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Short communication

Human micro- and macrovessel-derived endothelial cells: a comparative study on the effects of adrenaline and a selective adenosine A_2-type receptor agonist under normoxic and hypoxic conditions

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Abstract:
Adrenaline is a highly effective stimulator of cyclic AMP (cAMP) production in microvascular endothelial cells (ECs) – HMEC-1, showing only a moderate activity in macrovascular ECs – HUVEC. In both EC preparations, adrenaline acts *via* β-type receptors. Selective stimulation of adenosine A_2-type receptors resulted in comparable increases in cAMP formation in ECs lining micro- and macrovessels. Hypoxia largely suppressed the cAMP effects resulting from stimulation of both β-adrenoceptors and adenosine A_2-type receptors in ECs of microvessels (HMEC-1). In contrast, hypoxia had only slight effect on these responses in ECs of macrovessels (HUVEC). The present data provide further evidence of functional differences between microvessel- and macrovessel-derived ECs.

Key words:
human microvascular endothelial cells (HMEC-1), human umbilical vein endothelial cells (HUVEC), cyclic AMP, β-adrenoceptor, adrenaline, adenosine, hypoxia

Introduction

The endothelium represents the inner cellular lining of the blood and lymphatic vessels forming a metabolically active barrier that separates the circulation from underlying tissue [3, 6, 15, 27, 28, 34]. For a long time endothelial cells were considered a homogenous cell population, however, experimental evidence accumulating during the last decades provided arguments that the endothelium is not a passive inner lining of blood vessels. The endothelium plays a significant role in many physiological functions such as the control of vasomotor tone, homeostatic balance,

synthetic or secretory processes, transport or permeability. Due to its functional importance, the endothelium is more often treated like an organ. This "small organ" has a large surface of about 350 m^2 and total mass in the range of slightly above 100 g [3, 6, 15, 27, 28, 34].

Endothelial phenotypes differ between species, organs and vascular beds. Endothelial cells (ECs) from different organs demonstrate unique structural and functional properties as well as distinct developmental programs, roles in pathophysiology and potential for targeted therapy in patients with vascular diseases. According to many studies, the difference between ECs may also consist in distinct receptor levels and responses to endogenous ligands and exogenous drugs [3, 6, 15, 27, 28, 34].

In this work we focused on two types of ECs originating from micro- and macrovessels, i.e. human microvascular endothelial cells of dermal origin (HMEC-1) and human umbilical vein endothelial cells (HUVEC). The aim of current work was two-fold: (1) to check whether there is any difference in response of the tested cells to two physiologically meaningful signals resulting from activation of either adrenergic receptors (adrenaline) or adenosine A_2-type receptors (NECA), and (2) to see whether the observed cAMP responses in HMEC-1 and HUVEC cells are affected by hypoxic condition.

Materials and Methods

Chemicals

The following substances were used: MCDB 131 medium, M 199, fetal bovine serum, penicillin-streptomycin solution (5,000 units/ml penicillin and 5 mg/ml streptomycin sulfate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, California). Epinephrine (adrenaline), 5'-(N-ethylcarboxamido)adenosine (NECA), 3-isobutyl-1-methylxanthine (IBMX), forskolin, propranolol, prazosin and yohimbine were purchased from Sigma (St. Louis, MO, USA). Radioactive compounds comprised: 2,8-[^{3}H]adenine (specific activity 24.40 Ci/mmol) was from PerkinElmer Life Sciences, Inc. (Boston, MA, USA) and [^{14}C]cAMP (specific activity 56 mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA).

Cell culture

HMEC-1 (human microvascular endothelial cells) were kindly provided by Dr. F. Candal from Center for Disease Control and Prevention (Atlanta) [2]. The cells were used between passages 32–41 and cultured in 25 cm^3 flasks in MCDB 131 supplemented with 10% fetal bovine serum, 10 ng/ml EGF, 1 µg/ml hydrocortisone and penicillin-streptomycin solution, in humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Every third day cells were harvested in trypsin-EDTA (0.25% trypsin, 1 mM EDTA) solution.

HUVEC (human umbilical vein endothelial cells) were isolated from fresh umbilical cords. The cells were used between passages 2–4 and cultured in 25 cm^3 flasks in M 199 supplemented with 20% fetal bovine serum, 10 ng/ml EGF and penicillin-streptomycin solution, in humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. Every fourth day cells were harvested in trypsin-EDTA (0.25% trypsin, 1mM EDTA) solution.

