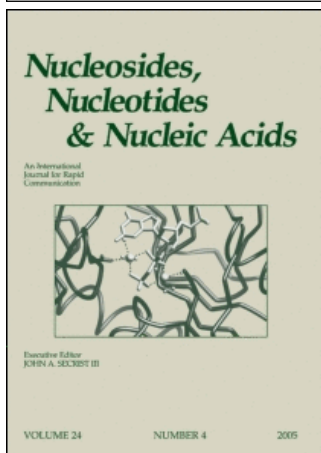


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### Decreased Ecto-Ntpdase1/Cd39 Activity Leads to Desensitization of P2 Purinoceptors Regulating Tonus of Corpora Cavernosa in Impotent Men with Endothelial Dysfunction

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## DECREASED ECTO-NTPDASE1/CD39 ACTIVITY LEADS TO DESENSITIZATION OF P2 PURINOCEPTORS REGULATING TONUS OF CORPORA CAVERNOSA IN IMPOTENT MEN WITH ENDOTHELIAL DYSFUNCTION

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□ Vascular responses to adenine nucleotides in human corpora cavernosa from men with vasculogenic erectile dysfunction were investigated. We also evaluated the catabolism of extracellular adenine nucleotides to probe its relevance to vascular hemodynamics in impotent men. Human corpora cavernosa have high NTPDase1/CD39 activity, converting ATP directly into AMP, without significant ADP formation. Extracellular ATP hydrolysis is slower in impotent patients. Adenine nucleotides have dual roles on phenylephrine-contracted strips of corpora cavernosa operated by P2X-contractant and P2Y-relaxant receptors. Prolonged exposure to endogenous ATP related to decreased NTPDase1/CD39 activity leads to P2-purinoceptor desensitization in impotent men. Shutting down ATP signaling in vasculogenic impotent men may represent a defense mechanism for preventing purinergic overstimulation.

**Keywords** Erectile dysfunction; human corpus cavernosum; P2 purinoceptors; ectonucleotidases; ecto-NTPDase1/CD39; adenine nucleotides

### INTRODUCTION

Purinergic transmission is important for initiation and maintenance of penile erection.<sup>[1]</sup> ATP released by blood elements, endothelium, and smooth muscle is a powerful regulator of vascular hemodynamics. In most vascular beds, ATP acts via P2X receptors located on vascular smooth muscle cells to mediate vasoconstriction. Alternatively, adenine nucleotides may cause vasodilatation either directly, through P2Y receptors located on endothelium and smooth muscle fibres, or indirectly, via adenosine gener-

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ated by ecto-nucleotidases. We recently demonstrated that adenosine regulates human corpus cavernosum (HCC) tone via CGS21680C-sensitive ( $A_{2A}$ ) and -insensitive ( $A_{2B}$ ) receptors located on smooth muscle and endothelial cells, respectively.<sup>[2]</sup> Because the functional significance of P2 purinoceptors in erectile tissue remains unclear, we investigated the vascular responses of HCC from control subjects and from impotent men with endothelial dysfunction to two subtype-selective P2-receptor agonists,  $\alpha,\beta$ -methylene ATP ( $\alpha,\beta$ -MeATP), and ATP $\gamma$ S.

The biological effects of adenine nucleotides may be cut-short by extracellular hydrolysis via the ecto-nucleotidase cascade. Many of the ecto-nucleotidases catalytically remove nucleotides in tandem with other ecto-enzymes to generate nucleosides at very high turnover rates. CD39, an apyrase or ecto-nucleoside triphosphate diphosphohydrolase (ecto-NTPDase 1), was identified as the first member of the ecto-nucleotidase family and its main function is to hydrolyse nucleoside tri- and diphosphates yielding monophosphates of both purine and pyrimidine nucleosides.<sup>[3]</sup> Ecto-NTPDase 1/CD39 is a plasma membrane-bound enzyme highly expressed in the vasculature (endothelium, smooth muscle, and cardiac cells), lymphocytes and platelets.<sup>[4]</sup> Transgenic mice with targeted disruption of ecto-NTPDase 1/CD39 exhibit alterations in hemostasis and in inflammatory and thrombotic reactions to reperfusion injury.<sup>[5]</sup>

