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Novel Paracrine Action of Endothelium Enhances Glucose Uptake in Muscle and Fat

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***Running title*:** Endothelial-Specific Insulin and IGF-1 Resistance

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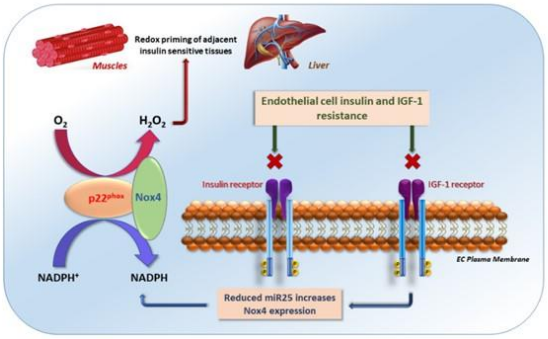
ABSTRACT

Rationale: A hallmark of type 2 diabetes is insulin resistance, which leads to increased endothelial cell production of superoxide and a simultaneous reduction in availability of the vasoprotective signalling radical, nitric oxide (NO). We recently demonstrated in preclinical models that type 2 diabetes simultaneously causes resistance to insulin like growth factor-1 (IGF-1) mediated glucose lowering and endothelial NO release.

Objective: To examine the effect of insulin and IGF-1 resistance specifically in endothelial cells *in vivo*.

Methods and Results: We generated mice expressing mutant IGF-1 receptors (mIGF-1R), which form non-functioning hybrid receptors with native insulin receptors (IR) and IGF-1R, directed to endothelial cells under control of the Tie2 promoter-enhancer. Despite endothelial cell insulin and IGF-1 resistance, mutant IGF-1R endothelial cell over-expressing mice (mIGFREO) had enhanced insulin and IGF-1 mediated systemic glucose disposal, lower fasting free fatty acids and triglycerides. In hyperinsulinaemic-euglycaemic clamp studies, mIGFREO had increased glucose disposal and increased glucose uptake into muscle and fat, in response to insulin. mIGFREO had increased NADPH oxidase 4 (Nox4) expression due to reduced expression of the microRNA, miR-25. Consistent with increased Nox4, mIGFREO endothelial cells generated increased hydrogen peroxide (H_2O_2), with no increase in superoxide. Treatment with catalase, a H_2O_2 dismutase restored insulin tolerance to wild type levels in mIGFREO.

Conclusion: Combined insulin and IGF-1 resistance restricted to the endothelium leads to a potentially favourable adaptation in contrast to pure insulin resistance, with increased Nox4-derived H_2O_2 generation mediating enhanced whole-body insulin sensitivity.



Nonstandard Abbreviations and Acronyms:

TG	transgenic
SVEC	saphenous vein endothelial cells
PTPs	Phospho-tyrosine phosphatases
PKC	Protein kinase C
PEC	pulmonary endothelial cells
NOX 2/4	NADPH oxidase 2/4
NO	Nitric oxide
mIR-25	microRNA-25
mIGF-1R	mouse IGF-1 receptor
L-NMMA	L-NG-monomethyl Arginine citrate
IR/IGF-1R	heterodimers of insulin receptor and IGF-1 receptor
IR	Insulin receptor
IGF-1	insulin-like growth factor-1
hIGF-1R	human IGF-1 receptor
HFD	High fat diet
GPrx	Glutathione peroxidase
ERK	MAP kinase 1/2
EndoRa	endogenous glucose production
ECs	Endothelial cells
WT	wild type

INTRODUCTION

A hallmark of type 2 diabetes mellitus is insulin resistance, defined as an inability of insulin to activate its complex intracellular signalling network appropriately (1). In addition to regulating glucose homeostasis, insulin activates the enzyme endothelial nitric oxide (NO) synthase (eNOS) in endothelial cells to stimulate generation of the signalling radical, NO (2,3). Acting via its tyrosine kinase receptor, which is structurally homologous to the insulin receptor (IR), insulin like growth factor-1 (IGF-1) also regulates metabolic and cellular responses to nutrient availability (4) and at the same time may stimulate eNOS in endothelial cells to generate NO (5).

We have shown that insulin resistance specific to the endothelium induced by a range of different mechanisms, leads to reduced availability of NO and generation of excess concentrations of the free radical superoxide, the principal enzymatic source of which is the Nox2 isoform of NADPH oxidase (6–9). However, we have also shown that diet-induced obesity leads to excess generation of the oxidant and dismutation product of superoxide, hydrogen peroxide (H_2O_2) (10). Moreover, we showed that diet-induced obesity leads to resistance to both insulin- and IGF-1 mediated glucose lowering and serine phosphorylation-mediated activation of eNOS (5). In order to improve our understanding of the synergistic impact of insulin and IGF-1 signalling in the endothelium, we generated a transgenic mouse expressing a mutant IGF-1 receptor (mIGF-1R), which forms non-functioning hybrid receptors with native insulin receptors and IGF-1R specifically in the endothelium (herewith described as: mIGFREO). When expressed exclusively in muscle, mIGF-1R induces resistance to both insulin and IGF-1 (11). Here, we describe for the first time the effect of endothelium-restricted insulin and IGF-1 resistance, secondary to expression of mIGF-1R, on whole body insulin sensitivity and endothelial cell homeostasis.

METHODS

Data Availability.
The authors declare that all supporting data and materials/protocol presented within this article and in the Data Supplement are available from the corresponding author by reasonable request. Materials and methods are described in detail, in the Online Data Supplement. Please see the Major Resources Table in the Supplemental Materials.

RESULTS

Endothelial cells from patients with type 2 diabetes are resistant to both insulin and IGF-1 mediated eNOS phosphorylation.