Assay of cAMP formation

Cells used in experiments were seeded in 12-well plates at a density of 250,000 cells/well in 500 µl of culture medium and cultured overnight. Next day, culture medium was removed, fresh serum-free culture medium was added and cells were incubated in the presence of [^{3}H]adenine for 2 h at 37°C. Next, the medium was removed, cells were rinsed two times with pre-warmed phosphate-buffered saline (PBS) and serum-free culture medium was added. Then, cells were preincubated for 20 min at 37°C in the presence of IBMX (0.1 mM). After the preincubation, cells were exposed to a proper agonists for additional 15 min. Antagonists, when tested, were applied 15 min before an agonist. The reaction was stopped by adding 500 µl of an ice cold 10% trichloroacetic acid. The resulting mixture was then transferred into test tubes, centrifuged and the formed cAMP was quantified in a supernatant fraction. The formation of [^{3}H]cAMP in [^{3}H]adenine-prelabeled cells was assayed according to the method described by Shimizu et al. [33] with some modifications [20]. The formed [^{3}H]cAMP was isolated by sequential Dowex-alumina column chromatography according to Salomon et

al. [31]. The results were individually corrected for percentage recovery with the aid of [^{14}C]cAMP added to each column system prior to the nucleotide extraction. The accumulation of cAMP during a 15-min stimulation period was assessed as a percentage of the conversion of [^{3}H]adenine to [^{14}C]cAMP.

Hypoxic conditions

Hypoxic conditions were evoked by cell culturing under 3% O_2, 92% N_2, 5% CO_2 (later on referred to as "3% oxygen") or in the presence of iron-chelating agent cobalt chloride ($CoCl_2$; 200 µM) for 24 h, as earlier described for "hypoxia" experiments with other cell types [26].

Data analysis

All data are expressed as the mean ± SEM values. For statistical evaluation of the results, analysis of variance (ANOVA) was used followed by the *post-hoc* Students Newman-Keuls test.

Results and Discussion

Our previous studies conducted on human microvascular endothelial cells (ECs), HMEC-1, have shown that the hormone adrenaline is capable of strongly stimulating the production of cAMP in this cell system. Isoprenaline, a selective β-adrenoceptor agonist, mimicked the action of adrenaline, whereas noradrenaline appeared to be a decisively less potent stimulator [25]. Thus, these findings extended an array of different ECs possessing the β-adrenoceptor-sensitive cAMP generating system [21, 23, 24, 32]. Although the observed by us cAMP responses to adrenaline in HMEC-1 cells were large and highly reproducible, the functional significance of this effect remains to be established. Based on recent reports showing on the one hand the ability of HMEC-1 cells to produce an angiogenic peptide, i.e. vascular endothelial growth factor (VEGF) [22], and on the other, a relationship between the β-adrenoceptor-linked cAMP-dependent mechanism and VEGF mRNA expression in some

