ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8): 745 - 749
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Hydrolysis of extracellular adenine nucleotides by cultured bovine endocardial endothelial cells

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KEY WORDS apyrase; endocardium; vascular endothelium; adenine nucleotides; sodium azide; sodium fluoride; ouabain; edetic acid; hydrogen peroxide

ABSTRACT

AIM: To characterize the ATP diphosphohydrolase (apyrase) of bovine endocardial endothelial cells, and to compare ecto-adeninenucleotidase activity between bovine endocardial and aortic endothelial cells (BEEC and BAEC). **METHODS**: The nucleotide was analyzed by reversed phase HPLC and apyrase activity was assayed by inorganic phosphate release. **RESULTS**: Apyrase inhibitors, both NaN_3 10 mmol·L^{-1} and NaF 20 mmol·L^{-1}, inhibited BEEC apyrase activity by 51 % and 38 %, respectively. The inhibitor for Na^+/K^+-ATPase, ouabain, did not affect the enzyme activity. Edetic acid 5 mmol·L^{-1} completely inhibited the enzyme activity. H_2O_2 0.5 mmol·L^{-1} downregulated BEEC apyrase activity in a time-dependent manner. The apyrases activities in BAEC were higher than those in BEEC, while the ecto-AMPase activity in BAEC was much weaker than that in BEEC. **CONCLUSION**: BEEC have NaN_3- and NaF-sensitive, ouabain-insensitive apyrase activity. BEEC had high ecto-AMPase activities, and low apyrases activities as compared with BAEC.

INTRODUCTION

Vascular endothelial cells are known to regulate local adenosine nucleotide concentrations by membrane ecto-enzymes termed ATP diphosphohydrolases (apyrase, EC 3.6.1.5), ecto-ATPase or ecto-ADPase that can hydrolyze both extracellular adenosine triphosphate (ATP) (now regarded as a apoptosis inductor of vessel endothelial cells) and adenosine diphosphate (ADP, a platelet agonist)[1-5]. The final product of apyrase, adenosine monophosphate (AMP), is a substrate for 5′-nucleotidases (AMPase) and ultimately generates adenosine, an important cardioprotective, anti-inflammatory and anti-aggregatory mediator[1,6,7]. While ATPases hydrolyze cytoplasm ATP to ADP but not to AMP, and participate in the ATP-dependent energetic processes[1].

Like vascular endothelial cells, endocardial endothelial cells (EEC) possess the ability to synthesize prostacyclin (PGI_2), endothelin, and nitric oxide [8,9]. Whether EEC have an apyrase activity has never been reported. The present work was to study the characteristics of BEEC-associated apyrase, and to compare ecto-adeninenucleotidase activity between bovine endocardial and aorta endothelial cells.

MATERIALS AND METHODS

Materials Adenosine 5′-diphosphate, adenosine 5′-monophosphate, adenosine 5′-triphosphate, M199 medium, bovine serum albumin (BSA), edetic acid, diadenosine pentaphosphate (Ap5A), sodium azide (NaN_3), and ouabain were obtained from Sigma Chemical Co, St Louis, MO, USA. Sodium fluoride (NaF) purchased from Beijing Chemicals Company was analytical grade. Fetal bovine serum (FBS) was purchased from Sijiqing Biologic Engineering Co, Hangzhou. Newborn bovine hearts and aortas were purchased from a local slaughterhouse.

Cell culture BAEC were isolated from the aortas of bovines and cultured as previously described[10]. BEEC were cultured from bovine hearts by the

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Received 1998-04-10 Accepted 1999-01-29

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

method of Mebazaa A[9]. Briefly, atria, valve tissue, and chordae tendineae were removed from freshly isolated newborn bovine hearts. The ventricles were filled with 0.1 % collagenase (type ⅠA) in phosphate-buffered saline (PBS) and incubated at 37 ℃ for 45 min. The inside of each ventricle was gently rubbed with a cell scraper to remove loosely attached cells. The cell suspension was removed, and spun at 100 × *g* for 10 min. The pellet was suspended in M199 medium supplemented with 20 % FBS, benzyl-penicillin 100 kU·L^{-1}, streptomycin 100 mg·L^{-1}. All cultures showed typical morphology. These cells were identified by electron microscopy. Cells (from passage 4 to 10) were plated for experiments in 24-well culture dishes at a density of 1×10^5 cells/cm^2, and incubated at 37 ℃ under air containing 5 % CO_2.