Patients with and without diabetes mellitus undergoing coronary bypass surgery were recruited. Total and serine phosphorylated eNOS and Akt in saphenous vein endothelial cells (SVEC) were quantified using Western blot under basal conditions and after stimulation with insulin or IGF-1. Basal eNOS and AKT was similar in SVEC from patients with and without type 2 diabetes mellitus (Figure 1A&B). Basal serine phosphorylated eNOS was similar in SVEC from patients with or without type 2 diabetes, as was basal serine phosphorylated Akt (Figure 1C&D). As we demonstrated in preclinical models (5), insulin and IGF-1 stimulated eNOS phosphorylation were blunted in SVEC from patients suffering from type 2 diabetes, compared to patients without type 2 diabetes (Figure 1E&G). Insulin and IGF-1 stimulated Akt phosphorylation were similar when comparing SVEC from patients with and without type 2 diabetes mellitus (Figure 1F&G). There was lower Nox4 expression in SVEC from patients with type 2 diabetes compared to patients without type 2 diabetes, whereas Nox2 protein levels were increased in SVEC from patients with type 2 diabetes compared to patients without diabetes (Figure 1H&I). Superoxide generation was higher in SVEC from patients with diabetes (Figure 1J).

Generation and characterisation of transgenic mice with endothelial specific expression of mutant IGF-1 receptors.

To examine the effect of disrupting both insulin and IGF-1 signalling in the endothelium, we generated a novel transgenic mouse expressing a human IGF-1R with an amino acid substitution in the ATPase domain (11) directed to the endothelium under control of the *Tie2* promoter-enhancer (Online Figure IA-C). Mutant IGF-1R endothelium over-expressing mice (described, as mIGFREO), were born with the same frequency as their wild type (WT) littermates (data not shown). There was no difference in body weight, organ weight, fat pad size or blood pressure between mIGFREO and their WT littermates (Figure 2A&B and Online Figure ID&E). We, next quantified the levels of human and native (mouse) IGF-1R mRNA expression in whole organs and pulmonary endothelial cells (PEC). While human IGF-1R mRNA was detected only in mIGFREO aorta and lungs (Figure 2C), native (mouse IGF-1R) mRNA was similar in both mIGFREO and WT aorta and lung (Online Figure IF). There was no human IGF-1R mRNA detectable in PEC from WT mice or non-endothelial cells from mIGFREO (Figure 2D), and there was almost no detectable expression of hIGF-1R in monocytes from mIGFREO (Online Figure IG). Human IGF-1R mRNA was not detected in pancreatic islets, pericytes or endothelium denuded aorta of mIGFREO mouse showing the specificity of the Tie-2 promoter in mIGFREO (Online Figure IH). Western blot analysis confirmed that total IGF-1R protein levels were significantly increased in mIGFREO endothelial cells compared to WT littermates, as were IR/IGF-1R heterodimers (hybrid receptors) (Figure 2E&F). Hybrid receptors in mIGFREO cells were resistant to insulin, as expected (12), IGF-1 stimulated activation of hybrids was also significantly reduced in mIGFREO (Figure 2G&H).

Disrupted insulin and IGF-1 signaling in mIGFREO endothelial cells.

There was no difference in total eNOS, total Akt or phosphorylated Akt during standard culture conditions in mIGFREO PEC compared to WT littermates (Figure 3A-D). Serine phosphorylated eNOS was significantly reduced in mIGFREO endothelial cells compared to WT littermates (Figure 3E). Basal and insulin-induced phosphorylation at Tyr657, an important residue in the negative regulation of eNOS was not significantly different between WT and mIGREO PEC (Online Figure IIA). While, as seen in humans with type 2 diabetes, mIGFREO PEC had reduced serine phosphorylation of eNOS in response to insulin and IGF-1, insulin-stimulated Akt phosphorylation was preserved (Figure 4A-C). Radioactive eNOS activity assay and phosphorylation of eNOS analysis of whole aorta also showed that mIGFREO endothelial cells were resistant to insulin and IGF-1 stimulation (Figure 4D&E and Online Figure IIB). We, therefore examined the possibility that eNOS and Akt demonstrate differential sensitivities to insulin-mediated serine phosphorylation in endothelial cells. We observed that in WT PEC, while AKT phosphorylation was induced on exposure to 50 nmol/L insulin, significant eNOS phosphorylation required a substantially higher concentration (150nmol/L) (Figure 4F&G). Similarly in insulin-resistant PEC from mIGFREO mice, although AKT phosphorylation was induced with 50 nmol/L insulin, eNOS was relatively resistant to phosphorylation at 150 nmol/L (Figure 4H). Next, we probed ERK and PKC - two key molecules involved in insulin signalling in endothelial cells. While we did not observe any difference in insulin-induced ERK phosphorylation between WT and mIGFREO PEC, PKC activity in response to insulin stimulation was reduced in mIGFREO PEC compared to WT cells (Online Figure IIC&D).

mIGFREO mice have normal glucose tolerance but enhanced glucose lowering in response to systemic insulin or IGF-1.

mIGFREO had similar fasting and fed capillary blood glucose, fasting and fed serum insulin, and random serum IGF-1 concentrations to WT littermates (Figure 5A&B and Online Figure IIIA). mIGFREO also had similar glucose tolerance as WT littermates, but had enhanced glucose disposal in insulin and IGF-1 tolerance tests compared to WT littermates (Figure 5C-G). It has been suggested, that by increasing delivery of glucose to its target tissues, insulin-induced vasodilation of small arteries is important for glucose uptake (13). In light of the enhanced insulin sensitivity at a whole-body level seen in mIGFREO, we examined insulin-induced relaxation in 2nd order mesenteric arteries. Consistent with the increased insulin-mediated glucose uptake seen in a range of tissues we observed an increase in insulin-induced vasorelaxation of 2nd order mesenteric arteries from mIGFREO (Figure 5H&I and Online Figure IIIB). Consistent with enhanced insulin sensitivity, fasting free fatty acids and triglycerides were significantly lower in mIGFREO compared to WT littermates (Figure 5J&K). Serum concentrations of the adipokines leptin and adiponectin were not different between mIGFREO and WT controls (Online Figure IIIC&D). In low-dose hyperinsulinaemic euglycaemic clamp studies, mIGFREO blood glucose was maintained throughout the clamp, glucose infusion rate was higher in mIGFREO, consistent with increased insulin sensitivity (Figure 6A&B). Fasting (basal) glucose turnover was no different between groups (Online Figure IVA). However, consistent with enhanced insulin sensitivity, the rate of glucose disappearance (Rd) was significantly higher in mIGFREO (Figure 6C). Endogenous glucose production (EndoRa) was no different between groups, indicative of no difference in hepatic gluconeogenesis (Online Figure IVB). In tracer studies, glucose uptake into brown adipose tissue, as well as skeletal muscle, was significantly increased in mIGFREO (Figure 6D-F and Online Figure IVC). Consistent with increased insulin sensitivity, *in vivo* insulin stimulation led to greater tyrosine phosphorylation of IR in liver and skeletal muscle in mIGFREO compared to WT littermates (Figure 6G&H).

