Cyclic Adenosine Monophosphate-Mediated Enhancement of Vascular Endothelial Growth Factor Released by Differentiated Human Monocytic Cells: The Role of Protein Kinase A

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Abstract

Objective: Our investigation was designed to examine the signaling pathway involved in the enhancement of vascular endothelial growth factor (VEGF) release by β-adrenoceptor agonists. Materials and Methods: Human U937 cells differentiated into macrophages were primed with lipopolysaccharide (LPS) in the absence or presence of β-adrenoceptor agonists and antagonists. The VEGF released and the intracellular cyclic adenosine monophosphate (cAMP) generated were assayed by ELISA. Where necessary, differences between mean values were tested for significance using Student’s t test. Results: Isoprenaline, procaterol and salbutamol concentration-dependently enhanced the release of VEGF induced by LPS in U937 cells. R*,R*-(-)-4-[2-[(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]phenoxyacetic acid (BRL 37344), a selective β3-adrenoceptor agonist, did not enhance VEGF release. Using isoprenaline as an agonist, propranolol, ICI 118551 and atenolol produced a parallel rightward shift of the concentration-response curve with no reduction in the maximum response. The –logKb values were 8.12 ± 0.17, 8.03 ± 0.05 and 7.23 ± 0.05 for propranolol, ICI 118551 and atenolol, respectively, indicating the possible involvement of both β1- and β2-adrenoceptor subtypes. Isoprenaline and prostaglandin E2 concentration-dependently increased cAMP generation in U937 cells. Isoprenaline, db-cAMP and 6-Bnz-cAMP, a protein kinase A (PKA) activator, all enhanced VEGF release induced by LPS, and this effect was abolished by KT 5720 and Rp-cAMPS, which are both selective PKA inhibitors, suggesting that PKA is the downstream effector of cAMP activity. 8-CPT-cAMP, a selective activator of the Epac system, had no effect on VEGF release induced by LPS, indicating that the Epac pathway played no role in the release process. Conclusion: In this study, we established that β1- and β2- but not β3-adrenoceptors mediated cAMP-dependent enhancement of VEGF release induced by LPS in differentiated U937 cells, and that PKA was the downstream effector of cAMP activity.

Key Words
Vascular endothelial growth factor · U937 cells · Protein kinase A · Cyclic adenosine monophosphate · β-Adrenoceptor agonists

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Introduction

β2-Adrenergic agonists are widely used as bronchodilators for the treatment of asthma [1, 2]. They relax the bronchial smooth muscles by a mechanism that involves the accumulation of cyclic adenosine monophosphate (cAMP) [1, 3]. In addition, this class of compounds has been shown, in vitro, to inhibit the release of proinflammatory mediators from eosinophils, neutrophils and macrophages [1–5]. However, chronic administration of these agents has been associated with a loss of bronchodilator function and exacerbation of the chronic inflammatory state. Some studies have suggested that this could be due to desensitization and/or downregulation of the β2-adrenoceptors located on bronchial smooth muscles [1, 3, 5–7].

Vascular endothelial growth factor (VEGF) plays an important role in angiogenesis in a variety of physiological and pathological conditions [8–12]. It contributes to the remodeling of airways smooth muscle associated with chronic asthma [13–16]. Bradbury et al. [17] reported induction of VEGF by prostaglandin E2 (PGE2) in human airway smooth-muscle cells by a mechanism involving cAMP. They also reported that isoprenaline, a nonselective β-adrenoceptor, and forskolin, a direct activator of adenylyl cyclase, similarly induced VEGF release by these cells. This observation has been reproduced in differentiated U937 cells by Verhoeckx et al. [18] who reported an upregulation of VEGF by β2-adrenoceptor agonists in U937 cells exposed to lipopolysaccharide (LPS). This was supported by the demonstration that the β2-adrenergic agonists, zilpaterol and clenbuterol, enhanced the release of VEGF by U937 cells primed with LPS [19]. These investigators also showed that the release of VEGF by these compounds was inhibited by ICI 118551, a selective β2-adrenoceptor antagonist; this indicates a role for β2-adrenoceptors in this release process. They suggested that the release of these proinflammatory proteins by β2-adrenoceptor agonists could account for the adverse effects associated with the chronic use of β2-adrenoceptor agonists. However, in these studies, the role of cAMP and its downstream pathway was not specifically investigated.

There are 3 subtypes of β-adrenoceptors, i.e. β1-, β2- and β3-adrenoceptors. The effect of activation of the β1- and β3-adrenoceptor subtypes on the release of VEGF has not been investigated. This study was designed to investigate the effect of activating the β1-, β2- and β3-adrenoceptor subtypes on the release of VEGF by LPS-primed U937 cells. Specifically, we examined the effect of isoprenaline (a nonselective agonist), salbutamol, pro-
Stimulation of cAMP Release by β-Adrenoceptor Agonists

Differentiated U937 cells were suspended at a concentration of 2 × 10^6/ml in reaction buffer. For these experiments, a 50-μl aliquot of cell suspension was added to each well of a 96-well sterile microplate. This was followed by the addition of 50 μl of isobutyl methyl xanthine (final concentration 250 μM) to prevent cAMP degradation. After 10 min of incubation at 37°C, 50 μl of the different concentrations of the β-adrenoceptor agonists or controls was added, followed by 50 μl LPS (1 μg/ml final concentration). The total volume in each well was 200 μl. The plate was then incubated for 15 min at 37°C, after which, the reaction was stopped by the addition of 22.5 μl of 1 N HCl to all wells. The plate was then further incubated, with frequent mixing, for 10 min at 37°C, centrifuged, and then the supernatant was stored at −40°C pending assay for VEGF.