Endothelial dysfunction is a cornerstone in the pathophysiology of both erectile dysfunction (ED) and cardiovascular diseases, with the two entities sharing a high degree of interdependence concerning severity.<sup>[6]</sup> We have demonstrated that endothelial dysfunction is correlated with a loss of adenosine  $A_{2B}$  receptor activity in penile vessels from men with vasculogenic ED.<sup>[2]</sup> Evidence that endothelial cell dysfunction may also be correlated with the loss of ecto-NTPDase 1/CD39 activity,<sup>[7]</sup> prompted us to investigate the pattern of extracellular catabolism of adenine nucleotides to probe its relevance to purinergic signaling in vasculogenic ED patients.

## MATERIAL AND METHODS

### Human Corpus Cavernosum (HCC) Tissues

HCC specimens were obtained from control subjects (bodies donated for harvesting organs, 18–50 years) and from patients suffering from 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 HGSA (University Hospital) and of ICBAS (Medical School) of the University of Porto. The investigation conforms to the principles outlined in the Declaration of Helsinki.

### Isometric Tension Assay

Longitudinal strips of corpus cavernosum tissue ( $3 \times 3 \times 7$  mm) were mounted in 12 ml organ chambers containing oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4) Tyrode's solution (composition in mM: NaCl 137, KCl 2.7, CaCl<sub>2</sub> 1.8, MgCl<sub>2</sub> 1, NaH<sub>2</sub>PO<sub>4</sub> 0.4, NaHCO<sub>3</sub> 11.9, glucose 11.2, and ascorbic acid, 100) at 37° C. HCC strips were connected to isometric force transducers, and changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments, Colorado Springs, CO, USA). Tissues were preloaded with 2 g of tension and allowed to equilibrate for 90 minutes in Tyrode's solution. Each strip was incrementally stretched to optimal isometric tension, as determined by maximal contractile response to 1  $\mu$ M phenylephrine (PE). After standardization, relaxation to acetylcholine (ACh, 1–10  $\mu$ M) was taken as a measure of endothelium integrity. To evaluate tissue relaxant responsiveness, HCC strips were contracted with 1  $\mu$ M PE, and once a stable contraction was achieved (15–20 minutes) the strips were challenged with cumulative additions of ATP $\gamma$ S and  $\alpha,\beta$ -MeATP to the chambers.

### Extracellular Metabolism of Adenine Nucleotides

To study the kinetics of ATP and AMP 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<sub>2</sub>/5% CO<sub>2</sub>, pH 7.4) Tyrode's solution at 37°C. After a 30 minutes equilibration period, the preparations were incubated with 30  $\mu$ M ATP or AMP (zero time). Samples of 75  $\mu$ l were collected from the organ bath at different times up to 45 min for HPLC (L-6200 intelligent pump with a L-4000 UV detector, Hitachi, Germany) analysis of the variation of substrate disappearance and product formation.<sup>[8]</sup>

### Presentation of Data and Statistical Analysis

The data are expressed as mean  $\pm$  S.E.M. from  $n$  number of individuals, and at least four strips were used for each experiment. The responses are expressed as percentage of PE (1  $\mu$ M) contractions. For multiple comparisons, results were analyzed by analysis of variance (ANOVA) followed by the Bonferroni's posttest. For comparison between two values, the unpaired Student's  $t$ -test was used. A value of  $P < 0.05$  was considered significant.