Increased vascular H_2O_2 in mIGFREO.

Aortic rings from mIGFREO mice had similar responses to the endothelium- and NO-dependent vasodilator acetylcholine (Online Figure VA), and the endothelium-independent vasodilator sodium nitroprusside, compared with WT littermates (Online Figure VIA). Vasoconstriction to phenylephrine remained unchanged in mIGFREO compared to their WT littermates (Online Figure VB). Bioavailable NO in response to isometric tension, assessed by measuring the constrictor response to the NOS inhibitor L-NMMA, was no different in mIGFREO compared to WT littermates (Online Figure VC). To examine the possibility that H_2O_2 generation may contribute to the vasorelaxation response to acetylcholine in mIGFREO, as we previously demonstrated in obese mice (10), we treated rings with the H_2O_2 dismutase, catalase (Online Figure VD&E). There was no difference in $\log EC_{50}$ of acetylcholine responses between mIGFREO and WT littermates after catalase treatment (note logarithmic scale; Online Figure VF). However, percent change in maximum relaxation was significantly increased in mIGFREO aorta compared to WT littermates (Online Figure VG) demonstrating increased hydrogen peroxide release in Ach-induced vasorelaxation in mIGFREO mice. Next to test whether inhibiting catalase can reverse catalase-induced reduction of ACh-induced relaxations in aorta, we performed vasorelaxation studies with the catalase inhibitor (3-amino-1,2,4-triazole) in the presence of catalase. The data confirmed the significance of catalase inhibitable H_2O_2 in Ach-induced relaxation in mIGFREO aorta (Online Figure VH). To confirm increased H_2O_2 production in mIGFREO aortic rings, we examined the amount of catalase-inhibited aortic H_2O_2 production using Amplex Red assay. mIGFREO aorta had significantly higher concentrations of H_2O_2 compared to WT littermates (Online Figure VI). We quantified superoxide generation in endothelial cells from mIGFREO using NADPH-dependent chemiluminescence and found no difference in superoxide generation between mIGFREO and WT littermates (Online Figure VJ).

Excessive endothelial H_2O_2 leads to enhanced whole-body insulin sensitivity.

H_2O_2 has previously been described as an insulin sensitizer in skeletal muscle and we too found that it can increase glucose uptake in muscle cells in culture in a dose-dependent manner (data not shown). Hence, we examined the effect of H_2O_2 quenching on insulin sensitivity *in vivo* in mIGFREO mice. Chronic treatment with catalase restored insulin sensitivity in mIGFREO to WT levels in insulin tolerance testing (Figure 7A-C). Moreover, catalase treatment reduced levels of tyrosine phosphorylated insulin receptor in mIGFREO liver (Figure 7D). Nox4 has been described as the primary source of H_2O_2 in endothelial cells. On the other hand, the related Nox isoform Nox2 is predominately responsible for superoxide generation. Nox2 mRNA expression in endothelial cells from mIGFREO was similar to WT littermates, whereas Nox4 mRNA was significantly higher in mIGFREO (Figure 7E&F). Endothelial cell Nox2 protein expression was lower in mIGFREO compared to WT littermates, whereas and consistent with mRNA expression, Nox4 protein expression was higher in mIGFREO compared to WT littermates (Figure 7G&H). No other ROS-related gene expression was changed in mIGFREO PEC (Online Figure VIB). To dissect how endothelial insulin and IGF-1 resistance contributes to whole body insulin sensitivity in the pathological context of diet-induced insulin resistance, we fed mIGFREO and WT littermates a high fat diet (HFD) for 10 days. We have previously shown that short term HFD feeding can result in resistance to insulin-induced glucose lowering before it causes insulin resistance in the endothelium (10,12). There was no significant difference in changes in body weight before and after HFD (Online Figure VIIA) or fasting glucose levels (Online Figure VIIB) between mIGFREO and WT animals upon HFD feeding. mIGFREO mice fed HFD, despite insulin resistance at the level of the endothelium, did not develop glucose intolerance compared to WT littermates (Online Figure VIIC&E). However, detailed exploratory GTT analysis at 30 minutes showed that glucose levels trended to be lower in mIGFREO ($p=0.05$) compared to WT littermates (Online Figure VIIC&D).

Mutant IGF-1R regulates Nox4 expression through miR-25 in endothelial cells from mIGFREO and patients with type 2 diabetes.

Unlike other Nox isoforms, Nox4 activity is dependent primarily on its abundance. Whilst transcriptional regulation of Nox4 is incompletely understood, a number of regulators of Nox4 expression, both negative and positive, have been proposed (14). One such transcriptional regulator is the microRNA mIR-25, which has been suggested to be a negative regulator of Nox4 transcription. We measured mIR-25 level in aorta and PEC of mIGFREO mice and found it was significantly reduced compared to WT littermates (Figure 8A&B). Moreover, miR-25 level was higher in SVEC of patients with diabetes (Figure 8C) and, importantly, when we transfected SVEC from patients without diabetes with mutant IGF-1R, miR-25 expression was significantly reduced with a reciprocal increase in Nox4 expression (Figure 8D-E). Finally, we used a synthetic miR-25 mimetic to elevate its level directly in SVEC and found it significantly decreased mRNA expression of Nox4 (Figure 8F). Taken together, these results show that disrupting IGF-1R and IR signalling by mutant IGF-1R expression leads to increased Nox4 expression, and H_2O_2 generation, by reducing miR-25 levels in the endothelial cells (Graphical Abstract).

