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-[(2-(3-chlorophenyl)-2-hydroxyethyl)amino]propyl]phenoxyacetic acid (BRL 37344), a selective β_3-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 $-\log K_B$ 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 β_1- and β_2-adrenoceptor subtypes. Isoprenaline and prostaglandin E_2 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 β_1- and β_2- but not β_3-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.

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Introduction

β_2-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 β_2-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 E_2 (PGE_2) 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 adenyl 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 β_2-adrenoceptor agonists in U937 cells exposed to lipopolysaccharide (LPS). This was supported by the demonstration that the β_2-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 β_2-adrenoceptor antagonist; this indicates a role for β_2-adrenoceptors in this release process. They suggested that the release of these proinflammatory proteins by β_2-adrenoceptor agonists could account for the adverse effects associated with the chronic use of β_2-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. β_1-, β_2- and β_3-adrenoceptors. The effect of activation of the β_1- and β_3-adrenoceptor subtypes on the release of VEGF has not been investigated. This study was designed to investigate the effect of activating the β_1-, β_2- and β_3-adrenoceptor subtypes on the release of VEGF by LPS-primed U937 cells. Specifically, we examined the effect of isoprenaline (a nonselective agonist), salbutamol, procaterol (both selective β_2-adrenoceptor agonists) and BRL 37344 (a β_3-adrenoceptor agonist) on VEGF release by U937 cells with and without priming with LPS. The signaling mechanism, specifically, the role of cAMP and the downstream pathway, either protein kinase A (PKA) or Epac, involved in the release process were also investigated.

Materials and Methods

Cell Culture and Differentiation

Human monocytic cells (U937) obtained from the American Type Culture Collection (Manassas, Va., USA), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 µg/l streptomycin and 100 U/ml penicillin at 37°C in 5% CO_2. Cells in passages 3–7 were differentiated into macrophages with 30 mM PMA for 24 h, followed by a 24-hour recovery period. Adherent cells were recovered by gentle scrapping and subsequently resuspended in the same culture medium at 1×10^6 cells/ml for the experiments.

Stimulation/Enhancement of VEGF Release

The effect of β-adrenoceptor agonists on LPS-induced VEGF release was examined as follows: an aliquot of 100 µl of cell suspension was added to each well of a 96-well microplate, followed by the addition of 50 µl of complete medium or the test drugs made up in the medium. The mixture was incubated for 10 min, followed by the addition of 1 µg/ml of LPS, and then incubated for 24 h, a time previously determined in preliminary experiments as being optimal for VEGF release. Some wells contained LPS only, to measure the unenhanced release of VEGF, and some contained β-adrenoceptor agonists but no LPS, to determine the effect of β-adrenoceptor agonists, on their own, on VEGF release. Different concentrations (10^{-9} to 10^{-5} M) of the agonists were tested to generate data for a concentration-response curve. At the end of the incubation, the supernatant was recovered by centrifugation (1,600 rpm for 5 min) and assayed for VEGF, using commercially available ELISA kits (R&D Systems Inc., Minneapolis, Minn., USA) according to the manufacturer's instructions. The limit of detection was 0.78 pmol/ml. Agonist potencies were expressed as pD_2 values, where pD_2 is the negative logarithm of the agonist concentration producing 50% of the maximum response.

In another series of experiments designed to determine which β-adrenoceptor subtype was involved in the enhancement of VEGF release, a 125-µl aliquot of the cell suspension was added to each well. This was followed by the addition of 25 µl of the antagonist or drug vehicle complete medium (control wells) and incubation for 30 min. Thereafter, 25 µl of different concentrations of isoprenaline were added and the mixture was incubated for 10 min, following which, the cells were stimulated with LPS (1 µg/ml) for 24 h. The reaction was stopped by centrifugation and the supernatant assayed for VEGF as described above. The pD_2 values for isoprenaline in the absence and presence of the antagonists were used to calculate the concentration ratios. Dissociation constants of the antagonists were calculated using the equation: K_B = [antagonist]/CR-1 [20], where CR is the concentration ratio.

