Supplemental Material

Animal housing, diet, handling

Mice were socially housed with no more than four other mice in Vanderbilt University's Division of Animal Care vivarium in a Level 6 room. They were kept on ventilated racks with a central water supply offered ad libitum, and in ventilated micro isolator cages that allowed for 10 to 15 air exchanges per hour, and contained a ¼ inch of Enrich-O-Cobs (The Andersons Inc, Maumee, Ohio, USA) bedding material along with cardboard huts as enrichment. Temperature set point in the room was 72°F with an acceptable range of 68° to 76°F. Humidity was maintained within a range of 30% to 70%. Automatic light timers were programmed for a standard twelve hour light/dark cycle, with normal light cycle times between 6:00am & 6:00pm, except during daylight saving time, where normal light cycle times were 7:00am to 7:00pm. Purina Rodent Diet 5L0D (Purina Lab Diet, St. Louis, Missouri, USA) was supplied as standard chow ad libitum by the Department of Animal Care. During experiments, animals were fed diets from Bio-Serv that had either standard or high fat content as described in the manuscript. The reason for adjusting the standard diet was to have sucrose-matched diets and to incorporate doxycycline (for transgene expression) into the chow.

Tissue sample collection and histology

Immediately following sacrifice, lung tissue was obtained for RNA (RNeasy Mini Kit, Qiagen, Valencia, CA), protein analysis, and a separate specimen was inflated with agarose

for histology. Fixed and inflated lung tissue was sectioned and utilized for histology. Lung sections were stained with fluorescence-labeled alpha smooth muscle actin and vessel count per 10x HPF was obtained. Hematoxylin and eosin stained lung sections were also prepared and counterstained with alpha smooth muscle actin. Pulmonary vessel morphometry was assessed by previously established methods[1] using these hematoxylin, eosin and alpha smooth muscle actin stained specimens. For morphometry, at least 4 animals per group and all vessels (viewed at 20x) on at least 15 slides per animal were assessed. RV was divided from the remainder of the heart and specimens were obtained for RNA and protein using similar technique to lung specimens above. Reactive oxygen mediators were quantified in lung tissue homogenates utilizing the OxiSelect In Vitro Assay Kit (Cell Biolabs Inc., San Diego, CA, USA) and following the manufacturer's protocol.

Metabolic testing

Biologic samples for metabolic testing were obtained after 4-6 hours of fasting. Whole blood glucose was tested by the oxidase method (LifeScan, Inc., Milpitas, CA, USA). Plasma insulin was measured via radioimmunoassay technique by the Hormone Assay Core at Vanderbilt University. Homeostatic model assessment insulin resistance (HOMA-IR) was calculated as previously described.[2]

Human pulmonary microvascular endothelial cells

Human pulmonary microvascular endothelial cells (PMVECs) were grown in culture utilizing Endothelial Cell Growth Medium MV (PromoCell, Heidelberg, Germany) in standard

culture conditions (humidified, 37°C, 5% CO_2). Stably transfected PMVECs were created and kept under G418S selection with empty vector (No mutation), or vector containing mutation in BMPR2. Two separate mutations were used for the included experiments, one in the kinase domain ($BMPR2^{R332X}$, referred to as Mutation1) and one in the cytoplasmic tail domain ($BMPR2^{2580delT}$, referred to as Mutation2).[3]

Real-time PCR

We measured relative expression of the two insulin receptor splice variants, isoform A (IR-A) and isoform B (IR-B) in PMVECs with and without mutation using the method developed by Pelletier[4] and the primers in **Supplementary Table S1**. Briefly, PMVECs were grown to 90-100% confluence, serum starved for 24 hours, and RNA isolated from whole cell lysate (RNeasy Kit, Qiagen, Hilden, Germany). Complimentary DNA was generated by reverse transcriptase (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany) and a common IR-specific primer. Expression of IR-A and IR-B was assessed by qPCR using a common 5' primer and two isoform-specific 3' primers. Experiments and PCR were each performed in triplicate.