DISCUSSION

Lower-order organisms have a single receptor (DAF-2) that transmits external cues to regulate glucose homeostasis, metabolism and growth (15), whereas mammals have evolved to have 2 separate receptors that regulate glucose homeostasis (the insulin receptor) and growth (the IGF-1 receptor). Insulin may act in synergy with IGF-1 to coordinate responses to nutrient availability. An unexplained paradox exists whereby, in worms and flies downregulation of DAF-2 has an advantageous effect on health, leading to stress resistance and extended lifespan (15), whereas in humans insulin resistance leads to a range of chronic disorders of health. To date, no studies have examined the effect of the combination of diminished insulin and IGF-1 actions in vascular cells on metabolic and vascular homeostasis in mammals. To examine the effect of the combination of prolonged and selective insulin and IGF-1 resistance at the level of the endothelium, we generated a transgenic mouse expressing mutant IGF-1R that form non-functioning hybrid receptors with native insulin receptors and IGF-1R, specifically in endothelial cells.

The following novel findings are reported: **1)** Endothelial cells from humans with advanced atherosclerosis and type 2 diabetes are resistant to both insulin- and IGF-1-mediated eNOS activation. **2)** Endothelial cell-specific expression of mutant IGF-1R leads to resistance to insulin- and IGF1-mediated eNOS serine phosphorylation in endothelial cells. **3)** Despite this, mIGFREO have enhanced glucose disposal in response to systemic insulin and IGF-1. **4)** In contrast to mice with endothelial cell-specific insulin resistance (7,8), mIGFREO have reduced Nox2 NADPH oxidase expression and increased Nox4 NADPH oxidase expression at an mRNA and protein level. **5)** Catalase, which reduces H_2O_2, restores insulin-mediated glucose lowering to WT levels in mIGFREO. **6)** mIGFREO mice reveal the microRNA miR-25 as an important regulator of Nox4 expression, this was recapitulated in endothelial cells from humans with type 2 diabetes and accelerated atherosclerosis.

Endothelial cells from humans with advanced atherosclerosis and type 2 diabetes are resistant to both insulin- and IGF-1-mediated eNOS serine phosphorylation.

As proof of principle, we examined the responses of saphenous vein endothelial cells (SVEC) from patients undergoing aortocoronary bypass surgery to insulin and IGF-1. We showed that, consistent with our preclinical studies, SVEC from humans with type 2 diabetes and advanced atherosclerosis, have

blunted serine phosphorylation of eNOS in response to both insulin and IGF-1. We also demonstrated decreased Nox4 NADPH oxidase expression and increased Nox2 expression. This is, perhaps not surprising when one considers that SVEC from patients with type 2 diabetes have been exposed to multiple systemic factors which are not present in mIGFREO (which have increased Nox4 and reduced Nox2 expression in EC) including, but not limited to; hyperglycaemia, hyperlipidaemia, hyperinsulinemia and excess circulating cytokines. This notwithstanding, we demonstrate here for the first time that type 2 diabetes in humans is accompanied by both insulin and IGF-1 resistance at the level of the endothelium, providing a conceptual framework for the present study.

Obesity leads to resistance to insulin *and* IGF-1 in the endothelium.

Obesity and type 2 diabetes mellitus induce defects at multiple points in the insulin signalling pathway, resulting in resistance to insulin-mediated glucose uptake into skeletal muscle and other insulin target tissues (1). We have shown that whole-body genetic (7) and diet-induced insulin resistance (5,10) also lead to insulin resistance at the level of the endothelium. During the development of obesity and simultaneous insulin resistance, we have also demonstrated a similar decline in IGF-1 actions at a whole-body level (5,16), and within the endothelium (5). Whilst we have a deep understanding of the effects of whole body and endothelial cell-specific insulin resistance on NO availability (6–9,17), the local and systemic consequences of prolonged insulin and IGF-1 resistance in the endothelium are unexplored. Moreover, the effects of endothelium-restricted insulin and IGF-1 resistance on systemic glucose homeostasis remain unclear.

mIGFREO mice reveal differential sensitivity of Akt and eNOS to insulin-mediated serine phosphorylation in endothelial cells.

Akt is a critical node (18) in the downstream insulin signalling pathway which lies proximal to eNOS. In endothelial cells from humans with advanced atherosclerosis and type 2 diabetes, we showed that at 100nM insulin-induced Akt phosphorylation was preserved, whereas eNOS phosphorylation was blunted. This scenario was recapitulated in mIGFREO mice. The role of Akt in insulin signalling in the endothelium in health and disease is of particular interest to the field. Elegant studies in adipocytes from the Accilli laboratory (19), raised the possibility that activation of different nodes in the insulin signalling pathway downstream of the insulin receptor require different concentrations of insulin, differing by as much as tenfold to phosphorylate the protein sufficiently. This has not been examined in endothelial cells from insulin sensitive or insulin resistant mammals. We, therefore examined the effect of different concentrations of insulin on Akt and eNOS phosphorylation in endothelial cells from mIGFREO mice and their WT littermates. Interestingly, we found that Akt was more sensitive to insulin than eNOS with a significant increase in phosphorylated Akt occurring at 50nM, which did not lead to an increase in serine phosphorylated eNOS in WT or mIGFREO. These data are important to our understanding of insulin signalling in the endothelium in health and disease. The contrast between blunted insulin-induced eNOS activation seen in endothelial cells from mIGFREO and the enhanced responses in resistance vessels most likely reflects the different mediators of relaxation in large and small arteries (20). Whereas NO has been shown to be most important in insulin-induced relaxation in large arteries (8), an endothelium derived hyperpolarising factor (EDHF) has been shown to be the principal mediator of insulin-induced relaxation of small arteries (21). Our data demonstrating increased release of H_2O_2, a putative EDHF, from mIGFREO in response to insulin fits with the enhanced relaxation seen in 2nd order mesenteric arteries from mIGFREO.

Insulin resistance in the endothelium and glucose intolerance.

