 doi:10.20944/preprints202105.0087.v1

Type of the Paper (Article)

Functional Characterization of the obesity-linked variant of the β3-adrenergic receptor

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Abstract: Adrenergic receptor β3 (ADRβ3) is a member of the rhodopsin-like G protein-coupled receptor family. The binding of the ligand to ADRβ3 activates adenylate cyclase and increases cAMP in the cells. ADRβ3 is highly expressed in white and brown adipocytes and controls key regulatory pathways of lipid metabolism. Trp64Arg (W64R) polymorphism in the ADRβ3 has been associated with the early development of type 2 diabetes mellitus, lower resting metabolic rate, abdominal obesity, and insulin resistance. It is unclear how the substitution of W64R affects the functioning of ADRβ3. This study was initiated to functionally characterize this obesity-linked variant of ADRβ3. We evaluated in detail the expression, subcellular distribution, and post-activation behavior of the WT and W64R ADRβ3 using a single cell quantitative fluorescence microscopy. When expressed in HEK 293 cells, ADRβ3 shows a typical distribution displayed by other GPCRs with a predominant localization at the cell surface. Unlike Adrenergic receptor β2 (ADRβ2), agonist induced desensitization of ADRβ3 does not involve loss of cell surface expression. WT and W64R variant of ADRβ3 displayed comparable biochemical properties and there was no significant impact of the substitution of Tryptophan with Arginine on the expression, cellular distribution, signaling, and post-activation behavior of ADRβ3. The obesity-linked W64R variant of ADRβ3 is indistinguishable from the WT ADRβ3 in terms of expression, cellular distribution, signaling, and post-activation behavior.

Keywords: G-Protein Coupled Receptors; beta-3-adrenergic receptor; receptor desensitization

1. Introduction

Beta-adrenergic receptors (ADRβ) belong to the family of seven transmembrane receptors called G-protein-coupled receptors (GPCRs) (1-3). They are expressed on the surface of several cell types and can bind epinephrine and norepinephrine as well as exogenously administered drugs, including beta-agonists and antagonists ('beta-blockers') (4-8). ADRβ play a key role in several important processes including fat and glucose metabolism and alterations in myocardial metabolism, heart rate, and systolic and diastolic function (9-14). Beta-adrenergic receptors are important drug targets for asthma and cardiovascular conditions including hypertension and congestive heart failure (15-17). There are three subtypes of Beta-adrenergic receptors; ADRβ1, ADRβ2 and ADRβ3 encoded by three separate genes. ADRβ1 and ADRβ2 have been comprehensively studied and have important effects on pulmonary and cardiac physiology. ADRβ3 is the newest isoform of this family and has been least studied to date (18, 19). ADRβ3 is predominantly expressed in white and brown adipose tissues, gastrointestinal tract and in the brain (19-22). Several studies have shown that ADRβ3 plays an important role in metabolic homeostasis. Activation of ADRβ3 with selective agonists stimulates lipolysis and release of fatty acids in white adipose tissue (WAT) and also activation of thermogenesis

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in brown adipose tissue (BAT) (19, 21, 23). ADRβ3 is now recognized as an attractive target for drug discovery and several recent efforts in this field have been directed toward the design of potent and selective ADRβ3 agonists (24, 25). Several groups have independently reported that the mutation of tryptophan to arginine at position 64 of human ADRβ3 (W64R) shows a strong association with obesity, glucose intolerance, hypertension, dyslipidemia, and early onset of Type 2 diabetes mellitus (26-34). Still, the functional significance of the mutation on the ADRβ3 functioning remains unclear and previous studies have shown contradictory results (35-37). No previous study has assessed sub-cellular distribution or post-activation behavior of the mutant receptor. Here we evaluated in detail the expression, membrane trafficking, signaling in response to agonist activation, and post-activation behavior of the receptor. Remarkably, the substitution of Tryptophan with Arginine (W64R) did not alter the expression or the membrane trafficking of ADRβ3. Besides, there was no difference in agonist-induced cAMP formation in between the WT and mutant ADRβ3. Even the post-activation behavior of the WT and mutant ADRβ3 was identical to the WT receptor.