## RESULTS

HCC from control subjects hydrolyzes extracellular ATP (30  $\mu$ M) with a half-degradation time of  $9 \pm 2$  minutes ( $n = 4$ ) (Table 1). The ATP

**TABLE 1** Half-degradation time of ATP (30  $\mu$ M) on HCC strips from control subjects and from vasculogenic impotent men with endothelial dysfunction

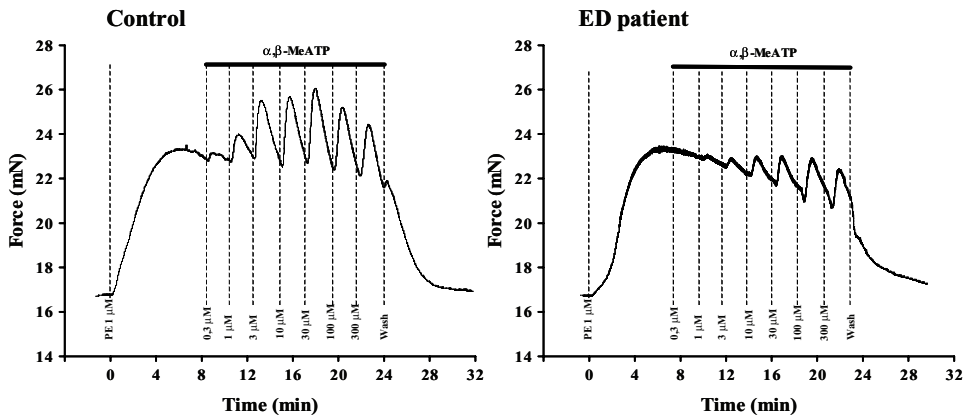
	Control individuals	Vasculogenic ED patients
ATP catabolism ( $t_{1/2}$ , minute)	9 $\pm$ 2 (4)	26 $\pm$ 1 (3)*
ACh (1 $\mu$ M) relaxation (% of maximal contraction)	23 $\pm$ 4% (21)	73 $\pm$ 7% (11)*

Half-degradation time ( $t_{1/2}$ , minute,  $\pm$  S.E.M.) of extracellular ATP (30  $\mu$ M) inactivation in HCC strips obtained from control subjects and from patients with vasculogenic ED. Relaxation to 1  $\mu$ M ACh was taken as measure of endothelium integrity; values are expressed as a percentage ( $\pm$ S.E.M.) of maximal contraction caused by 1  $\mu$ M PE. The number of individual human specimens is shown between parentheses.

\* $P < 0.05$  as compared with data obtained in control individuals.

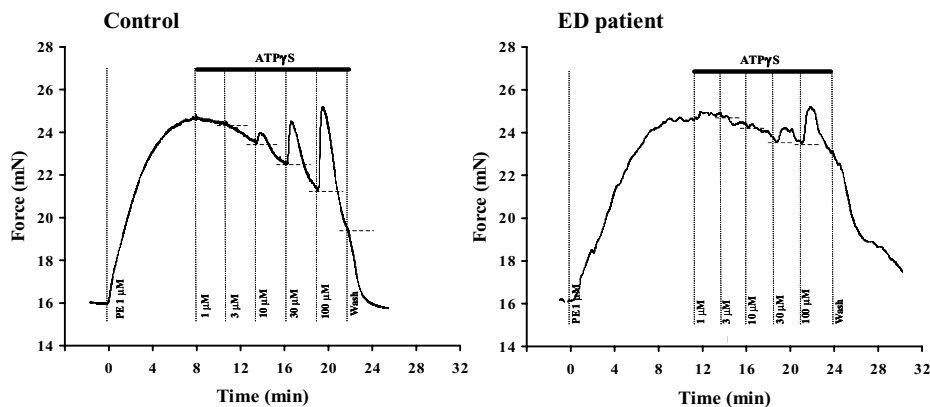
metabolites detected in the bath were AMP, whose accumulation was detected within the first min and reached a maximum of  $6.8 \pm 1.5 \mu$ M at 15 minutes, adenosine, whose maximum concentration ( $22.1 \pm 3.8 \mu$ M) was reached at 45 minutes, and inosine, whose maximum concentration ( $8.9 \pm 0.7 \mu$ M) was also reached at 45 minutes. Surprisingly, small amounts of ADP ( $<1.5 \mu$ M) were detected only after a 5-minute incubation period. This pattern of extracellular catabolism of ATP suggests the presence of ecto-NTPDase 1/CD39 enzyme converting ATP directly into AMP, which is then sequentially inactivated into adenosine and inosine by ecto-5'-nucleotidase (CD73) and ecto-adenosine deaminase, respectively (see, e.g., Faria et al.<sup>[2]</sup>). Table 1 shows that the extracellular catabolism of ATP is significantly ( $P < 0.05$ ) slower in HCC from vasculogenic ED patients ( $t_{1/2} = 26 \pm 1$  minute;  $n = 3$ ) than in control subjects. The rate of AMP (30  $\mu$ M) metabolism was similar when comparing the two groups of samples (data not shown).

