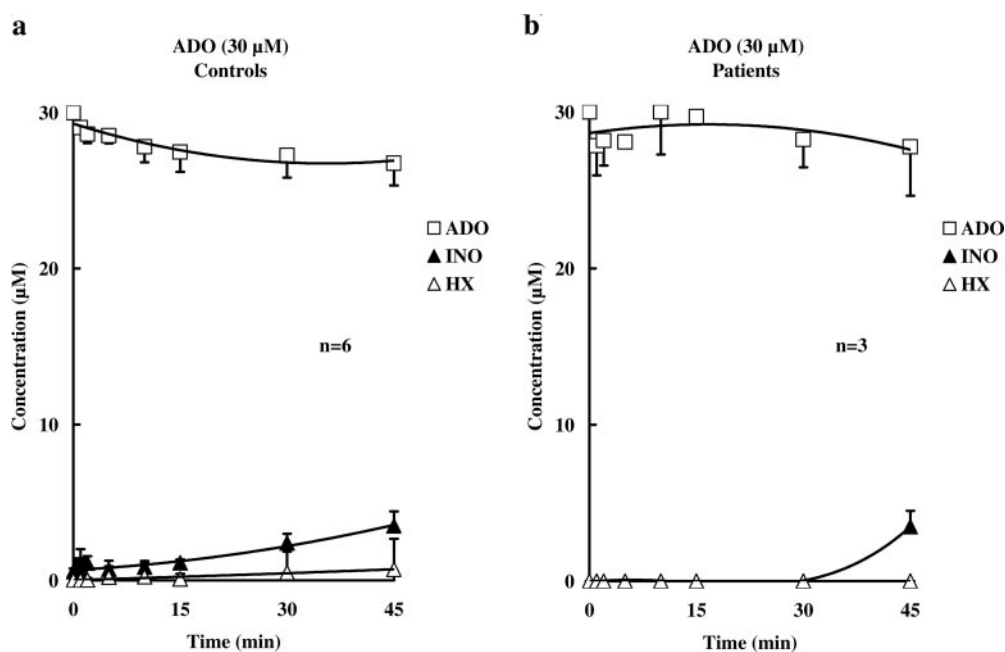
Fig. 8. Extracellular inactivation of 30 μM adenosine in HCC strips obtained from control subjects (a) and from patients with vasculogenic ED (b). Adenosine (30 μM) was added at zero time to the preparations. Bath samples were collected at the times indicated on the abscissa, for high-performance liquid chromatography analysis to separate and quantify the substrate and its metabolites. The adenosine metabolites were mainly inosine (INO) and a negligible amount of hypoxanthine (HX). The data are means $\pm$ S.E.M. of an n number of separated human specimens.

sine receptors in the human cavernosal tissue. The agonist efficacy profile to cause relaxation of HCC strips from control subjects was NECA > adenosine $\gg$ CGS21680C. NECA and adenosine relaxed HCC strips by about 80% of the maximal contractile response to PE, whereas CGS21680C produced only a 30 to 50% relaxation. Suspicion of the existence of two populations of adenosine receptors acting cooperatively to cause relaxation of HCC strips was further confirmed by the additive effects of NECA and CGS21680C, when the latter compound was used in a concentration (10 μM) high enough to saturate A$_{2A}$ receptors. Moreover, blockade of A$_{2B}$ receptors with 10 nM MRS1706 shifted the concentration-response curve of NECA to the right, without significantly modifying CGS21680C relaxation. The nonxanthine antagonist ZM241385 (50 nM), exhibiting a high affinity for the A$_{2A}$ receptor (pK_B of 9) (Poucher et al., 1995), attenuated the relaxing effects of NECA and CGS21680C, because it has only 22-fold selectivity for human A$_{2A}$ over A$_{2B}$ receptors (Linden et al., 1999). As far as we know, this is the first study to suggest the involvement of two subtypes of adenosine receptors, high-affinity A$_{2A}$ and low-affinity A$_{2B}$, in the relaxation of cavernosal vessels of the human penis. Receptor heterogeneity might explain some controversies found in the literature concerning the efficacy profile of nonselective adenosine agonists (e.g., NECA) on erectile tissues of human and other species (Mantelli et al., 1995; Filippi et al., 2000; Andersson, 2001; Noto et al., 2001).