2.1. Results

2.1.1 ADRβ3 (W64R) variant shows normal protein expression and subcellular distribution

To evaluate the impact of Tryptophan to Arginine substitution on ADRβ3 expression and function, we engineered ADRβ3 construct with an HA tag at the extracellular N-terminus and a GFP tag at the intracellular C-terminus (Fig 1A). To ensure HA and GFP tags do not interfere with the functioning of ADRβ3, the receptor construct was functionally validated in HEK 293 cells (Fig 1B). Cells expressing empty vector, untagged ADRβ3 or HA-ADRβ3-GFP were stimulated with ADRβ3 agonist SR-58611A and cAMP was measured using chemiluminescence based immunoassay kit. Stimulation with SR-58611A induced cAMP formation in a dose dependent manner in cells expressing ADRβ3. cAMP formation was comparable in cells expressing untagged or tagged version of ADRβ3 indicating that HA and GFP tags have no effect on receptor function (Fig 1C). Next, we generated W64R HA-ADRβ3-GFP variant in the HA-ADRβ3-GFP construct using site-directed mutagenesis. The mutation was confirmed by DNA sequnsing. WT and W64R HA- ADRβ3-GFP constructs were stably expressed in HEK 293 cells and the effect of W64R mutation on the expression and subcellular distribution of ADRβ3 was assessed. ADRβ3 showed a typical GPCR pattern in HEK 293 cells and the receptor showing predominantly plasma membrane expression (Fig 2A). Interestingly, W64R mutation did not affect the total cellular expression nor the surface expression of ADRβ3 (Fig 2B and 2C). The total cellular and surface expression were determined using single-cell quantitative fluorescence and therefore included a broad range of receptor expression. Remarkably, irrespective of the level of expression the W64R mutation did not alter the cellular distribution of ADRβ3.

2.1.2. W64R mutation does not alter the agonist-induced cAMP formation

ADRβ3 belongs to the family of G-protein coupled receptors (GPCRs) that signal through the G- protein complex. ADRβ3 signals through the activation of the G-protein subunit (Gαs) that activates adenylate cyclase leading to the formation of cAMP. Disease-linked GPCR variants are often associated with impaired receptor signaling leading to cellular defects. To evaluate if W64R mutation affects the signaling of ADRβ3, we measured the agonist-induced cAMP formation in cells expressing WT or W64R ADRβ3. Cells were stimulated with various concentrations of ADRβ3 agonist (SR-58611A) and cAMP was measured as described in the method section (Fig 3A). SR-58611A induced a dose-dependent increase in cellular cAMP in cells expressing WT or W64R ADRβ3 (Fig 3B). Interestingly, W64R mutation had no impact on the agonist induced cAMP formation, suggesting no alteration of receptor signaling because of W64R substitution.

2.1.3. Agonist induced desensitization of ADRβ3 does not involve loss of surface receptor expression

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One of the significant features of G protein signaling systems is that they show a memory of the previous activation. Agonist stimulation of a particular GPCR rapidly activates effector pathways downstream of the receptor resulting in the formation of secondary messengers like cAMP, calcium, and diacylglycerol. This response is rapid and occurs within a few minutes of agonist stimulation. The majority of the GPCRs undergo "desensitization" in response to the agonist stimulation (38). The desensitization of a G protein-coupled receptor (GPCR) response can be described as the loss of response after prolonged or repeated administration of an agonist. Agonist induced desensitization of ADRβ3 was determined by measuring cAMP formation in response to consecutive challenges of β3 agonist, SR-58611A. ADRβ2 was used as a control since its signaling and post-activation behavour has been comprehensively studied (39-41). The scheme of the experiment is depicted in Fig 4 A. Cells expressing HA-ADRβ2-GFP and HA-ADRβ3-GFP reporter constructs were stimulated with indicated agonists once or twice and cAMP was measured as described. Activation of ADRβ2 and ADRβ3 lead to a robust increase in cAMP levels after 1st challenge. There was a drastic reduction of cAMP formation after 2nd challenge as compared to the 1st challenge in both ADRβ2 and ADRβ3 expressing cells indicating a comparable level of desensitization (Fig 4 B and 4 C).