Endothelial dysfunction of HCC strips isolated from vasculogenic ED patients was confirmed in vitro by a significant ( $P < 0.05$ ) reduction of ACh (1  $\mu$ M)-induced relaxation (Table 1). The two metabolically stable adenine nucleotides exerted a dual role on HCC strips pre-contracted with 1  $\mu$ M PE. The P2X agonist,  $\alpha,\beta$ -MeATP (0.3–30  $\mu$ M), transiently increased the HCC tonus in a concentration-dependent manner (Figure 1); when applied in concentrations higher than 100  $\mu$ M,  $\alpha,\beta$ -MeATP also increased HCC tonus but the increase was smaller than that observed with 30  $\mu$ M ( $32 \pm 3\%$ ,  $n = 3$ ). Contractile responses to  $\alpha,\beta$ -MeATP (0.3–300  $\mu$ M) were sensitive to blockade with pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 10  $\mu$ M) and were significantly ( $P < 0.05$ ) attenuated in vasculogenic ED patients; this compound failed to increase HCC tension beyond  $16 \pm 2\%$  ( $n = 3$ ), even when a higher concentration (100  $\mu$ M) was used. The nonselective P2 purinoceptor agonist, ATP $\gamma$ S (1–100  $\mu$ M), had a biphasic effect on HCC tonus in control individuals (Figure 2). ATP $\gamma$ S (1–100  $\mu$ M) transiently increased HCC tension, which was then followed by a concentration-dependent relaxing effect ( $IC_{25} = 10 \mu$ M). At the highest



**FIGURE 1** Contractile responses of pre-contracted (with 1  $\mu$ M PE) HCC strips from control subjects and from patients with vasculogenic ED to  $\alpha,\beta$ -MeATP (0.3–300  $\mu$ M). Shown are typical recording traces of increased HCC tension by cumulative application of  $\alpha,\beta$ -MeATP (0.3–300  $\mu$ M).

concentration (100  $\mu$ M) tested, ATP $\gamma$ S caused only a mild relaxing effect of  $23 \pm 6\%$  ( $n = 5$ ) in patients with vasculogenic ED. Pyrimidine nucleotides (such as UTP and UDP) were virtually devoid of effect on HCC tension (data not shown). Overall these results indicate that both P2X- and pyrimidine-insensitive P2Y-mediated responses of HCC from men with vasculogenic ED are markedly depressed.



**FIGURE 2** Biphasic responses of pre-contracted (with 1  $\mu$ M PE) HCC strips from control subjects and from patients with vasculogenic ED to ATP $\gamma$ S (1–100  $\mu$ M). Shown are typical recording traces of tension changes induced by cumulative application of ATP $\gamma$ S (1–100  $\mu$ M) on HCC strips. Horizontal dashed lines are shown to facilitate distinction between transient increases in tension and the following relaxant component for a given drug concentration.