In experiments in which the cAMP signaling pathway was investigated, 50 μl of cell suspension was added to all wells, followed by 50 μl of the PKA antagonists KT 5720 or Rp-cAMPS or drug vehicle in complete medium (control) to the assigned wells. The plate was then incubated for 30 min at 37°C. Thereafter, 50 μl of isoprenaline (0.1 μM final concentration) or different concentrations of the agonists, i.e. 6-Bnz-cAMP and 8-CPT-cAMP, or 30 μM db-cAMP or vehicle in complete medium (control) were added to their corresponding wells.

The plate was then incubated for 15 min at 37°C, after which, 50 μl of LPS (1 μg/ml final concentration) was added to all wells except the spontaneous wells, to which complete medium was added. The volume in each well was 200 μl. The plate was then incubated for 24 h at 37°C. After incubation, the reaction was stopped by centrifugation at 1,600 rpm for 5 min at 5°C. The supernatant was taken and stored at –40°C pending assay for VEGF.

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 cAMP assay. PGE$_2$ was studied simultaneously for comparison. The concentration of cAMP in the supernatant was determined using an ELISA kit (Eazo Life Sciences, Farmingdale, N.Y., USA) and the experiment was performed according to the manufacturer's instructions. The limit of detection was 0.037 pmol/ml.

Statistical Analysis

Experimental data are presented as means ± SEM for n, where n represents the number of independent experiments. A potency comparison of β$_2$-agonists was performed, using the concentrations that gave 50% maximal response (EC$_{50}$) or their pD$_2$ 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 Bonferonni post hoc test. p < 0.05 was considered significant.

Results

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

By themelves, isoprenaline and procaterol (both 10^{-9} to 10^{-5} M) induced a concentration-dependent release of
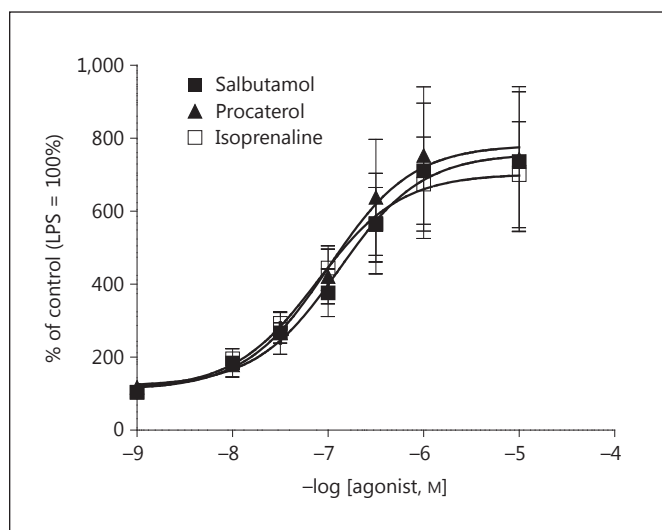

Fig. 1. Effect of salbutamol, isoprenaline and procaterol on LPS-induced VEGF release from differentiated U937 cells. Values are means ± SEM of 5 separate experiments.

VEGF by differentiated U937 cells with pD$_2$ 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 pD$_2$ value was 7.19 ± 0.10 (n = 5). Like isoprenaline, salbutamol and procaterol (both 10^{-9} to 10^{-5} M) concentration-dependently enhanced VEGF release induced by LPS (fig. 1), with pD$_2$ values of 6.96 ± 0.07 (n = 5) and 7.08 ± 0.08 (n = 5), 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 –logK$_B$ value was calculated to be 8.12 ± 0.17 (n = 4). Atenolol, a selective β$_1$-adrenoceptor antagonist (at a concentration of 3×10^{-7} M) and ICI
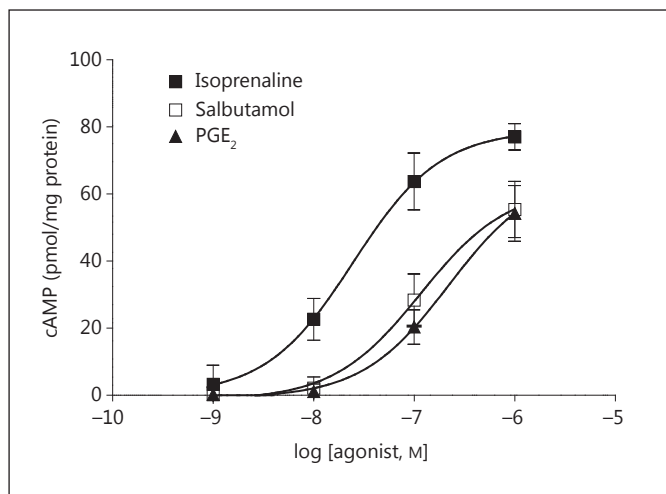
Fig. 2. Effect of isoprenaline, salbutamol and PGE2 on generation of cAMP by differentiated U937 cells. Values are means ± SEM of 4 separate experiments.