Insulin signalling in the endothelium has been suggested to be important in glucose uptake into skeletal muscle (22). *In vitro* studies have shown divergent results addressing the question of whether

insulin signaling regulates glucose transport and metabolism in endothelial cells. It has been shown that insulin signaling does not regulate glucose transport in human micro- and macrovascular endothelial cells (23), bovine brain and retinal endothelial cells (24,25), whereas glucose transport and glycogen synthesis are increased by insulin in bovine endothelial cells isolated from adipose tissue or retinas and rabbit ECs (26–28). Consistent with the mIGFREO phenotype we have described here, seminal *in vivo* studies from Vicent *et al* demonstrate that mice with endothelial cell depletion of IR display no changes in fed and fasting blood glucose levels and in fact, at 6 months of age have better glucose tolerance than WT littermates; suggesting that normal glucose uptake is not dependent on insulin signaling in endothelial cells (29). These data suggest that the presence of endothelial insulin and IGF-1 resistance may represent a potentially favourable adaptation to metabolic stress, leading to enhanced glucose uptake in response to insulin and a favourable effect on lipid profile.

Reactive oxygen species as signalling molecules in glucose homeostasis.

Some biomolecules may be modified by oxidation (30). Once specific types of oxidant are generated at a given time and place, they can mediate reversible and irreversible modifications in a range of molecules. In relation to insulin signalling, it is thought that inhibition of phosphotyrosine phosphatases (PTPs) by H_2O_2-mediated oxidation of cysteine residues is necessary for optimal signalling (31–34). It has also been shown that mild oxidative conditions enhance the activation of IGF-1R (35), suggesting that optimal insulin and IGF-1R responsiveness involves redox priming. Mice deficient in glutathione peroxidase (GPrx), which reduces H_2O_2 to water, provide evidence that H_2O_2 is important in insulin sensitivity. GPrx-deficient mice are protected against HFD induced insulin resistance (36). H_2O_2 and Nox4 may therefore, be components of a complex system of receptor tyrosine kinases/PTPs and oxidants that regulate insulin-mediated glucose lowering. Consistent with this hypothesis, we showed that the enhanced insulin-stimulated glucose disposal of mIGFREO was blunted by infusion of the H_2O_2 degrading enzyme, catalase. Moreover, we show that insulin receptors in liver from mIGFREO treated with systemic insulin have increased tyrosine phosphorylation, which is reduced to WT levels by catalase.

A number of mechanisms may underpin endothelial cell release of H_2O_2 at levels that enhance whole body insulin sensitivity in mIGFREO. We have shown increased expression of Nox4 NAD(P)H oxidase in endothelial cells from mIGFREO. Nox4 is unique amongst the NADPH oxidases in that it generates H_2O_2 (37) and is constitutively active, generating H_2O_2 at concentrations proportionate to its expression (38). Consistent with this, we have shown that increased endothelial cell expression of Nox4 leads to increased basal H_2O_2 release (39). In mIGFREO, as discussed above, this enhances insulin sensitivity by redox priming of insulin receptors. Nox4 is also an insulin-responsive enzyme (40). When insulin binds to its receptor, it rapidly activates Nox4 to generate a transient burst of H_2O_2 (33,34). This short-lived increment in H_2O_2 enhances insulin sensitivity by inhibition of PTP1B and PTEN both of which are negative regulators of insulin signalling (33).

NADPH oxidase derived oxidants as signalling molecules.

The NADPH oxidases are a group of enzymes whose specific function is to generate superoxide (41). Members of the family are named after the transmembrane protein Nox. All 7 Nox proteins share highly conserved structural features; despite this, Nox proteins differ in their mode of activation, their interaction with the transmembrane protein $p22^{phox}$ and the requirement for additional maturation and activation factors (41). Nox4 is highly expressed in human endothelial cells (42) and unlike other Nox isoforms, is constitutively active and independent of cytosolic activator proteins or regulatory domains. Recent studies demonstrate that H_2O_2 is the principal oxidant generated by Nox4, rather than $O_2^{\cdot-}$ (37). In the present report, we identify a previously unidentified pathway, activated in the presence of an evolutionarily conserved response to cellular stress i.e. downregulation of insulin and IGF-1 signalling. We show that the combination of insulin and IGF-1 resistance increases Nox4 expression and leads to

increased generation of the signalling oxidant H_2O_2, which in turn leads to redox priming of insulin receptors in canonical insulin target tissues, enhancing whole body insulin sensitivity and reducing fasting free fatty acid levels (See schematic representation in the Graphical Abstract).

mIGFREO mice reveal the microRNA miR-25 as an important transcriptional regulator of Nox4 that is dysregulated in humans with type 2 diabetes mellitus and advanced atherosclerosis.

Nox4 is thought to be unique amongst the NADPH oxidase isoforms, in that the principal mechanism of regulation is transcriptional (43). Amongst a number of potential transcriptional regulators of Nox4 expression, the microRNA miR-25 has emerged as potentially important in diabetes (44). mIR-25 having been shown to negatively regulate Nox4 expression in a number of studies (45,46). miRNAs are a class of non-coding RNA that play a critical role in cell differentiation, proliferation and survival by binding to complementary target mRNAs, leading to transcriptional inhibition or degradation (47). miRNAs are found to be dysregulated in a range of disorders associated with abnormal cellular growth and/or metabolism (48). We examined expression of mIR-25 in endothelial cells and whole aorta from mIGFREO mice, consistent with increased Nox4 expression, we found mIR-25 to be decreased in both aorta and endothelial cells. We, then examined expression of mIR-25 in saphenous vein endothelial cells from patients with advanced atherosclerosis and type 2 diabetes, showing increased mIR-25 consistent with the reduced Nox4 seen in endothelial cells from these patients. To take this a step further, we expressed mutant IGF-1R in saphenous vein endothelial cells from patients with advanced atherosclerosis and demonstrated a reduction in mIR-25 expression and an increase in Nox4 expression. Another intriguing finding in the present study was the reduced Nox2 in mIGFREO mice. Consistent with our findings, studies have shown that Nox4 may inhibit Nox2 expression (49,50). Our dataset raise the possibility that by manipulating miR-25, it may be possible to change expression of Nox4 and insulin sensitivity at a whole body level. An interesting initial experiment would be to administer miR-25 mimetic to mIGFREO mice, where the initial proof of concept would be a reduction Nox4 and a commensurate decline in whole body insulin sensitivity.