## DISCUSSION

The concentration of extracellular nucleotides is regulated by a variety of surface-located enzymes known as ecto-nucleotidases; most of them are members of the ecto-NTPDase family.<sup>[3,9]</sup> The most widely expressed NTPDase on the vasculature, NTPDase 1/CD39, exhibits a tissue distribution (e.g., endothelium and smooth muscle cells) that coincides with the distribution of several P2X and P2Y receptors. Therefore, vascular hemodynamics may be tightly regulated by NTPDase 1/CD39 activity. As in knockout mice, deletion of NTPDase 1/CD39 should favor accumulation of nucleotides and therefore enhance purinergic signaling. In contrast, we found the opposite in HCC strips from impotent men with endothelial dysfunction, which exhibit decreased NTPDase 1/CD39 activity. Indeed, we found that vascular responses of HCC from men with vasculogenic ED to exogenously applied stable ATP analogues,  $\alpha,\beta$ -MeATP and ATP $\gamma$ S, were markedly depressed as compared to control individuals. Similar results were obtained in thrombogenesis, angiogenesis and immune cell models, confirming that impairment of NTPDase 1/CD39 activity leads to P2-purinoreceptor desensitization.<sup>[10–13]</sup> Thus, purinoreceptor desensitization as a result of NTPDase 1/CD39 inactivation appears to be a general phenomenon with potential clinical significance under conditions of inflammation or oxidative stress, thereby causing profound shifts in vascular hemodynamics (see, e.g., Robson et al.<sup>[9]</sup>).

Not only was the  $\alpha,\beta$ -MeATP-sensitive (which distinguishes P2X<sub>1</sub> and P2X<sub>3</sub> receptors from other homomeric forms) contractile response reduced in HCC strips from vasculogenic impotent men, but also the relaxant effect of ATP $\gamma$ S mediated by P2Y receptors was blunted as compared with control individuals. The P2X<sub>1</sub> receptor seems to be the predominant P2X receptor expressed in vascular smooth muscle cells.<sup>[14]</sup> Although the P2X receptor profile of endothelial cells is less well characterized and there is evidence for regional variation in endothelial P2X receptor expression throughout the vasculature,<sup>[15]</sup> the predominant receptor on this cell type is probably the P2X<sub>1</sub> receptor.<sup>[16]</sup> The P2X<sub>1</sub> receptor undergoes rapid desensitization,<sup>[17]</sup> but recovery from desensitization is extremely slow.<sup>[18]</sup> Nanomolar ATP concentrations drive significant fractions of the rapidly desensitized P2X<sub>1</sub> receptor pool into a long lasting refractory state.<sup>[19]</sup> Therefore, attenuation of contractile responses of HCC strips from vasculogenic ED patients to  $\alpha,\beta$ -MeATP is most likely due to PPADS-sensitive P2X<sub>1</sub> desensitization. Since ATP concentrations can reach  $\sim 1$ – $20\ \mu\text{M}$  in the absence of cell lysis and persistent elevated levels of the nucleotide can lead to cell toxicity, P2X<sub>1</sub> receptor desensitization may actually be a protective phenomenon.

On the other hand, P2Y receptors do not readily desensitize. Instead, as with other G protein-coupled receptors, desensitization of P2Y receptors may have a slower kinetics involving receptor phosphorylation by protein kinases and/or uncoupling from the associated G protein. Homolo-

gous desensitization of IP<sub>3</sub> formation by P2Y<sub>1</sub>- and P2Y<sub>2</sub>-like receptors was observed in cultured bovine aortic endothelial cells<sup>[20]</sup> as well as in rabbit mesenteric arterial smooth muscle.<sup>[17]</sup> Thus, persistent accumulation of ATP in HCC from men with vasculogenic ED due to impairment of NTPDase 1/CD39 activity might disturb purinergic signaling in erectile tissue also as a consequence of P2Y-purinoceptor desensitization. This might explain the shift to the right of the concentration-response curve for the relaxing effect of ATPγS. Another possibility is a differential distribution of relaxant P2Y<sub>1</sub>/P2Y<sub>2</sub> receptor subtypes on vascular endothelium and smooth muscle cells, which might be unbalanced by endothelial cell dysfunction.

In conclusion, we have demonstrated that HCC from men with vasculogenic ED exhibit decreased NTPDase 1/CD39 activity leading to persistent extracellular ATP accumulation. As a consequence, attenuation of vascular responses of HCC strips from impotent patients to stable ATP analogues may be due to P2-purinoceptor desensitization.

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