118551, a selective β_2-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 $-\log K_B$ 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 PGE$_2$ was studied simultaneously for comparison. As shown in figure 2, both isoprenaline and PGE$_2$ significantly increased cAMP levels in LPS-primed U937 cells in a concentration-dependent manner. PGE$_2$ 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
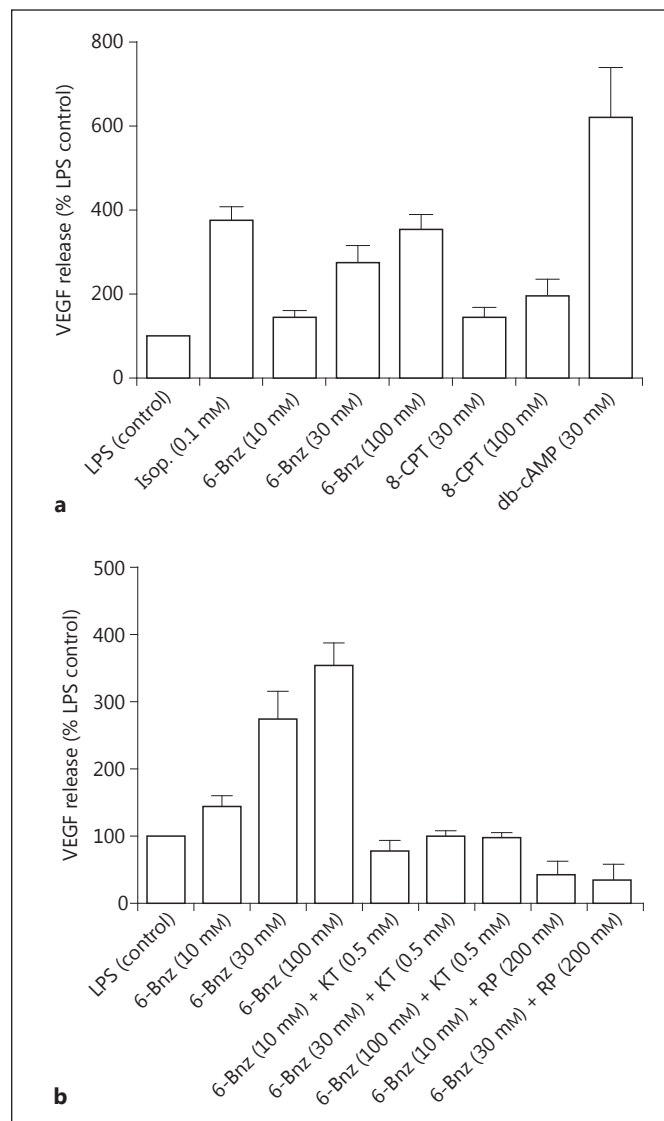

Fig. 3. a The effect of different cAMP pathway activators on LPS-induced VEGF release from differentiated U937 cells. Values are means ± SEM of 4 separate experiments. **b** Effect of PKA inhibitors on 6-Bnz-cAMP-induced VEGF release from LPS-stimulated U937 cells. Values are means ± SEM of 6 (Bnz) and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

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).