Study limitations.

The Tie-2 promoter has been shown on occasions to drive expression in populations of myeloid cells (51), although using a similar approach to generate mice over-expressing the insulin receptor in endothelial cells (8), we did not demonstrate significant off-target expression. Consistent with this, we did not demonstrate significant expression of mIGF-1R in monocytes from mIGFREO (Online Figure I). While one could argue that the changes in glucose tolerance in mIGFREO mice are relatively small, in the context of normal glucose homeostasis, they remain striking and of therapeutic and physiological relevance.

Conclusions.

Here, we show that in the setting of insulin and IGF-1 resistance, the endothelium undergoes a phenotypic change underpinned by increased endothelial cell Nox4 NADPH oxidase expression that augments H_2O_2 generation. This H_2O_2 release is likely to act in a paracrine fashion to enhance insulin-mediated glucose lowering in skeletal muscle and brown adipose tissue; therefore, revealing novel crosstalk between the endothelium and insulin sensitive tissues. Thus, this dataset significantly contributes to our understanding of the nature and mechanism of mammalian responses to metabolic stress and provides a new perspective in understanding of the regulation of the insulin/IGF-1 pathways under normal conditions and in the context of disease.

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DISCLOSURES
None.

SUPPLEMENTAL MATERIALS
Supplemental Materials
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FIGURE LEGENDS

Figure 1. Endothelial cells from humans with type 2 diabetes and advanced atherosclerosis are resistant to both insulin and IGF-1. (A&B) Unstimulated, basal total eNOS (**A**; No Diabetes n=5, Diabetes n=8) and AKT (**B**; No Diabetes n=12, Diabetes n=5) expression in saphenous vein endothelial cells (SVEC) from patients with and without type 2 diabetes mellitus. **(C&D)** Serine-phosphorylated eNOS (**C**; No Diabetes n=12, Diabetes n=7) and serine-phosphorylated Akt (**D**; No Diabetes n=13, Diabetes n=7) in SVEC under basal condition. **(E)** Insulin or IGF-1 stimulated eNOS serine phosphorylation (Ins and IGF-1; 100nM, 10 minutes) in SVEC from patients with diabetes (No Diabetes n=12, Diabetes n=7). **(F)** Akt serine-phosphorylation upon insulin (Ins, 100nM; 10 minutes) and IGF-1 (IGF, 100nM; 10 minutes) treatment in SVEC from patients with and without diabetes (No Diabetes n=11, Diabetes n=6). **(G)** Representative blots for the data showed in **E&F**. **(H&I)** Nox4 (**H**; No Diabetes n=7, Diabetes n=5) and Nox2 (**I**; No Diabetes n=7, Diabetes n=7) expression in SVEC from patients with (Diab) or without diabetes (No-Diab) (Representative blots presented on the right of Figure **H**. **(J)** Mean superoxide level in SVEC from patients with and without diabetes (No Diabetes n=5, Diabetes n=6). All SVEC used after isolation were at passage 2 to 5. Data expressed as mean±sem * denotes p<0.05, no diabetes vs diabetes in Figures **H-J**. In Figure **E**, * denotes p<0.05, Control vs insulin-stimulation in patients without diabetes; ** denotes p<0.05, Control vs IGF-1 stimulation in patients without diabetes, $^{\#}$ denotes p<0.05, insulin-stimulation in diabetes vs without diabetes and $^{\#\#}$ denotes p<0.05, IGF-1 stimulation in diabetes vs without diabetes. In Figure **F** * denotes p<0.05, Control vs insulin-stimulation in patients without diabetes; ** denotes p<0.05, Control vs IGF-1 stimulation in patients without diabetes, $^{\#}$ denotes p<0.05, Control vs insulin-stimulation in diabetes and $^{\#\#}$ denotes p<0.05, Control vs IGF-1 stimulation in diabetes. *('B' denotes basal unstimulated cells grown in full growth medium for total protein expression. 'C' denotes control cells in 0.2% serum-containing medium*). Data in **A-D** and **H-J** were analysed using unpaired Students t test. Others were analysed using one way ANOVA Fisher's test.

Figure 2. Generation and characterisation of transgenic mice over-expressing mutant IGF-1 receptor in the endothelium. (**A**) Body (n=11 each group) and (**B**) Organ weights of mIGFREO and wild type littermate (WT) mice (WT n=5, mIGFREO n=8). **(C&D)** Mutant human IGF-1R mRNA expression in aorta and lungs (**C**; WT aorta and mIGFREO aorta n=10. WT lungs n=9, mIGFREO lungs n=11) in mIGFREO mice and in pulmonary endothelial cells (PEC) and non-endothelial cells (non-PEC, described in Methods) (**D**; WT n=4, mIGFREO n=4). **(E&F)** IGF-1 receptor (IGF-1R) expression (**E**; WT n=5, mIGFREO n=6) and hybrid receptor (**F**; WT n=8, mIGFREO n=5) protein expression in PEC of mIGFREO and WT. **(G&H)** Hybrid receptor activation at insulin receptor IR-pY1334, upon insulin (Ins, 150nM, 10 min) (**G**; WT n=5, mIGFREO n=5) and IGF-1 stimulation (IGF-1, 150nM, 10 min) (**H**; WT n=5, mIGFREO n=6) in PEC of mIGFREO and WT. Data expressed as mean±sem. * denotes p<0.05. In Figure **C - H**, * denotes p<0.05, WT vs mIGFREO. Data in **A** was analysed using 2-way ANOVA, followed by Bonferroni's multiple comparisons test, **B** were analysed by multiple t test, Data in **C, D**, **E** were analysed by unpaired Students t test with Mann-Whitney comparison, **F-H** were analysed by Unpaired Students t test.