In the majority of GPCRs including ADRβ2, agonist-induced desensitization occurs via loss of receptors from the cell surface. Stimulation with the ligands leads to phosphorylation of the receptor with G-protein regulated kinases (GRKs) that eventually leads to the internalization of the receptor. Previous studies have shown that ADRβ3 does not have the consensus phosphorylation sequence, and is resistant to agonist-induced internalization and therefore shows no loss of cell surface receptor post activation (42). To determine the subcellular distribution of ADRβ2 and ADRβ3, cells were stimulated with respective agonists, fixed under non-permeabilizing conditions and stained with antibodies as described in the method section. Cells were imaged and image processing was done to determine cells surface and total expression of ADRβ2 and ADRβ3 in each cell before and after stimulation with the agonist. As shown in Fig 4 D and 4E activation of ADRβ2 leads to a loss of receptor post-stimulation whereas ADRβ3 surface expression remained unchanged after stimulation. This indicated that unlike ADRβ2, agonist induced desensitization of ADRβ3 is not a result of loss of surface receptor and possibly involves a different mechanism.

2.1.4. W64R mutation does not affect the post-activation behavior of ADRβ3

One of the possible impacts of W64R mutation on ADRβ3 could involve post activation behavior of the receptor. Earlier studies have demonstrated that disease-linked mutants of GPCRs can impact post-activation behavior of the receptor and undergo excessive desensitization leading to metabolic abnormalities. We evaluated the extent of desensitization in cells expressing WT or W64R ADRβ3 and compared it with that ADRβ2, a well-studied and closely related GPCR. ADRβ2 and ADRβ3 showed reduced cAMP formation in response to the second challenge with the agonist, indicating receptor desensitization. The reduction of cAMP formation in response to second challenge of agonist stimulation of W64R ADRβ3 was comparable to the WT ADRβ3 suggesting that the mutation has no impact on the extent of desensitization of ADRβ3. (Fig 5A). We further evaluated the post activation behavior of WT and W64R ADRβ3 by determining surface and total expression of the receptor before and after stimulation with the agonist. In the unstimulated state ADRβ2 and ADRβ3 are predominant expressed at the plasma membrane. After stimulation, ADRβ2 redistributed and accumulated in intracellular spaces whereas both WT and W64R ADRβ3 do not undergo redistribution and maintain the plasma membrane expression (Fig 5B). Image analysis and quantification of the fluorescence reveled that agonist stimulation does not alter the cell surface expression of both WT and W64R variant of ADRβ3 (Fig 5C). In contrast, agonist stimulation resulted in a significant loss of surface expression of the ADRβ2 (Fig 5C).Taken together, these results indicate that W64R mutation has no impact on the post-activation behavour of ADRβ3.

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2.2. Figures

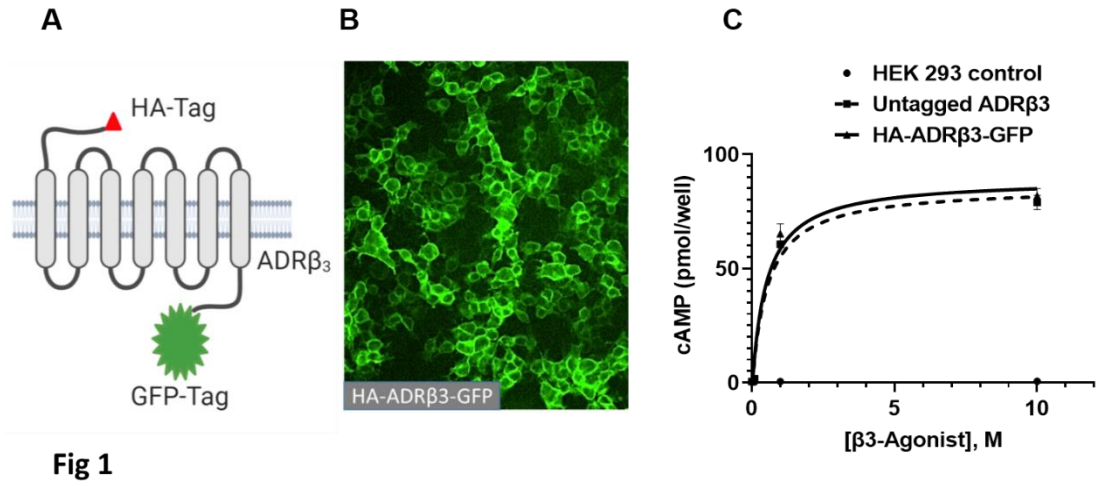

Fig 1

Fig. 1 Engineering of HA-ADRβ3-GFP reporter construct and its functional validation

A) HA-ADRβ3-GFP reporter construct was generated by PCR cloning as described in the method section. B) HEK293 cells stably expressing HA-ADRβ3-GFP. C) β3 agonist induced cAMP formation in cells expressing empty vector, untagged ADRβ3 or HA-ADRβ3-GFP.