Measurement of ectonucleotidase activities with HPLC[11] The ability to hydrolyze nucleotide represented ectonucleotidase activities. The nucleotide was analyzed by reversed phase HPLC. The chromatograph consisted of K510 pump, Lambda-Max Model 490 ultraviolet detector, Spherisorb C18 column (250 mm × 4.6 mm ID, 5 μm particle size), K501 injector valve, and Waters system interface module. Except for C18 column (Dalian Institute of Chemicophysics, Chinese Academy of Sciences), the other above apparatus were purchased from Waters (USA). The detector was set at 254 nm. Samples (25 μL injection) were eluted with KH_2PO_4 200 mmol·L^{-1} (adjusted to pH 6 with NH_4OH) for 45 min. The solvent flow rate was 0.4 mL/min.

Enzymic assays by inorganic phosphate release Intact BEEC were plated in 24-well culture dishes, each well was incubated in 1 mL of 50 mmol·L^{-1} Tris/HCl (pH 7.4) containing $CaCl_2$ 1 mmol·L^{-1} and ADP 500 μmol·L^{-1} for 30 min, and free phosphate release was determined[12-15]. One U of apyrase activity corresponds to the amount of enzyme which catalyzes the liberation of 1 nmol inorganic phosphate/min at 30 ℃.

Statistical analysis Results were presented as $\bar{x} \pm s$ and analyzed by *t* test.

RESULTS

Effects of selected inhibitors on BEEC apyrase activity The inhibitors were added to BEEC just before ADP addition. The inhibitor for Na^+/K^+-ATPase, ouabain, did not affect apyrase activity. The addition of edetic acid 5 mmol·L^{-1} completely inhibited the activities. Ap5A, inhibitor of adenylate kinase, had a little effect on apyrase activity, whereas both NaN_3 and NaF could markedly inhibit the enzyme activity (Tab 1).

Tab 1. Effect of inhibitors on BEEC apyrase activity. Results were the $\bar{x} \pm s$ of 5 independent experiments each in triplicate. The apyrase activity in control group was (1.63 ± 0.24) U per 10^5 cells.

Inhibitor	Concentration	Inhibition/%
Ouabain	3 mmol·L^{-1}	2.0 ± 2.5
Ap5A	0.25 mmol·L^{-1}	13 ± 5
NaF	20 mmol·L^{-1}	38 ± 9
NaN_3	10 mmol·L^{-1}	51 ± 8
Edetic acid	5 mmol·L^{-1}	99 ± 5

Modulation of BEEC apyrase activity by H_2O_2 Stimulating BEEC by H_2O_2 0.5 mmol·L^{-1} resulted in a rapid loss of the apyrase activity. Apyrase activity, as determined by inorganic phosphate release from ADP, showed comparable patterns of inhibition at the time intervals examined. A trend towards inhibition was observed as early as 60 min after H_2O_2 stimulation, and was maximal by 8 h (Tab 2).

Tab 2. Modulation of BEEC apyrase activity by H_2O_2 0.5 mmol·L^{-1}. Results were $\bar{x} \pm s$ of 5 independent experiments (each in triplicate). $^bP < 0.05$, $^cP < 0.01$ *vs* 0 time.