Insulin signaling via western blotting

After 4 hours of serum starvation of cells at 80-90% confluence, cells were treated with serum-free culture media with or without 100 nM recombinant human insulin (Sigma-Aldrich, St. Louis, MO) for 20 minutes. Total cell lysates were collected and analyzed by electrophoresis and Western blotting using standard techniques. Antibodies for phospho-

Akt(Ser473), phospho-Akt(Thr308), pan-Akt, phospho-AMPKα (Thr172), pan-AMPKα, phospho-IKKα/β (Ser176/180), phospho-SAPK/JNK(Thr183/Try185), SAPK/JNK, phospho-GSK-3β(Ser9), and β-actin were obtained from Cell Signaling Technology, Inc. (Danvers, MA). Antibody for GSK-3β was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX). Antibody to HSP70 was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). Tissue homogenates from animal experiments were also analyzed by Western blotting utilizing these antibodies. All protein amounts assessed by Western blotting were normalized to a housekeeping protein (beta-actin or HSP70).

Immunohistochemistry

PMVECs with and without BMPR2 mutation were plated on chamber slides appropriate for immunofluorescence. After 4-hour serum deprivation, cells were stimulated with insulin (100nM) then washed with cold PBS, fixed and permeabilized at various time points. After incubation with anti-GLUT4 antibody (ab654, Abcam plc, Cambridge, England) and fluorescent secondary antibody, GLUT4 localization was assessed by immunofluorescence microscopy and the predominant distribution of immunofluorescence categorized as either perinuclear or cytoplasmic for at least 100 cells with each condition. Assessments were made by in a blinded fashion.

Glucose Substrate Uptake

Two methods were utilized to assess insulin-mediated glucose uptake in PMVECs with and without mutation in BMPR2. First, flux was assessed by measurement of glucose in media

supernatant. Using equal numbers of cells and media containing 100mg/dL glucose, 5% serum and 100 nM human insulin, glucose was measured (glucose oxidase method, LifeScan, Inc., Milpitas, CA) in supernatant media over 24 to determine flux. Additionally, uptake of 2-deoxyglucose was quantified by an enzymatic recycling reaction (Glucose Uptake Colorimetric Assay Kit, BioVision Inc., Milpitas, CA) with and without insulin stimulation.

Supplementary Table

Supplementary Table 1:

PCR primers for insulin receptor mRNA isoform amplification

Primer description	Primer sequence
IR-specific primer for cDNA generation	5′-TTG GGG AAA GCT GCC ACC GT-3′
Common PCR (“Primer C”)	5’-CCA AAG ACA GAC TCT CAG AT-3’
IR-A specific; crosses exon 10-12 junction (“Primer A”)	5’-CCG AGA TGG CCT GGG GAC GA-3’
IR-B specific; internal to exon 11 (“Primer B”)	5’-GGG TCC TCG GCA CCA GTG CC-3’

Table 1 Note: Adapted from methods developed by Pelletier, et al.[4]

Supplementary References

Online Supplement

1. Chazova I, Loyd JE, Zhdanov VS, Newman JH, Belenkov Y, Meyrick B. Pulmonary artery adventitial changes and venous involvement in primary pulmonary hypertension. *Am. J. Pathol.* 1995;146(2):389-397.
2. Mather K. Surrogate measures of insulin resistance: Of rats, mice, and men. *Am. J. Physiol. Endocrinol. Metab.* 2009;296(2):E398-399.
3. Lane KL, Talati M, Austin E, Hemnes AR, Johnson JA, Fessel JP, Blackwell T, et al. Oxidative injury is a common consequence of bmpr2 mutations. *Pulmonary circulation.* 2011;1(1):72-83.
4. Pelletier R, Hamel F, Beaulieu D, Patry L, Haineault C, Tarnopolsky M, Schoser B, Puymirat J. Absence of a differentiation defect in muscle satellite cells from dm2 patients. *Neurobiol. Dis.* 2009;36(1):181-190.