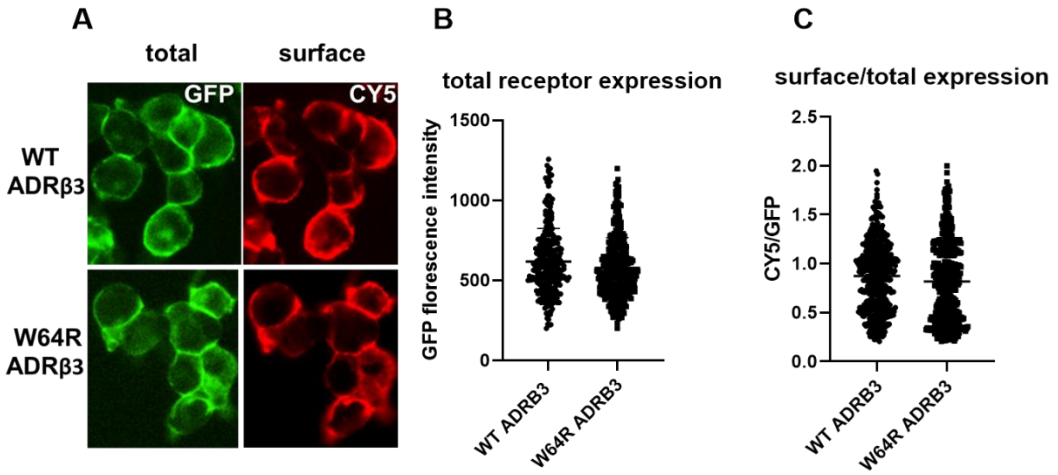

Fig 2

Fig. 2. Total and cell surface expression of WT and W64R variant of ADRβ3

A) Imaging showing GFP (total) and CY5 (cell surface) expression of WT and W64R ADRβ3 B and C) Single cell analysis showing total (B) and cell surface expression (C) of WT and W64R ADRβ3. Each graph represents analysis of at least 100 cells across multiple experiments.

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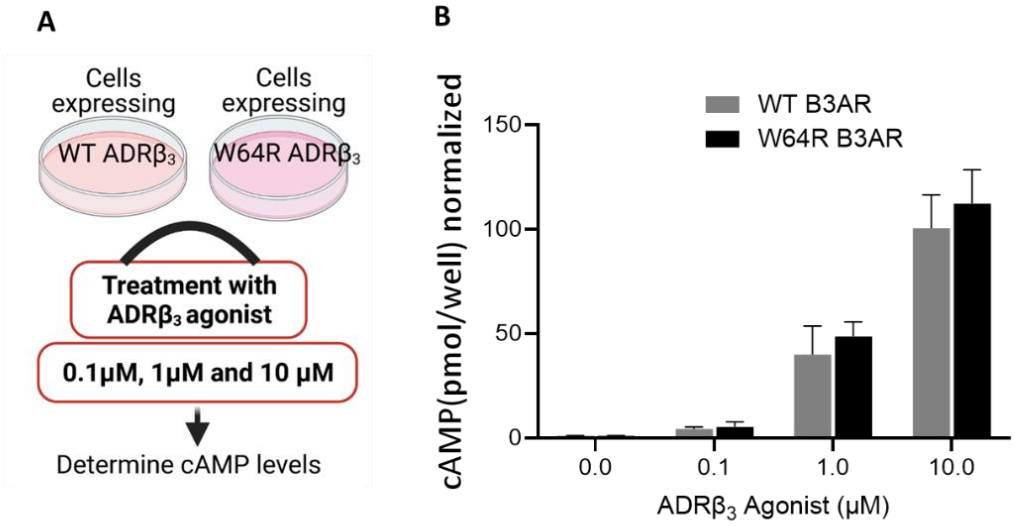

Fig 3

Fig 3. Agonist induced cAMP formation in cells expressing WT and W64R variant of ADRβ3

A) Outline of the experiment and B) dose dependent cAMP formation in cells expressing WT and W64R ADRβ3. Each bar represents an average of three independent experiments. Statistical analysis carried out by students t-test $^{ns}p>0.05$