Time/h	Activity/U per 10^5 cells
0	1.63 ± 0.24
1	1.50 ± 0.29
2	1.25 ± 0.20^b
4	0.98 ± 0.20^c
8	0.84 ± 0.21^c

Comparison of ecto-adeninenucleotidase activity between bovine endocardial and aorta endothelial cells In both types of cells, the concentration of ATP and ADP decreased rapidly with time until almost 60 min of incubation. No transient accumulation of ADP was seen and the concentration of

AMP increased rapidly (Fig 1). After incubation with nucleotides 500 $\mu mol \cdot L^{-1}$ for 2 h, BEEC decreased ATP to the concentration [(155 ± 21) $\mu mol \cdot L^{-1}$ *vs* (60 ± 19) $\mu mol \cdot L^{-1}$ for BAEC group, $n = 6$ independent experiments, $P < 0.01$], decreased ADP to the concentration [(110 ± 30) $\mu mol \cdot L^{-1}$ *vs* (20 ± 13) $\mu mol \cdot L^{-1}$ for BAEC group, $n = 6$ independent experiments, $P < 0.01$], and decreased AMP to the concentration [0 $\mu mol \cdot L^{-1}$ *vs* (346 ± 29) $\mu mol \cdot L^{-1}$ for BAEC group, $n = 6$ independent experiments, $P < 0.01$]. These results showed that apyrases activities in BAEC were higher than that in BEEC, while the ecto-AMPase activity in BAEC was much weaker than that in BEEC.

DISCUSSION

Apyrase activity has been reported in vascular endothelial cells[11]. The results reported here demonstrated for the first time that bovine endocardial endothelial cell also had apyrase. Our results showed that well-known apyrase inhibitors, both NaN_3 and NaF, could markedly inhibit BEEC apyrase activity. The inhibitor for Na^+/K^+-ATPase, ouabain, did not affect apyrase activity. Edetic acid completely inhibited the activities, suggesting that BEEC apyrase activity depended on divalent cations (Tab 1). Our these results were closely in accordance with the results reported in various other tissues[11,13-15].

So far there are a few report regarding the modulation of apyrase[16-18]. Studies showed that vascular EC apyrase activity was rapidly lost following ischemia-reperfusion injury and during xenograft rejection[16,17], and that aspirin could upregulate human endothelial cells apyrase[18]. The present experiments showed that H_2O_2 could downregulate BEEC apyrase activity, which was in agreement with the studies[17]. These researchers showed that loss of rat glomerular apyrase activity during reperfusion injury was associated with oxidative stress reactions.

We compared the kinetics of extracellular nucleotide hydrolysis in BAEC with that in BEEC.