Figure 3. Disrupted insulin and IGF-1 signalling in mIGFREO PEC. (A) Representative blots for figures **B** to **E. (B&C)** Total Akt (**B**; WT n=5, mIGFREO n=5) and total eNOS (**C**; WT n=6, mIGFREO n=6) expression in wild type (WT) and mIGFREO pulmonary endothelial cells (PEC). **(D&E)** Basal serine phosphorylated AKT (**D**; WT n=8, mIGFREO n=7) and serine phosphorylated eNOS (**E**; WT n=8, mIGFREO n=9) expressions in WT and mIGFREO PEC. *WT denotes wild type mice and mIGFREO / TG*

denotes mutant human IGF1-R transgenic mice. Data expressed as mean±sem. * denotes p<0.05, WT vs mIGFREO. Data in **B-E** were analysed using Unpaired Students t test.

Figure 4. mIGFREO mice had blunted eNOS phosphorylation while sustained Akt phosphorylation in response to insulin or IGF-1. (A) Representative blots for Figures **B** and **C**. **(B&C)** Insulin (Ins, 150nM, 10 min) and IGF-1 (IGF-1; 150nM, 10 min) stimulated Akt (**B**; WT n=7, mIGFREO n=7), eNOS (**C**; WT n=6, mIGFREO n=9) serine-phosphorylation in WT and mIGFREO PEC. **(D&E)** eNOS activity in WT and mIGFREO (TG) PEC upon insulin (150nM; 30 min; **D**; WT n=5, mIGFREO n=7) and IGF-1 (150nM; 30 min; **E**; WT n=5, mIGFREO n=7) stimulation. **(F)** Representative blots for figures **G** and **H**. **(G&H)** Dose-dependent response to insulin (Ins; 50, 150nM, 10 min) induced serine-phosphorylation of Akt and eNOS in WT endothelial cells (PEC; **G**; WT n=21, mIGFREO n=22). Dose-dependent response to insulin (Ins; 50, 150nM, 10 min) induced serine-phosphorylation of Akt and eNOS in mIGFREO endothelial cells (**H**; WT n=21, mIGFREO n=22). *WT denotes wild type mice and mIGFREO / TG denotes mutant human IGF1-R transgenic mice*. Data expressed as mean±sem. (In Fig **B** *denotes p<0.05, Control vs insulin-stimulated Akt phosphorylation and **denotes p<0.05, Control vs IGF-1-stimulated Akt phosphorylation in WT littermates, $^{\#}$ denotes p<0.05, Control vs insulin-stimulated Akt phosphorylation and $^{\#\#}$ denotes p<0.05, Control vs IGF-1-stimulated Akt phosphorylation in mIGFREO cells; In Fig **C** † denotes p<0.05, Control vs insulin-stimulated eNOS phosphorylation and †† denotes p<0.05, Control vs IGF-1-stimulated eNOS phosphorylation in wild type cells. $^{\#}$ denotes p<0.05, WT+Insulin vs TG+Insulin for eNOS phosphorylation, $^{\#\#}$ denotes p<0.05 WT+IGF-1 vs TG+IGF-1 for eNOS phosphorylation; In Fig **D** and **E** * denotes p<0.05, WT Control vs WT+Insulin or WT Control vs WT+IGF-1. $^{\#}$ denotes p<0.05 WT+Insulin vs TG+Insulin or WT+IGF-1 vs TG+IGF-1; In Fig **G** and **H** * denotes p<0.05, serine-phosphorylation of Akt or eNOS at 50nmol/L insulin in WT or mIGFREO. $^{\#}$ denotes p<0.05 serine-phosphorylation of Akt or eNOS at 150nmol/L insulin in WT or mIGFREO). Data **B-H** were analysed using one-way ANOVA, followed by Fisher's test.

Figure 5. mIGFREO mice have normal glucose tolerance but enhanced glucose lowering in response to systemic insulin or IGF-1. (A&B) Blood glucose (**A**; WT n=18, mIGFREO n=18) and insulin concentration (**B**; WT n=5, mIGFREO n=7) in mIGFREO and WT littermates in fasting and fed state. **(C-G)** Glucose tolerance (**C**; WT n=20, mIGFREO n=22), insulin tolerance (**D;** WT n=20, mIGFREO n=22), area under curve for insulin tolerance tests **(E;** WT n=20, mIGFREO n=21**)**, IGF-1 tolerance (**F;** WT n=16, mIGFREO n=17), and area under the curve for IGF-1 tolerance tests **(G;** WT n=16, mIGFREO n=17**)** in mIGFREO mice and WT littermates. **(H)** Insulin-induced vasorelaxation in 2nd order mesenteric vessels from WT (n=17) and mIGFREO (n=20). **(I)** Mean area under curve of the vasorelaxation data presented in **(H) (J&K)** Plasma free fatty acid (**J;** WT n=16, mIGFREO n=16) and triglyceride concentration in mIGFREO and WT littermates (**K**; WT n=11, mIGFREO n=18). Data expressed as mean±sem. * denotes p<0.05, WT vs mIGFREO. Data in **A, B, E, G**, **I** were analysed using unpaired Students t test. Data in **C, D, F** were analysed using 2-way ANOVA, followed by Fisher's test. Data in **H** analysed using -way ANOVA, followed by Bonferroni's multiple comparisons test. Data in **J** and **K** were analysed using unpaired t test, followed by Mann Whitney test.

Figure 6. Enhanced insulin sensitivity in mIGFREO mice. (A-C) Data from low-dose hyperinsulinaemic euglycaemic clamp studies on mIGFREO and wild type (WT) littermates showing blood glucose (**A**), glucose infusion rate (**B**) and rate of glucose disappearance (Rd; **C**) during hyperinsulinaemic euglycaemic clamp (WT n=8, mIGFREO n=8). **(D-F)** Tissue-specific glucose uptake into brown adipose tissue (**D**; WT n=10, mIGFREO n=9), gastrocnemius skeletal muscle (**E**; WT n=7, mIGFREO n=6) and vastus skeletal muscle (**F**; WT n=8, mIGFREO n=7). **(G&H)** Insulin (intraperitoneal injection; 0.75U/kg; 15 min) stimulated tyrosine phosphorylation of IR (pY-IR) upon) in liver (**G**; WT n=8, mIGFREO n=8) and skeletal muscle (**H**; WT n=5, mIGFREO n=6). Data expressed as mean±sem. In Figure **A** and **B**, $^{\#}$ denotes p<0.05, total glucose flux WT vs mIGFREO mice. In Figure **D** – **H** *

denotes p<0.05 WT vs mIGFREO. Data in Fig **A, B** were analysed using 2-way ANOVA, followed by Bonferroni's multiple comparisons test. All other data were analysed by unpaired Students t test.

