Dissecting the pathways leading to murine free-fatty acid receptor 4 (FFAR4) and arrestin interactions in HEK293 cells lacking β-arrestin-1/2: consequences for receptor internalisation, desensitisation and signalling

The murine free-fatty acid receptor 4 (mFFAR4) responds to long chain fatty acids (LCFAs) or synthetic agonist ligands by rapidly phosphorylating specific residues of its C-terminus (Thr347, Thr349, Ser350, Ser357 and Ser361) (1). This in turn, promotes β-arrestin-2 recruitment to the receptor and subsequent receptor internalisation (1). Mutations on these residues in the C-terminus have been shown to impair β-arrestin recruitment to the receptor in both the human and mouse receptor orthologues (1, 2). Activation of mFFAR4 also triggers the activation of $G_{q/11}$ heterotrimeric G-proteins, which has been associated with the stimulation of GLP-1 release in enteroendocrine L cells where FFAR4 can be found (3), whereas β-arrestin mediated signalling has been suggested to modulate anti-inflammatory effects in adipose tissue via infiltrated macrophages where FFAR4 expresses abundantly (4). It is therefore proposed that the balance between G-protein and β-arrestin mediated signalling dictates the final outcome of receptor activation. In this study we have used HEK293 cells lacking functional β-arrestin-1 and 2 proteins (β-arrestin1/2-KO cells) generated by CRISPR-Cas9-mediated genome editing expressing constitutively a FLAG-mFFA4-eYFP construct, to analyse the dynamics of agonist mediated receptor internalisation, desensitization and signalling.

Initial characterisation of the parental HEK293 and β-arrestin1/2-KO cells by Western blot using specific antibodies raised against either form of β-arrestin showed a 50 kDa band consistent with either β-arrestin1 or 2 present in the parental HEK293 cell lysates that was absent in the β-arrestin1/2-KO cells. These cells were subsequently transfected with wild type versions of mFFA4 (FLAG-mFFA4-eYFP) and clones showing stable expression of the receptor at the cell membrane were selected in DMEM supplemented with 1.5μg.ml^{-1} of geneticin.

Imaging experiments together with quantitative ELISA assays were carried out on the selected clones to monitor receptor internalisation. These showed that the FLAG-mFFAR4-eYFP receptor was efficiently internalised upon stimulation with 10 μM of the synthetic agonist TUG-891 ([(3-(4-((4-fluoro-4'-methyl-[1,1'-biphenyl]-2-yl)methoxy)phenyl)propanoic acid]) in the parental HEK293 cells whereas this was markedly impaired in the β-arrestin1/2-KO cellular background. Here only 25% of receptor internalisation was detected after 60 min agonist treatment compared to over 50% of internalised receptor in the parental HEK293 cells. In addition to this, the FLAG-mFFAR4-eYFP receptor failed to desensitise efficiently when challenged repeatedly with TUG-891 in the β-arrestin1/2-KO cells.

To further analyse the mode of arrestin-receptor interaction site directed mutagenesis was used to replace serine or threonine residues that are phosphorylated upon agonist treatment (1) with alanines, generating a "phospho-deficient" mutant. Moreover, using this as template, C-terminal acidic residues (Glu341, Asp348, and Asp355) were further mutated and both mutated forms of the receptor were used in BRET based β-arrestin recruitment and desensitisation assays. We found that in both cases the ability of the receptor to both desensitise and efficiently recruit β-arrestin-2 was greatly reduced compared with the wild type consistent with the findings on the β-arrestin1/2-KO cells.

(1) Prihandoko *et al*., (2016) *Mol Parmacol* (in press)
(2) Butcher *et al*., (2014) *J Biol Chem* **289**: 18451–65
(3) Hirasawa *et al*., (2005) *Nat Med* **11**: 90–4
(4) Oh *et al*., (2010) *Cell* **142** :687–98