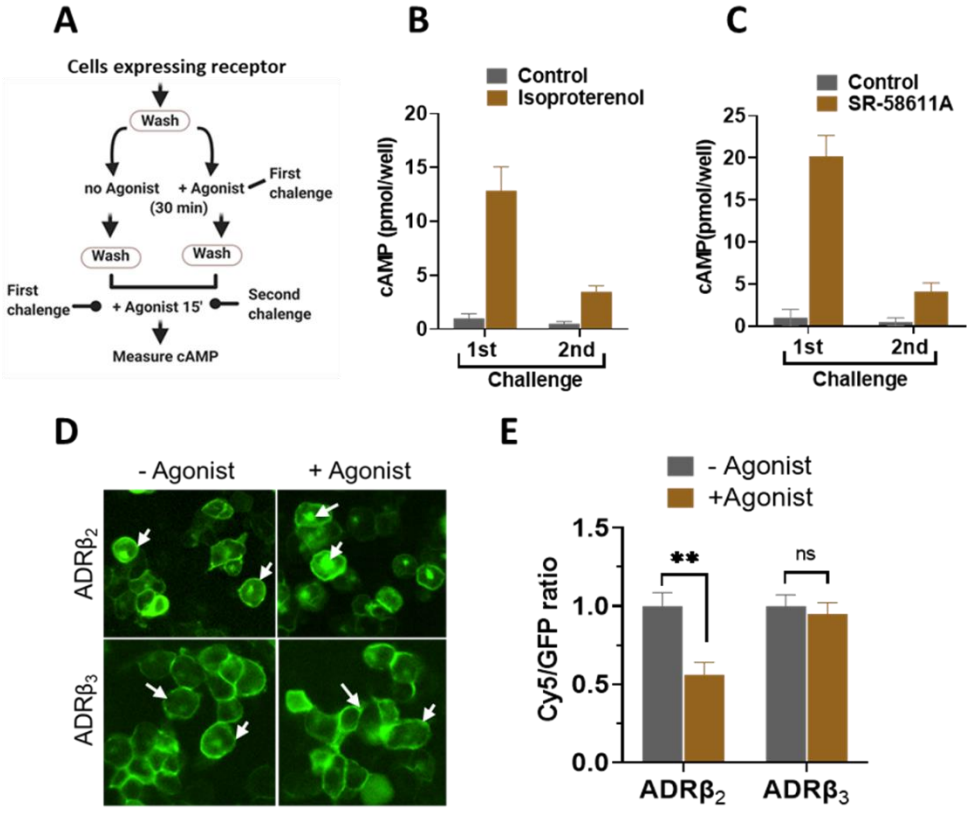

Fig 4

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Fig 4. **Agonist induced desensitization of ADRβ2 and ADRβ3**

A) Experimental outline to measure agonist-induced desensitization of ADRβ2 and ADRβ3. B) cAMP formation in cells expressing ADRβ2 after 1st or 2nd challenge with isoproterenol. C) cAMP formation in cells expressing ADRβ3 after 1st or 2nd challenge with SR-58611A. D) Fluorescent images of of cells showing expression of HA-ADRβ2-GFP and HA-ADRβ3-GFP without and with agonist stimulation. E) Graph showing surface to total cellular expression (Cy5/GFP) of ADRβ2 and ADRβ3 with or without agonist stimulation.

Each bar represents an average of three independent experiments. **p<0.01 and nsp>0.05