The etiology of vasculogenic ED has been associated to reduced NO formation either from nonadrenergic noncholinergic nerve fibers or from endothelial cells of cavernosal vessels (Gonzalez-Cadavid et al., 1999; Cashen et al., 2002). Penile smooth muscle relaxation produced by some endogenous mediators, such as adenosine via A$_{2B}$ receptors (Chiang et al., 1994), is operated by endothelial NO formation triggered by activation of NO synthase secondary to phosphorylation by protein kinase A or to increases in intracellular Ca^{2+}. In this context, we investigated whether adenosine-induced relaxation of HCC strips could be mediated by NO production and what would be the receptor subtype involved.

Data suggest that activation of A$_{2B}$ receptors, which are insensitive to CGS 21680C, requires endothelial NO production to cause relaxation of HCC strips. Adenosine may also relax HCC strips acting directly through A$_{2A}$ receptors on smooth muscle fibers in an NO-independent manner, like that observed in the rabbit penile vessels (Mantelli et al., 1995) and in the anesthetized dog (Noto et al., 2001).

HCC tissue can synthesize prostanoids (for review, see Andersson, 2001; Jeremy et al., 1986), and their actions may be impaired in tissues previously treated with the cyclooxygenase inhibitor indomethacin (10 μM) (Angulo et al., 2002). Prostanoids are involved in the regulation of penile smooth muscle contractility by way of specific receptors. Thromboxane A$_2$ (TP) receptors mediate contraction, whereas relaxation of HCC strips and human penile resistant arteries are mediated by prostaglandin E$_2$ (EP) and/or prostacyclin (IP) receptors, respectively. Enhancement of HCC strips contractility from control individuals to 10 μM indomethacin suggests a high output of relaxant prostanoids in this tissue, whereas the synthesis of contractile prostanoids (thromboxane A$_2$ and prostaglandin F$_{2\alpha}$) might not play a relevant role (Angulo et al., 2002). Although cases of indomethacin-associated sexual dysfunction have been reported previously (Miller et al., 1989), treatment with nonsteroidal anti-inflammatory drugs is not as commonly implicated in ED as might be predicted. This discrepancy may result from increased production of endogenous prostanoids when oxygen tension in the buffer solution is near saturation (95% O$_2$) (Daley et al., 1996). Our results showed that, although A$_{2B}$ receptor-mediated relaxation can be partially dependent on the production of NO and prostanoids by the endothelium, inhibition of the two pathways with 100 μM L-NOARG plus 10 μM indomethacin did not further reduce NECA activity, because they might be mutually exclusive. Hemodynamic studies in men showed that penile tumescence due to intracavernosal application of prostaglandin E$_1$ for treating impotence could be enhanced by adenosine (Chiang et al., 1994), since the nucleoside acts synchronously via smooth muscle fibers and endothelial cells.