Statistical Analysis

Experimental data are presented as means ± SEM for n, where n represents the number of independent experiments. A potency comparison of β2-agonists was performed, using the concentrations that gave 50% maximal response (EC50) or their pD2 values, derived from the nonlinear regression analysis of their respective dose-response curves made with GraphPad Prism software (GraphPad Software, Philadelphia, Pa., USA). Where data were expressed as a percentage of the LPS value, differences between mean values were analyzed using a 1-sample Student t test. Data from different treatment groups were subjected to analysis of variance (ANOVA), followed by the Bonferroni post hoc test. p < 0.05 was considered significant.

Results

Effect of β-Adrenoceptor Agonists on LPS-Induced VEGF Release

By themselves, isoprenaline and procaterol (both 10^-9 to 10^-5 M) induced a concentration-dependent release of VEGF by differentiated U937 cells with pD2 values of 7.79 ± 0.17 (n = 3) and 8.01 ± 0.39 (n = 3), respectively. Isoprenaline also concentration-dependently enhanced LPS-induced VEGF release (Fig. 1). The maximum increase (571 ± 116% of the LPS value) was obtained at a concentration of 10^-6 M and the pD2 value was 7.19 ± 0.17 (n = 3) and 8.01 ± 0.39 (n = 3), respectively. The maximum increase in VEGF release was 560 ± 124 and 603 ± 149% for salbutamol and procaterol, respectively. The maximum increase in VEGF release did not differ significantly between the agonists, suggesting that like isoprenaline, both salbutamol and procaterol were full agonists in this preparation. In contrast, BRL 37344 (10^-6 M) did not enhance VEGF release by these cells treated or not treated with LPS.

Characterization of β-Adrenoceptor Subtype Involved in Enhanced VEGF Release

Propranolol, a nonselective β-adrenoceptor antagonist, at a concentration of 3 × 10^-8 M, produced a surmountable and parallel rightward shift of the isoprenaline concentration-response curve with no reduction in the maximum response. The -logKb value was calculated to be 8.12 ± 0.17 (n = 4). Atenolol, a selective β1-adrenoceptor antagonist (at a concentration of 3 × 10^-7 M) and ICI

El-Zohairy/Oriowo/Ezeamuzie

Med Princ Pract 2015;24:548–554
DOI: 10.1159/000433540

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118551, a selective β₂-adrenoceptor antagonist (at a concentration of 3 × 10⁻⁸ M) also produced parallel rightward shifts of the isoprenaline concentration-response curves. As with propranolol, the antagonism was surmountable because no reduction in the maximum response to isoprenaline was observed. The –logK_B values were calculated to be 7.23 ± 0.05 (n = 4) and 8.03 ± 0.05 (n = 4) for atenolol and ICI 118551, respectively. In an attempt to confirm the selectivity of atenolol, the effect of atenolol on the salbutamol-induced increase in VEGF was examined. Atenolol (3 × 10⁻⁷ M) had no significant effect on salbutamol-induced responses.

Role of Intracellular cAMP Generation in VEGF Release

In these experiments, designed to assess the role of cAMP and its downstream signaling pathway in the release of VEGF, isoprenaline and salbutamol were used as agonists while PGE₂ was studied simultaneously for comparison. As shown in figure 2, both isoprenaline and PGE₂ significantly increased cAMP levels in LPS-primed U937 cells in a concentration-dependent manner. PGE₂ appeared to be more potent and more efficacious than the β-agonists.

Analysis of the pathway by which cAMP may cause enhancement of VEGF release revealed that the PKA agonist, 6-Bnz-cAMP, produced a concentration-dependent increase in VEGF release, but that 8-CPT, an activator of the Epac pathway, had no effect on VEGF release at concentrations of 3 × 10⁻⁵ and 10⁻⁴ M (fig. 3). The release of VEGF induced by 6-Bnz-cAMP was significantly reduced by KT 5720 (5 × 10⁻⁷ M) and Rp-cAMP (2 × 10⁻⁴ M), both inhibitors of PKA (fig. 3). The isoprenaline- and db-cAMP-induced increase in VEGF release was abolished by KT 5720 (5 × 10⁻⁷ M) and Rp-cAMP (2 × 10⁻⁴ M), indicating a role for PKA activation in the release of VEGF (fig. 4).
Discussion

Our findings confirmed and extended the initial observations of Verhoeckx et al. [19], i.e. that isoprenaline (nonselective) and the selective β₂-adrenoceptor agonists, salbutamol and procaterol (but not BRL 37344, a selective β₃-adrenoceptor agonist) concentration-dependently released VEGF from differentiated U937 cells and enhanced the VEGF release induced by LPS. The rank order of potency was isoprenaline > procaterol ≥ salbutamol. BRL 37344 had no significant effect on LPS-induced VEGF release, indicating that the activation of β₃-adrenoceptors was not involved in the release of VEGF by isoprenaline.