β-Adrenoceptor Agonists and VEGF Release

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.
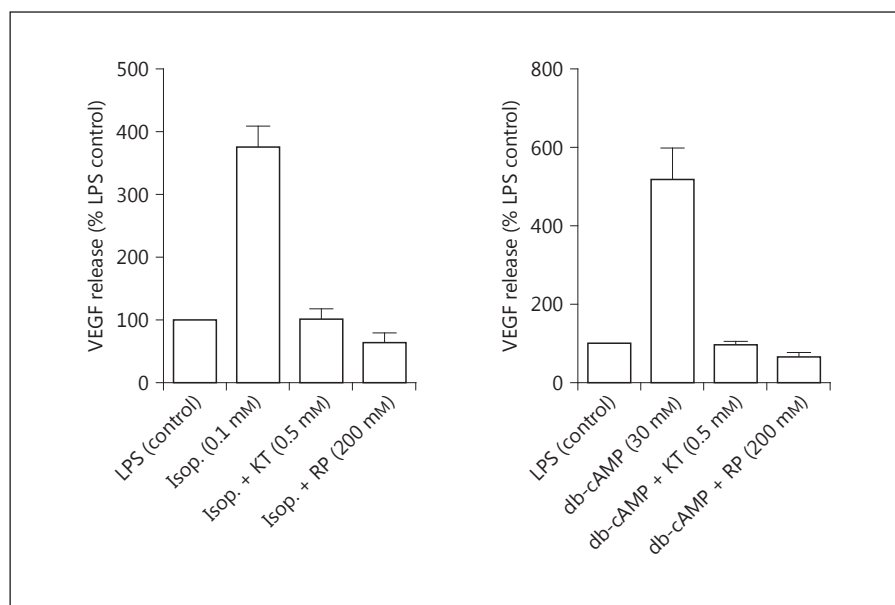
Discussion

Our findings confirmed and extended the initial observations of Verhoeckx et al. [19], i.e. that isoprenaline (nonselective) and the selective β_2-adrenoceptor agonists, salbutamol and procaterol (but not BRL 37344, a selective β_3-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 β_3-adrenoceptors was not involved in the release of VEGF by isoprenaline. We investigated if β_1- and β_2-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 β_2-adrenoceptor antagonist) and atenolol (a selective β_1-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 $-\log K_B$ value of 8.12 ± 0.17. ICI 118551 antagonized isoprenaline-induced enhancement of VEGF release with a $-\log K_B$ value of 8.03 ± 0.05, which is within the range of values expected of an action on β_2-adrenoceptors [21].

These results, in agreement with a previous report by Verhoeckx et al. [19], would confirm that β_2-adrenoceptors play a role in the isoprenaline-induced enhancement of VEGF release. However, atenolol, a selective β_1-adrenoceptor antagonist, also antagonized the effect of isoprenaline, with a $-\log K_B$ value of 7.23 ± 0.05 which is within the range of values reported for an action on β_1-adrenoceptors [21], thus suggesting that β_1-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 β_1-adrenoceptors do indeed play a role in the isoprenaline-induced enhancement of VEGF release.

Activation of β-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-

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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 β_2-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 β_2-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 β_1- and β_2- but not β_3-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