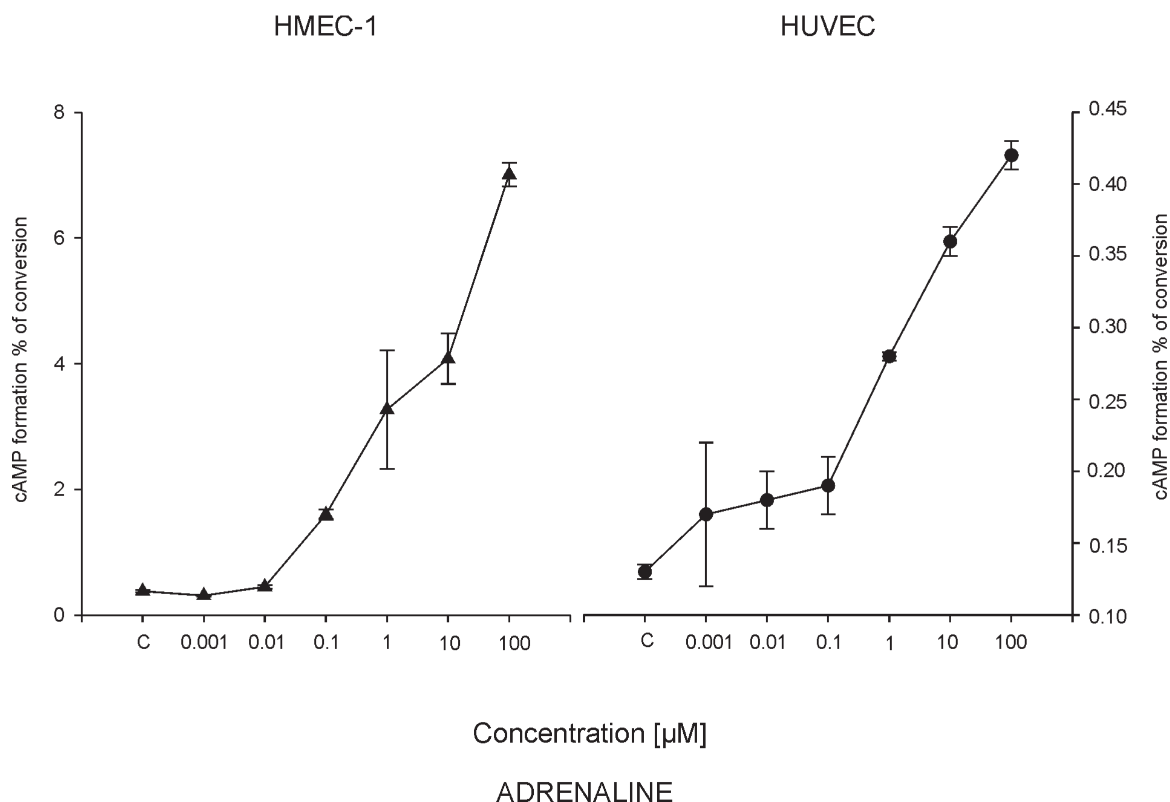

Fig. 1. Concentration-dependent effects of adrenaline on cAMP synthesis in HMEC-1 and HUVEC cells. Note that y-axis scales in left and right figure are different. Data represent the means ± SEM of 9–18 experiments; C – control values

cells [4, 12, 30], there was a possibility that the adrenergic mechanism may be involved in the molecular cascade leading to VEGF synthesis by HMEC-1 cells. HMEC-1 cells are in fact able to produce VEGF, and this process can be initiated under hypoxic condition evoked by both reduced oxygen supply [1% O_2] or the use of the hypoxia-mimicking agent $CoCl_2$ [22, 25], but we were unable to detect any significant effect of adrenaline (when used up to 100 μM; 3–24 h) on VEGF expression in these microvascular ECs [25].

The β-adrenoceptor-dependent cAMP generating system operating in various ECs seems to regulate different aspects of cell physiology, including nitric oxide generation, for instance, in porcine aortic endothelial cells (PAEC), stimulation of von Willebrand factor release in HUVEC cells, or vasorelaxation tested on umbilical vein rings, indicating a functional importance of the cAMP signal in these "macrovascular" cells/tissues [11, 16, 17, 29, 30]. Yet, till now there are no firm data linking β-adrenoceptor-driven adenylyl cyclase (AC)-cAMP signaling and any functional response in "microvascular" HMEC-1 cells.

This work carried out in parallel on ECs originating from microvessels (HMEC-1) and macrovessels (HUVEC) was designed to gain a closer insight into the cAMP generating system and its possible role in ECs physiology. These cell systems were tested for their ability to produce cAMP when exposed to adrenaline or the selective adenosine A_2-receptor agonist NECA under normoxic and hypoxic conditions. Adrenaline is a well known, intensively studied hormone, whose blood levels physiologically go up in specific situations [14], which means that under such conditions adrenaline has access to ECs lining all vessels in the body, including micro- and macrovessels. Adenosine is an intermediate product of adenine nucleotide metabolism and occurs in tissues, being prominent in extracellular space, especially in situations where oxygen supply is decreased [5, 18, 35]. Adenosine is a ubiquitous nucleoside and its diverse biological effects are mediated *via* four types of adenosine receptor (A_1, A_{2A}, A_{2B}, A_3), of which both A_2 types are positively linked to cAMP production [8, 13, 19] and both occur in ECs, including HMEC-1 and HUVEC [1, 9, 10].