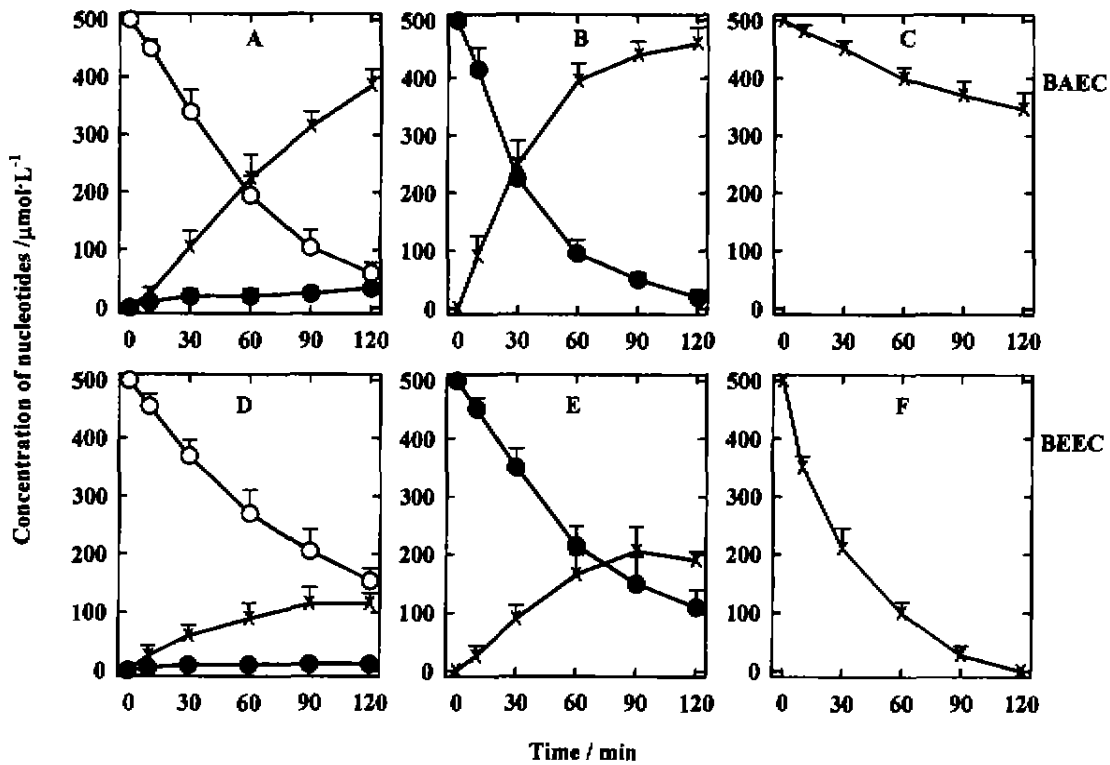

Fig 1. Hydrolysis of extracellular adenine nucleotides (500 $\mu mol \cdot L^{-1}$) by cultured BEEC (D, E, F) and BAEC (A, B, C). $\bar{x} \pm s$ of 6 independent experiments. Substrates: A and D: ATP (○), B and E: ADP (●), C and F: AMP (×).

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 Aug; 20 (8)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Our results showed that the ecto-ATPase and ecto-ADPase activities in BEEC were weaker than those in BAEC, while the ecto-AMPase activity in BEEC was much higher than that in BAEC. The difference may be due to tissue particularity.

The physiological function of BEEC apyrase is not known. One possible function is to prevent thrombotic diseases such as endocarditis and rheumatic valvulitis, and to prevent rejection of discordant xenograft, because platelet activation and aggregation are important factors in the mediation of vascular and cardiac inflammation and are specifically associated with the rejection of discordant xenograft[3,16,19,20]. While ADP is the most important platelet agonist, and now endothelial cell apyrase is known as most important vascular "thromboregulatory" systems[2,3]. Moreover, it has been shown that apyrase administration could prolong discordant xenograft survival[16]. Another possible function of BEEC apyrase is to protect BEEC from apoptosis or death, because that ATP and ADP could induce vascular endothelial cell apoptosis through the activation of P_2 receptors and the activation of NF-κ B[4], and that apyrase could prevent macrophage death induced by ATP[21].

REFERENCES

1 Komoszynski M, Wojtczak A. Apyrase (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. Biochim Biophys Acta 1996; 1310: 233 – 41.

2 Marcus AJ, Safier LB. Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. FASEB J 1993; 7: 516 – 22.

3 Marcus AJ, Safier LB, Broekman MJ, Islam N, Fliessbach JH, Hajjar KA, *et al*. Thrombosis and inflammation as multicellular processes: significance of cell-cell interactions. Thromb Haemost 1995; 74: 213 – 7.

4 von Albertini M, Palmetshofer A, Kaczmarek E, Koziak K, Stroka D, Grey ST, *et al*. Extracellular ATP and ADP activate transcription factor NF-κ B and induce endothelial cell apoptosis. Biochem Biophys Res Commun 1998; 248: 822 – 9.

5 Dawicki DD, Chatterjee D, Wyche J, Rounds S. Extracellular ATP and adenosine cause apoptosis of pulmonary artery endothelial cells. Am J Physiol 1997; 273 (2 Pt 1): L485 – 94.

6 Ely SW, Berne RM. Protective effects of adenosine in myocardial ischemia. Circulation 1992; 85: 893 – 904.

7 Mubagwa K, Mullane K, Flameng W. Role of adenosine in the heart and circulation. Cardiovasc Res 1996; 32: 797 – 813.

8 Redmond EM, Cherian MN, Wetzel RC. 17 β-Estradiol inhibits flow- and acute hypoxia-induced prostacyclin release from perfused endocardial endothelial cells. Circulation 1994; 90: 2519 – 24.

9 Mebazaa A, Martin LD, Robotham JL, Maeda K, Gabrielson EW, Wetzel RC. Right and left ventricular cultured endocardial endothelium produces prostacyclin and PGE_2. J Mol Cell Cardiol 1993; 25: 245 – 8.