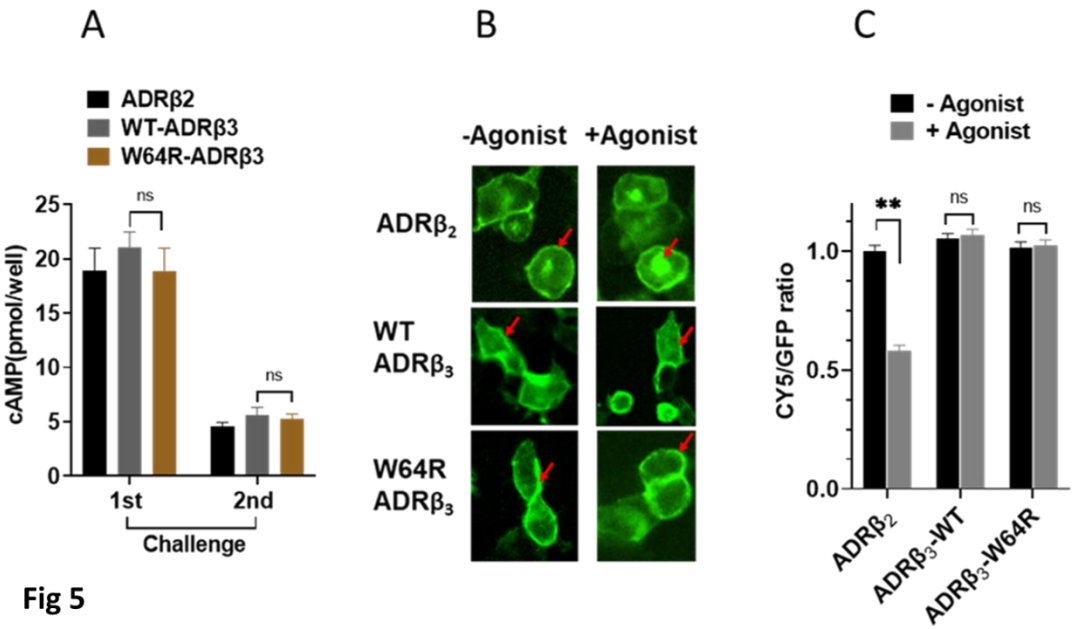

Fig 5

Fig 5. **Agonist induced desensitization of WT and W64R ADRβ3**

A) Agonist induced cAMP formation in cells expressing ADRβ2, WT ADRβ3 and W64R ADRβ3. B) Fluorescent images of cells showing expression HA-ADRβ2-GFP, WT HA-ADRβ3-GFP and W64R HA-ADRβ3-GFP without and with agonist stimulation. C) Graph showing surface to total cellular expression (Cy5/GFP) of ADRβ2, WT ADRβ3 and W64R ADRβ3 with or without agonist stimulation. Each bar represents an average of three independent experiments. **p<0.01 and nsp>0.05

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3. Discussion

GPCRs are the largest family of surface receptors widely expressed in the body and play a vital role in multiple biological processes (43-47). ADRβ3 is one of the members of this family and is highly expressed in adipose tissue. Ligand-induced stimulation of ADRβ¬3 activates adenylate cyclase leading to the formation of cAMP. This activates cAMP-dependent protein kinase (PKA) and downstream signaling pathway to control key functions in adipose tissues including thermogenesis and lipid homeostasis. ADRβ¬3 agonists have generated considerable interest as potential anti-obesity drugs (9, 11). Several independent studies have reported that the missense variant of ADRβ3 (W64R) correlates with obesity, glucose intolerance, hypertension, dyslipidemia, and early onset of non-insulin-dependent diabetes mellitus (29-34). The impact of the substitution of tryptophan with Arginine (W64R) on the ADRβ3 function has remained a mystery. Previous studies have given conflicting results, with some studies reporting that the biochemical properties of the W64R ADRβ3 are comparable to that of the WT ADRβ3, while others reported either impaired or enhanced signaling in cells expressing W64R ADRβ3 (35-37). This discrepancy has been attributed to the expression systems (stable vs transient) and variable expression levels of the receptor. In the present study, we compared the behavior of the WT and mutant (W64R) ADRβ3 in human embryonic kidney cells (HEK-293) using stable expression of the receptor.

Disease linked GPCRs often have impaired cellular expression and/or cell surface expression (48). We used single-cell analysis to determine the total cellular and surface expression of the ADRβ3 and address the fundamental issue of whether W64R mutation affects the expression and/or subcellular distribution of ADRβ3. The single-cell analysis allowed us to determine the distribution of ADRβ3 in wide-ranging expression levels. Our results show that W64R mutation does not affect the expression or the subcellular distribution of ADRβ¬3. We further demonstrate that WT and W64R ADRβ3 show no difference in agonist-induced cAMP formation, which is consistent with earlier reports. Some disease-linked GPCR variants undergo exaggerated down-regulation resulting in metabolic abnormalities. We have previously reported that E354Q variant of Gastric Inhibitory peptide receptor (GIPR) that is associated with an increased incidence of insulin resistance, type 2 diabetes, and cardiovascular disease in humans undergoes exaggerated down-regulation from the plasma membrane after stimulation with GIP (49). Therefore, we studied in detail the post-activation behavior of W64R and WT ADRβ3. Our results show no difference in the extent of receptor desensitization between WT and W64R variant of ADRβ¬3. Unlike ADRβ2, agonist- activation of ADRB3 is not accompanied by receptor internalization so there is not loss of surface ADRβ¬3 expression post-activation. Agonist-activation didn't induce loss of surface expression in both WT and W64R mutant ADRβ3.