We investigated if β₁- and β₂-adrenoceptors play a role in the isoprenaline-induced enhancement of VEGF release, by studying the effects of propranolol (a nonselective β-adrenoceptor antagonist), ICI 118551 (a selective β₂-adrenoceptor antagonist) and atenolol (a selective β₁-adrenoceptor antagonist) on the VEGF release induced by isoprenaline. Only one concentration of these antagonists was used, based on concentrations reported in the literature as having been used previously [21]. The results showed that, on their own, none of the antagonists had any effect on VEGF release. Propranolol antagonized isoprenaline-induced enhancement of VEGF release with a –logKᵦ value of 8.12 ± 0.17. ICI 118551 antagonized isoprenaline-induced enhancement of VEGF release with a –logKᵦ value of 8.03 ± 0.05, which is within the range of values reported for an action on β₂-adrenoceptors [21], thus suggesting that β₁-adrenoceptors could also be involved. The investigation of whether or not the antagonist effect of atenolol against isoprenaline was due to a nonspecific effect, using the same concentration of atenolol on salbutamol-induced enhancement of VEGF release, showed that atenolol did not significantly reduce salbutamol-induced enhancement of VEGF release. This confirmed that the effect of atenolol was not nonspecific, i.e. that β₁-adrenoceptors do indeed play a role in the isoprenaline-induced enhancement of VEGF release.

Activation of β₁-adrenoceptors is linked to adenylate cyclase activation and generation of cAMP. Our results also showed that isoprenaline increased cAMP production in U937 cells with a potency that was very similar to that for VEGF release, confirming that the effect of isoprenaline on VEGF release was due to cAMP. This confirmed previous reports by Bradbury et al. [17] and Verhoeckx et al. [19]. PKA and Epac are two known downstream effectors mediating the activities of cAMP. These pathways have been shown to either act cooperatively or independently to mediate cAMP actions. Previous stud-
ies [22–24] show that both PKA and Epac are involved in the release of proinflammatory mediators in human lung tissue [22] and airway smooth-muscle cells [23] and in cAMP-mediated mitogenesis [24]. While it has been reported that the PKA pathway is involved in norepinephrine-induced expression of the VEGF gene in brown adipocytes [25] and the proasthmatic effect associated with chronic administration of β2-agonists [26], Epac appears to be involved in cAMP-mediated cell adhesion [27], airway smooth-muscle cell proliferation [28] and the production of proinflammatory cytokines by macrophages after stimulation by β2-agonists [26]. In this study, we determined that the PKA pathway, rather than the Epac pathway, was responsible for the cAMP-dependent enhancement of VEGF release by isoprenaline in U937 cells. This conclusion was based on the fact that 6-Bnz-cAMP, a selective activator of PKA [28, 29], but not 8-CPT-cAMP, the Epac agonist [29, 30], significantly enhanced LPS-induced VEGF release, i.e. suggesting a role for the PKA pathway but not the Epac pathway in VEGF release.

This was confirmed by the observation that KT 5720 and Rp-cAMPS, selective PKA antagonists, abolished the effect of isoprenaline and db-cAMP at the same concentrations that they abolished the effect of 6-Bnz-cAMP on VEGF release.

### Conclusion

In this study, we established that β1- and β2- but not β3-adrenoceptors mediated the enhancement of VEGF release by β-adrenoceptor agonists in LPS-primed U937 cells, and that this release was via cAMP-dependent signaling through the activation of PKA.

### Acknowledgement

This study was supported by a Research Sector, Kuwait University grant (No. YM 14/09).

### References


Erratum

In the article by El-Zohairy et al., entitled 'Cyclic adenosine monophosphate-mediated enhancement of vascular endothelial growth factor released by differentiated human monocytic cells: the role of protein kinase A' [Med Princ Pract 2015;24:548–554, DOI: 10.1159/000433540], the labeling of the x-axis in figures 3 and 4 is wrong. The units should be micromolar (μM) and not millimolar (mM), as shown below.

Fig. 3. **a** The effect of different cAMP pathway activators on LPS-induced VEGF release from differentiated U937 cells. Values are means ± SEM of 4 separate experiments. **b** Effect of PKA inhibitors on 6-Bnz-cAMP-induced VEGF release from LPS-stimulated U937 cells. Values are means ± SEM of 6 (Bnz) and 4 (Rp-cAMP and KT 5720) separate experiments. Isop. = Isoprenaline; KT = KT 5720; RP = Rp-cAMP.

Fig. 4. Effect of PKA inhibitors (Rp-cAMP and KT 5720) on isoprenaline- and db-cAMP-induced VEGF release from LPS-stimulated U937 cells. Values are means ± SEM of 6 (isoprenaline) and 4 (db-cAMP) separate experiments. Isop. = Isoprenaline; KT = KT 5720; RP = Rp-cAMP.