Pharmacological heterogeneity and differential distribution of adenosine receptors through distinct layers of the penile vessel wall prompted us to investigate adenosine-induced relaxation in HCC strips from patients with ED presenting multiple risk factors for endothelial lesion. HCC strips from vasculogenic ED patients were partially resistant to NECA but kept relaxation to CGS21680C, and they were sensitive to blockade by the A_{2A} antagonist ZM241385 (50 nM). In contrast to control subjects, maximal relaxation by NECA had a similar magnitude (30–50%) to that caused by the selective A_{2A} receptor agonist CGS21680C. Although the A_{2B} receptor antagonist MRS1706 (10 nM) antagonized NECA-induced relaxation in HCC strips from control subjects, it was devoid of effect on tissues isolated from patients with vasculogenic ED. Likewise, Chiang et al. (1994) showed that adenosine action (probably via A_{2B} receptors) was greater in intact than in endothelium-denuded rabbit corpora cavernosa, suggesting the involvement of an endothelium-derived relaxing factor. The ability of adenosine analogs to cause relaxation of HCC strips from age-matched patients with nonvasculogenic ED was also evaluated, but their effects were not significantly different from controls. These results constitute the first experimental evidence of a close relationship between endothelial dysfunction and the loss of A_{2B} receptors activity in penile vessels from vasculogenic ED patients. Whether this reflects a reduction of A_{2B} receptor binding sites or results from impairment of distal effectors pathways needs further investigation. Dysfunctional blood vessels can alter the balance between relaxant and constrictor actions of endogenous prostanoids (Azadzoi et al., 1998; Behr-Roussel et al., 2003). This, together with the reduced NO-mediated component, might explain the difficulty in relaxing HCC strips from vasculogenic ED patients in response to NECA and ACh. Maintenance of relaxation to CGS21680C opens new perspectives for the pharmacological management of severe forms of vasculogenic ED. Targeting adenosine A_{2A} receptors located on the smooth muscle layer may be an interesting strategy to overcome resistance to common vasodilators (e.g., sildenafil and alprostadil) requiring NO production by intact endothelial cells (Rosen and Kostis, 2003).

Extracellular inactivation by cellular uptake and extracellular deamination may restrict adenosine actions to the release/production sites and may limit diffusion of the exogenously added nucleoside toward the receptor zones (Daly, 1982; Duarte-Araújo et al., 2004). This could be one of the factors for the limited time window of adenosine responses in corpora cavernosa and for the lack of systemic blood pressure repercussions following intracavernous injection of the nucleoside. Because we hypothesized that human penile vessels possess two subtypes of adenosine receptors acting cooperatively to regulate smooth muscle tone, a high-affinity receptor (A_{2A}) located on smooth muscle fibers and a low-affinity receptor (A_{2B}) present at endothelial cells, and the functional equilibrium between these two receptors may be altered in patients with severe vasculogenic ED, we investigated the kinetics of adenosine inactivation in HCC strips to probe its role in the pathophysiology of ED. Extracellular adenosine was slowly, but stoichiometrically, converted into inosine by ectoadenosine deaminase with a similar kinetics in HCC strips from both control subjects and patients with vasculogenic impotence. These results also indicate that the nucleoside transport system does not play a major role in adeno-

sine inactivation in human cavernosal vessels, which is in contrast with the findings obtained in many other vascular beds. Therefore, adenosine clearance to the main bloodstream and/or the nucleoside inactivation by several blood elements might be the major contributors for adenosine disappearance in the human penis. The slow inactivation kinetics in HCC implies that adenosine might play a significant role to control blood supply to penile vessels in men with vasculogenic impotence. This hypothesis is in keeping with clinical studies showing that intracavernosal application of adenosine maintains competent penile erection that exclusively depends on the activity of membrane-bound adenosine receptors (Takahashi et al., 1992) for a period of 5 to 13 min, which is enough to facilitate performance of hemodynamic tests (Kilic et al., 1994). It remains, however, to be elucidated whether endogenous adenosine reaches high enough levels to activate the low-affinity A_{2B} receptor. The relative contribution of A_{2B} receptors might be greater during initiation of penile tumescence following a period of flaccidity, where oxygen supply is much lower (30–40 mm Hg) than that observed on full erection (100 mm Hg).

In conclusion, this work is pioneering 1) in studying the kinetics of extracellular inactivation of adenosine in HCC and 2) in characterizing the adenosine receptor subtypes responsible for relaxation of penile vessels. By comparing the magnitude of adenosine relaxation of HCC strips from control subjects and from vasculogenic ED patients, it is concluded that endothelial dysfunction may be correlated with the loss of adenosine A_{2B} receptors activity, keeping unaltered relaxation of cavernosal vessels via A_{2A} receptors probably located on the smooth muscle layer. Although direct evidence of regional adenosine receptors distribution in human penile vessels requires further studies, the information from the present study may help to delineate new pharmacological strategies to manage severe vasculogenic impotence resistant to common vasodilators.

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