In the present study, we confirmed a powerful action of adrenaline (0.001–100 μM) on cAMP formation in HMEC-1 cells, and showed also a similar but much weaker effect of this hormone in HUVEC cells (Fig. 1). The effects of adrenaline applied at 100 μM

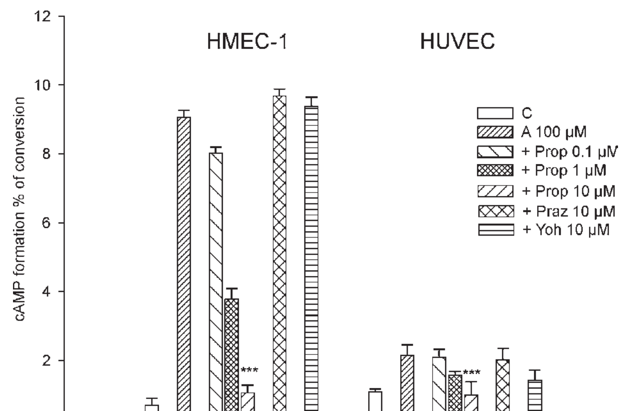

Fig. 2. Effects of adrenergic receptor antagonists propranolol (Prop), prazosin (Praz) and yohimbine (Yoh) on 100 μM adrenaline (A)-evoked stimulation of cAMP accumulation in HMEC-1 and HUVEC cells. Bars represent the means ± SEM of 4–22 experiments, *** $p < 0.001$; C – control values

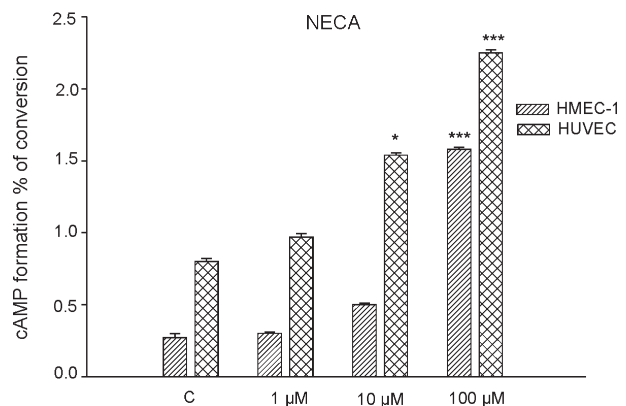

Fig. 3. Concentration-dependent effect of the A_2-receptor selective adenosine derivative (NECA) on cAMP synthesis in HMEC-1 and HUVEC cells. Bars represent the means ± SEM of 4–9 experiments, * $p < 0.05$, *** $p < 0.001$; C – control values

concentration expressed in percent of respective control value (representing basal activity) were: 1845% for HMEC-1 cells and 323% for HUVEC cells. In both cell types, the effect of adrenaline was sensitive to a β-blocker propranolol, and was unaffected by a selective α_1-adrenoceptor blocker prazosin, indicating the role of β-adrenergic receptors (Fig. 2). The α_2-adrenergic receptor does not seem to be involved in adrenaline action on HMEC-1 cells (no effect of an α_2-blocker yohimbine), yet such a component cannot be excluded in HUVEC cells, where yohimbine tended to attenuate the adrenaline action (Fig. 2).

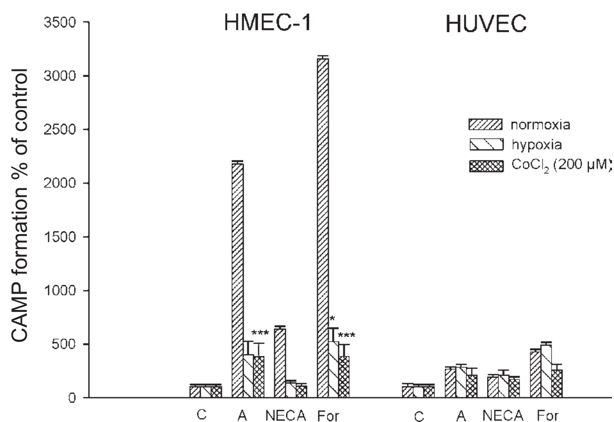

Fig. 4. Effects of adrenaline (A), A_2-receptor selective adenosine derivative (NECA) and forskolin (For) on cAMP formation under normoxic and hypoxic conditions in HMEC-1 and HUVEC cells. Bars represent the means ± SEM of 5–27 experiments, * $p < 0.05$, *** $p < 0.001$; C – control values

The cAMP-enhancing effect of adrenaline in the tested ECs was mimicked by the adenosine derivative NECA, yet the effects of the latter drug were comparatively small (Fig. 3, 4). It should be stressed, however, that in all experiments the cAMP assay system was supplemented with IBMX used to inhibit the activity of phosphodiesterase (PDE), a cAMP catabolizing enzyme. The omission of IBMX from the assay system resulted in marked decreases in cAMP accumulation due to accelerated breakdown of the newly synthesized cyclic nucleotide (results not shown; [25]). In addition to their PDE inhibiting potential, methylxanthine derivatives, including IBMX, are also endowed with some adenosine receptor blocking activity [13], a feature that likely contributed to the observed attenuation of NECA effects.