10 Guo ZG, Niu XL, Guo X. Oxidized low-density lipoproteins induce apoptosis in aortic and endocardial endothelial cells. Acta Pharmacol Sin 1997; 18: 525 – 8.

11 Yagi K, Shinbo M, Hashizume M, Shimba LS, Kurimura S, Miura Y. ATP diphosphohydrolase is responsible for ecto-ATPase and ecto-ADPase activities in bovine aortic endothelial and smooth muscle cells. Biochem Biophys Res Commun 1991; 180: 1200 – 6.

12 Geladopoulos TP, Sotiroudis TG, Evangelopoulos AE. A malachite green colorimetric assay for protein phosphatase activity. Anal Biochem 1991; 192: 112 – 6.

13 Christoforidis S, Papamarcaki T, Galaris D, Kellner R, Tsolas O. Purification and properties of human placental ATP diphospholydrolase. Eur J Biochem 1995; 234: 66 – 74.

14 Picher M, Cote YP, Beliveau R, Potier M, Beaudoin AR. Demonstration of a novel type of ATP-diphosphohydrolase (EC 3.6.1.5) in the bovine lung. J Biol Chem 1993; 268: 4699 – 703.

15 Moodie FD, Baum H, Butterworth PJ, Peters TJ. Purification and characterisation of bovine spleen ADPase. Eur J Biochem 1991; 202: 1209 – 15.

16 Koyamada N, Miyatake T, Candinas D, Hechenleitner P, Siegel J, Hancock WW, *et al*. Apyrase administration prolongs discordant xenograft survival. Transplantation 1996; 62: 1739 – 43.

17 Candinas D, Koyamada N, Miyatake T, Siegel J, Hancock WW, Bach FH, *et al*. Loss of rat glomerular ATP diphosphohydrolase activity during reperfusion injury is associated with oxidative stress reactions. Thromb Haemost 1996; 76: 807 – 12.

18 Cheung PK, Visser J, Bakker WW. Upregulation of antithrombotic ectonucleotidases by aspirin in human endothelial cells *in vitro*. J Pharm Pharmacol 1994; 46: 1032 – 4.

19 Harasaki H, Hanano H, Tanaka J, Tokunaga K, Torisu M. Surface structure of the human cardiac valve. A comparative study of normal and diseased valves. J Cardiovasc Surg (Torino) 1978; 19: 281 – 90.

20 Carter G, Gavin JB. Endocardial damage induced by lactate, lowered pH and lactic acid in non-ischemic beating hearts. Pathology 1989; 21: 125 – 30.

21 Chiozzi P, Murgia M, Falzoni S, Ferrari D, Di Virgilio F. Role of the purinergic P2Z receptor in spontaneous cell death in J774 macrophage cultures. Biochem Biophys Res Commun 1996; 218: 176 – 81.

培养的牛心内膜内皮细胞水解细胞外腺苷酸

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关键词 腺苷三磷酸双磷酸酶；心内膜；血管内皮；腺苷酸类；叠氮钠；氟化钠；哇巴因；依地酸；过氧化氢

目的：研究牛心内膜内皮细胞（BEEC）腺苷三磷酸双磷酸酶（apyrase）的特性并比较牛心内膜及血管内皮细胞的胞外腺苷酸酶活性. **方法**：以反相HPLC测定核苷酸含量，以磷离子释放法检测apyrase活性. **结果**：叠氮钠 10 mmol·L^{-1}和氟化钠 20 mmol·L^{-1}分别抑制 BEEC apyrase 51 % 及 38 %活性，而 Na^+/K^+-ATP 酶抑制剂哇巴因则对 BEEC apyrase 活性无影响. 过氧化氢 0.5 mmol·L^{-1}呈时间依赖性地抑制心内膜内皮细胞 apyrase 活性. BEEC 的 apyrase 活性低于主动脉内皮细胞的 apyrase 活性，而 BEEC 的胞外 AMP 酶则远较血管内皮细胞的活性高. **结论**：BEEC apyrase 具有叠氮钠和氟化钠敏感的、哇巴因不敏感的特性；与血管内皮细胞相比，BEEC apyrase 活性较高而胞外 AMP 酶活性较低.

（责任编辑 朱倩蓉）