β_2-ADRENOCEPTOR ACTIVATION CAUSES PROTEIN KINASE A- AND AKT-DEPENDENT PHOSPHORYLATION OF NITRIC OXIDE SYNTHASE TYPE 3 IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Stimulation of β_2-adrenoceptors (β_2AR) on vascular endothelium causes vasorelaxation through endothelial cell release of nitric oxide, with no change in endothelial cell Ca^{2+} concentration (Ferro *et al.*, 1999). We hypothesised that this might be explained by β_2AR-mediated serine phosphorylation of endothelial-type nitric oxide synthase (NOS3). The aim of the present work was to examine changes in serine phosphorylation of NOS3 in response to β_2AR stimulation in human umbilical vein endothelial cells (HUVEC), and whether such changes could be explained by the action of protein kinase A (PKA) and/or protein kinase B (PKB, Akt).

HUVEC were isolated from umbilical cords derived from healthy uncomplicated pregnancies, and cultured to confluence at passage 3, as previously described (Ferro *et al.*, 1999). Following extensive washing, and equilibration in Krebs buffer, HUVEC were incubated with the NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA) 10^{-4}M or corresponding vehicle. Additionally, incubations were performed with the PKA inhibitor H-89 10^{-7}M, the phosphatidylinositol 3-kinase inhibitor wortmannin 500nM, Akt inhibitor 10^{-5}M, or corresponding vehicle. All incubations were for 15 min, after which the selective β_2AR agonist albuterol 10^{-5}M or the direct-acting adenylyl cyclase activator forskolin 10^{-5}M were added for a further 10 min. Reactions were terminated by the addition of ice-cold lysis buffer, cells were sonicated and disrupted using a cell scraper; the lysates were immunoprecipitated using a rabbit polyclonal anti-NOS3 antibody, and NOS3 expression as well as serine phosphorylation of NOS3 were analysed by western blotting, as previously described (Xu *et al.*, 2003). Data are expressed as the densitometric ratio of the phosphoserine to the total NOS3 bands, and are mean ± s.e.m. of 5 experiments. Statistical analysis was by repeated measures one-way ANOVA, with $P<0.05$ (two-tailed) taken as indicating statistical significance.

The phosphoserine/total NOS3 densitometric ratio in untreated cells was 0.41±0.07 arbitrary units, and this increased to 0.82±0.04 in response to albuterol ($P<0.01$). This increase was abolished when H-89 was co-incubated (0.47±0.02, $P<0.05$ as compared with albuterol alone), but only partially attenuated in the presence either of wortmannin or of Akt inhibitor (densitometric ratios 0.64±0.01 and 0.65±0.05 respectively; $P<0.05$ for each as compared with vehicle and also as compared with albuterol alone). Forskolin also increased phosphoserine/total NOS3 densitometric ratio, to 0.72±0.02 ($P<0.05$ as compared with vehicle). The increases to albuterol and to forskolin were not attenuated by L-NMMA.

In conclusion, β_2AR stimulation with albuterol or adenylyl cyclase activation with forskolin each cause an increase in serine phosphorylation of NOS3 in HUVEC. The increase in response to β_2AR stimulation is not affected by NOS inhibition, but is partially attenuated by inhibition of the Akt pathway and is abolished by PKA inhibition, suggesting that both PKA and Akt are important in this response.

Ferro, A. *et al*. (1999) *Br. J. Pharmacol.* **126,** 1872-1880.
Xu, B. *et al.* (2003) *FASEB J.* **17,** 1289-1291.