In contrast to the observed cell type-dependent differences in adrenaline-evoked cAMP effects, the adenosine derivative NECA produced roughly similar cAMP responses in both HMEC-1 and HUVEC cells (Fig. 3). When the results are expressed in percent of respective control, 1, 10 and 100 μM of the drug produced increases above basal activity by 11, 85 and 485% for HMEC-1 cells, and 21, 93 and 181% for HUVEC cells. Such a picture would suggest that the density and/or responsiveness of adenosine A_2-type receptors (i.e. A_{2A} and A_{2B}, which both are positively coupled to AC and are targets for NECA) in "microvascular" HMEC-1 and "macrovascular" HUVEC cells are similar.

Recently, Feoktistov et al. [9] reported the expression of adenosine receptors in HMEC-1 and HUVEC cells. Of the four classified receptors (A_1, A_{2A}, A_{2B}, A_3), they found only A_2 types to be expressed in both cell systems. Interestingly, HMEC-1 cells expressed preferentially A_{2B} type, whereas HUVEC cells mainly A_{2A} type. According to these authors, the adenosine-sensitive cAMP generating system in HMEC-1 cells is to be linked nearly exclusively to A_{2B} type receptor, while in HUVEC cells both A_{2A} and A_{2B} receptors may mediate the cAMP generation, with A_{2A} receptor component being more important. Feoktistov et al. [9] also reported that NECA concentration (10 and 100 μM)-dependently stimulated expression of VEGF in HMEC-1 cells but not in HUVEC cells. These observations have recently been confirmed in our laboratory [25; unpublished data].

In the last series of experiments, HMEC-1 and HUVEC cells were subjected to hypoxic conditions (either 3% oxygen or 200 μM $CoCl_2$, each for 24 h) under which both cell systems were tested for their ability to generate cAMP in response to adrenaline (100 μM), NECA (100 μM) and forskolin (10 μM), a direct stimulator of AC. The obtained results are shown in Figure 4. HMEC-1 cells appeared to be highly sensitive to hypoxic conditions and their cAMP responses to all tested drugs were dramatically suppressed. In contrast, the cAMP generating system(s) operating in HUVEC cells appeared to be resistant to hypoxia, irrespective of the fact whether it was due to reduced supply of oxygen or to the presence of $CoCl_2$ in culture medium, although in the latter case there was some tendency to suppression of the forskolin effect. The effect of hypoxic condition on receptor-driven cAMP generation in ECs may be dependent on duration of hypoxia. In contrast to our findings coming from experiments with 24-h hypoxic conditions, Feoktistov et al. [10] have found that 3-h hypoxia did enhance (compared to normoxia) the effectiveness of NECA to stimulate cAMP formation in HUVEC cells. These data considered together may rise a question when there is a switch from activation to inhibition of the stimulated cAMP response of the ECs subjected to hypoxic conditions, and more detailed study with different times of exposure to hypoxia is needed to solve this problem.

In conclusion, the present study carried out in parallel on ECs originating from microvessels (HMEC-1) and macrovessels (HUVEC) have shown the existence of both quantitative and qualitative differences

in biological responses of the two cell systems to adrenaline and adenosine. The hormone adrenaline appeared to be a highly effective stimulator of cAMP production in microvascular ECs, but it displayed only a moderate activity in macrovascular ECs. In contrast to that, selective stimulation of adenosine A_2-type receptors by NECA resulted in comparable increases in cAMP formation in ECs lining micro- and macrovessels. Twenty four-hour hypoxia largely suppressed the cAMP effects resulting from stimulation of both β-adrenoreceptors and adenosine A_2-type receptors in ECs of microvessels, whereas hypoxia had none or only slight effect on these responses in ECs of macrovessels. The obtained findings further confirm the results reported by other scientists showing morphological and functional heterogeneity of ECs, with the greatest differences between those cells residing in macro- and microcirculation as documented in a variety of tissues [3, 7, 27, 28].

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