4. Materials and Methods

4.1. Chemicals Reagents and Antibodies

Isoproterenol, SR-58611A, IBMX, Peroxidase labeled anti-HA antibodies, were from Sigma-Aldrich, mouse anti-HA antibodies were from Biolegend (Berkley, CA);, Cy5-conjugated anti-mouse IgG were from Jackson Immuno-Research Laboratories (West Grove, PA); DNA oligonucleotides were purchased from Macrogen; pLenti-C-mGFP vector was from Origene (Rockville, MD); restriction enzymes were from Promega (Madison, WA); Phusion High-Fidelity DNA Polymerase Mix was from Thermofisher (Waltham, MA)

4.2. DNA reporter constructs and mutagenesis

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Human ADRβ3 cDNA in the pCDNA3 vector was purchased from Origene. The HA epitope (YPYDVPDYA)-tagged ADRβ3 was produced by PCR amplification with High-Fidelity DNA Polymerase using ADRβ3- pCDNA3 plasmid as a template. Purified PCR product was digested and ligated into the pLenti-C-mGFP vector to generate HA- ADRβ3-GFP, which contains the HA tag at the extracellular N-terminus and GFP tag at the intracellular C-terminus of ADRβ3 constructs. Site-directed mutagenesis was used to generate W64R variant of ADRβ3 using specific primers. The mutation was confirmed by DNA sequencing. Generation of of HA-ADRβ2-GFP reporter construct has been described before (50).

4.3. Cell Culture and Generation of stable cell lines.

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin-streptomycin. Cells were transfected with WT or W64R HA-ADRβ3-GFP plasmids using lipofectamine 3000. 48 h after the transfection cells were cultured with complete media supplemented with blasticidin (5μg/ml). The expression of HA- ADRβ3-GFP was confirmed by fluorescent microscopy.

4.4. cAMP Assay

cAMP assay has been previously described (50). Briefly, cells were washed with serum-free DMEM and incubated in the same medium for 1 h with 0.5 mM IBMX. This was followed by stimulation with the indicated agonists in the continued presence of IBMX. Cells were lysed and total cellular cAMP was measured by using chemiluminescence based cAMP Immunoassay Kit (Applied Biosystems) following the manufacturer's instructions.

4.5. Quantification of cell surface β3-Adrenergic receptor

Single-cell quantification cell surface β2-Adrenergic receptor has been previously described (50). Briefly, HEK-293 cells stably expressing WT or W64R HA-ADRβ3-GFP were fixed with 4% formaldehyde under non-permeabilizing conditions followed by incubation with mouse monoclonal anti-HA antibodies and anti-mouse Cy5 conjugated secondary antibodies. Cells were imaged using the MetaXpress High-Content Image Acquisition system (Molecular Devices) and image analysis was done to determine fluorescent intensity using image processing software Metamorph (Molecular Devices). Cy5/GFP ratio was determined for each cell, which specifies surface/total ratio ADRβ3. GFP fluorescence intensity represents the total cellular expression of ADRβ3. An average of at least 100 cells was used to quantify the expression of surface and total cellular expression of ADRβ3.

5. Conclusions

The biochemical properties of obesity-linked variant of ADRβ3 (W64R) are indistinguishable from the WT ADRβ3. In addition to the normal expression and subcellular distribution, the ligand stimulation results in similar levels of cAMP formation in WT and W64R ADRβ3. Besides, the post-activation behavior of the mutant receptor is indistinguishable from that of the WT ADRβ3.

Supplementary Materials: None

Author Contributions: FEH, SAM and YA performed most of the experiments with assistance from SM. SM and SSM contributed to the data analyses; SM and SSM wrote the manuscript; EH and SAM edited the manuscript. All authors read and agreed to the published version of the manuscript.

Funding: This study was supported by King Abdullah International Medical Research Center (KAIMRC) through research grant RC13/268/R awarded to SM

Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable

 doi:10.20944/preprints202105.0087.v1

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest".

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