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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.
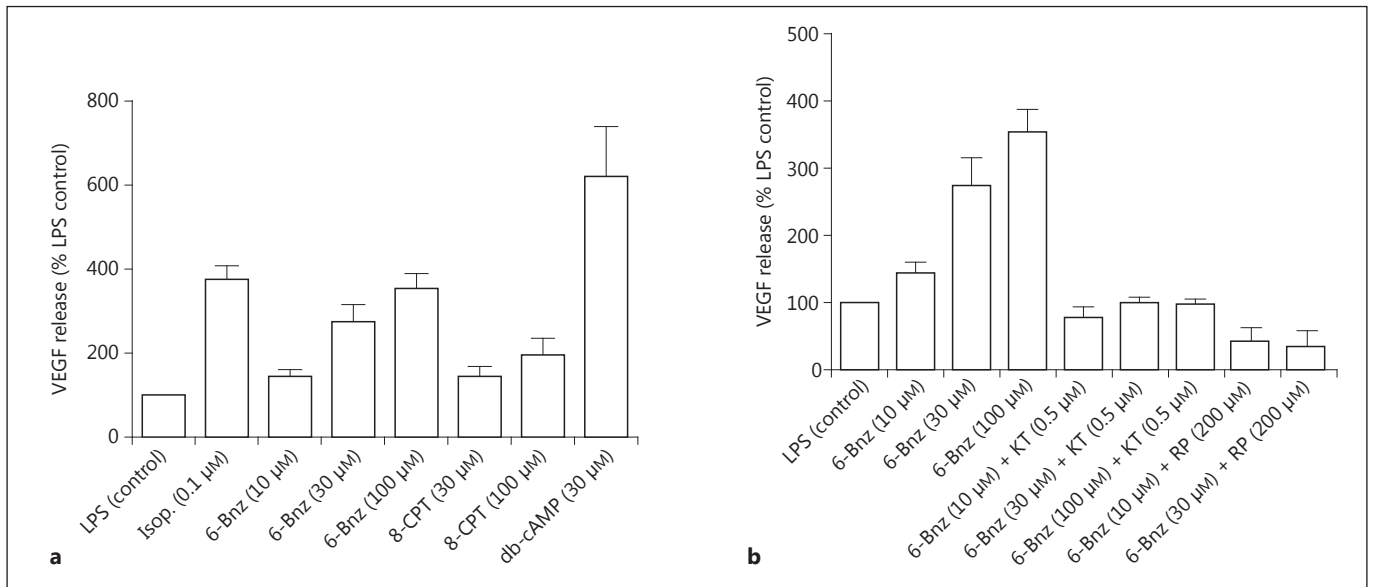

Fig. 3. a The effect of different cAMP pathway activators on LPS-induced VEGF release from differentiated U937 cells. Values are means ± SEM of 4 separate experiments. **b** Effect of PKA inhibitors on 6-Bnz-cAMP-induced VEGF release from LPS-stimulated U937 cells. Values are means ± SEM of 6 (Bnz) and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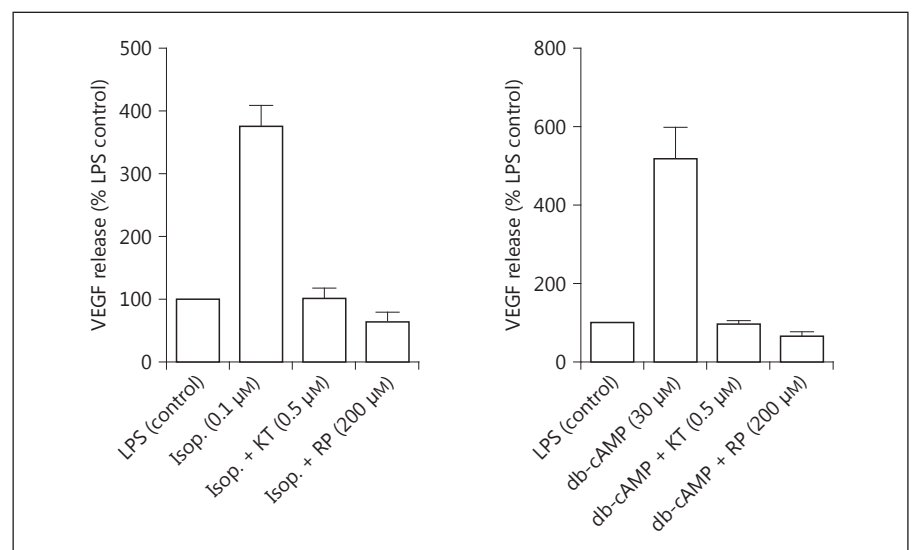

Fig. 4. Effect of PKA inhibitors (Rp-cAMP and KT 5720) on isoprenaline- and db-cAMP-induced VEGF release from LPS-stimulated U937 cells. Values are means ± SEM of 6 (isoprenaline) and 4 (db-cAMP) separate experiments. Isop. = Isoprenaline; KT = KT 5720; RP = Rp-cAMP.