Figure 7. Excess generation of hydrogen peroxide in mIGFREO mice. (A&B) Insulin tolerance testing after catalase infusion in wild type (WT) **(A)** and mIGFREO **(B)** mice (WT n=17, mIGFREO n=20). **(C)** Area under the curve for ITT presented in **A&B** showing mead data for WT, mIGFREO and mIGFREO post-catalase infusion (10,000U/kg/day; 5 days) (WT, n=18, mIGFREO n=21). **(D;** WT n=12, mIGFREO n=12**)** Tyrosine phosphorylation of IR upon insulin stimulation (Ins, 0.75U/kg; 15 min) with and without catalase infusion in liver (n=8; representative blots shown on the right). **(E&F)** Relative mRNA expression of Nox2 (**E**; WT, n=3, mIGFREO n=4) and Nox4 (**F**; WT n=6, mIGFREO n=9) in endothelial cells of mIGFREO and WT littermates. **(G&H)** Protein expression of Nox2 (**G**; WT n=7, mIGFREO n=6) and Nox4 (**H**; WT n=7, mIGFREO n=5) in endothelial cells of mIGFREO and WT littermates. Data expressed as mean±sem. In Figure **B** * denotes p<0.05 mIGFREO vs mIGFREO+Catalase. In Figure **C**, ** denotes p<0.05 mIGFREO vs mIGFREO+Catalase. In Figure **D**, * denotes p<0.05, WT vs mIGFREO; $^{\#}$ denotes p<0.05 mIGFREO vs mIGFREO+catalase. In Figure **F, G, H** * denotes p<0.05 WT vs mIGFREO. Data in **A and B** were analysed using multiple t test. Data in **C** and **D** were analysed by one-way ANOVA with Fisher's test. Data in **E** were analysed using unpaired Student t test. All others were analysed using unpaired Student t test, Mann Whitney test.

Figure 8. miR-25 levels in mIGFREO and saphenous vein endothelial cells (SVEC). (A&B) Aortic (**A**; WT n=6, mIGFREO n=9) and PEC (**B**; WT n=7, mIGFREO n=5) levels of miR-25 mRNA expression in mIGFREO and WT littermates (WT). **(C)** miR-25 mRNA level in SVEC from patients with and without diabetes (No Diabetes n=7, Diabetes n=5). **(D&E)** miR-25 mRNA levels (**D**; No Diabetes n=5, Diabetes n=5) and Nox4 protein (**E**; WT n=7, mIGFR n=6) levels in SVEC from patients without diabetes, transfected with mutant IGF-1 receptor (mIGF-1R) cDNA compared to WT cDNA. **(F)** Nox4 mRNA expression in SVEC from patients without diabetes transfected with miR-25 mimetic or scrambled control (Scrambled n=5, mimetic n=5). **(Graphical Abstract)** Schematic representation showing conceptual framework for insulin/IGF-1 resistance in endothelial cells leading to decreased miR-25 and a concomitant increase in Nox4, hydrogen peroxide (H_2O_2), leading to enhanced whole-body insulin sensitivity. Data expressed as mean±sem * denotes p<0.05, WT vs mIGFREO or no diabetes vs diabetes or WT-cDNA vs mIGF-1R-cDNA or scrambled vs miR-25 mimetic. Data were analysed using unpaired Student t test. Data in **D** were analysed using unpaired Student t test, Mann Whitney test.

NOVELTY AND SIGNIFICANCE

What Is Known?

- Type 2 diabetes mellitus is characterized by an inability of both insulin and insulin like growth factor-1 (IGF-1) to appropriately activate their intracellular signalling networks in the endothelium and other tissues.

- The combination of insulin and IGF-1 resistance in skeletal muscle in mice, due to expression of a mutant IGF-1 receptor (mIGF-1R) which forms non-functioning hybrids with native insulin (IR) and IGF-1 receptors (IGF-1R), leads to type 2 diabetes.

- The effect of the combination of insulin and IGF-1 resistance specific to the endothelium at a cellular and systemic level remains unexplored.

What New Information Does This Article Contribute?

- To examine the combination of reduced insulin and IGF-1 sensitivity in endothelial cells (EC), we generated transgenic mice expressing mIGF-1R specifically in EC (mIGF-1R endothelium overexpressing mice: mIGFREO).

- Despite resistance to insulin and IGF-1, stimulated activation of endothelial nitric oxide synthase in EC mIGFREO demonstrated enhanced insulin-stimulated glucose uptake due to redox priming of IR by NOX4 NADPH oxidase (NOX4)-derived H_2O_2.

- The increase in NOX4 in mIGFREO EC was due to a reduction in expression of the inhibitory microRNA mIR-25.

Type 2 diabetes mellitus in humans and rodents is associated with whole-body and EC resistance to the actions of the closely related hormones insulin and IGF-1. While the effects of reduced insulin or IGF-1 sensitivity in EC on vascular and systemic metabolic homeostasis are well established, the effect of the combination of insulin and IGF-1 resistance in EC on vascular function and glucose homeostasis remains unexplored. To examine this question, we generated mIGFREO mice. mIGFREO had enhanced insulin-mediated glucose lowering, enhanced insulin-induced glucose uptake in muscle and fat and greater insulin-mediated vasodilatation of 2nd order mesenteric arteries. This potentially advantageous effect of EC specific insulin *and* IGF-1 resistance was due to redox priming of IR in liver and muscle by NOX4 derived H_2O_2. NOX4 activity is dependent on its expression levels, consistent with this we showed increased NOX4 expression in mIGFREO EC due to a reduction in expression of the microRNA mIR-25. Treating EC from humans with a mIR-25 mimetic reduced, whereas transfection of EC from humans with mIGF-1R increased NOX4 expression. Our data reveal a hitherto unrecognized cross talk between EC and insulin target tissues leading to enhanced insulin sensitivity.

Figure 1

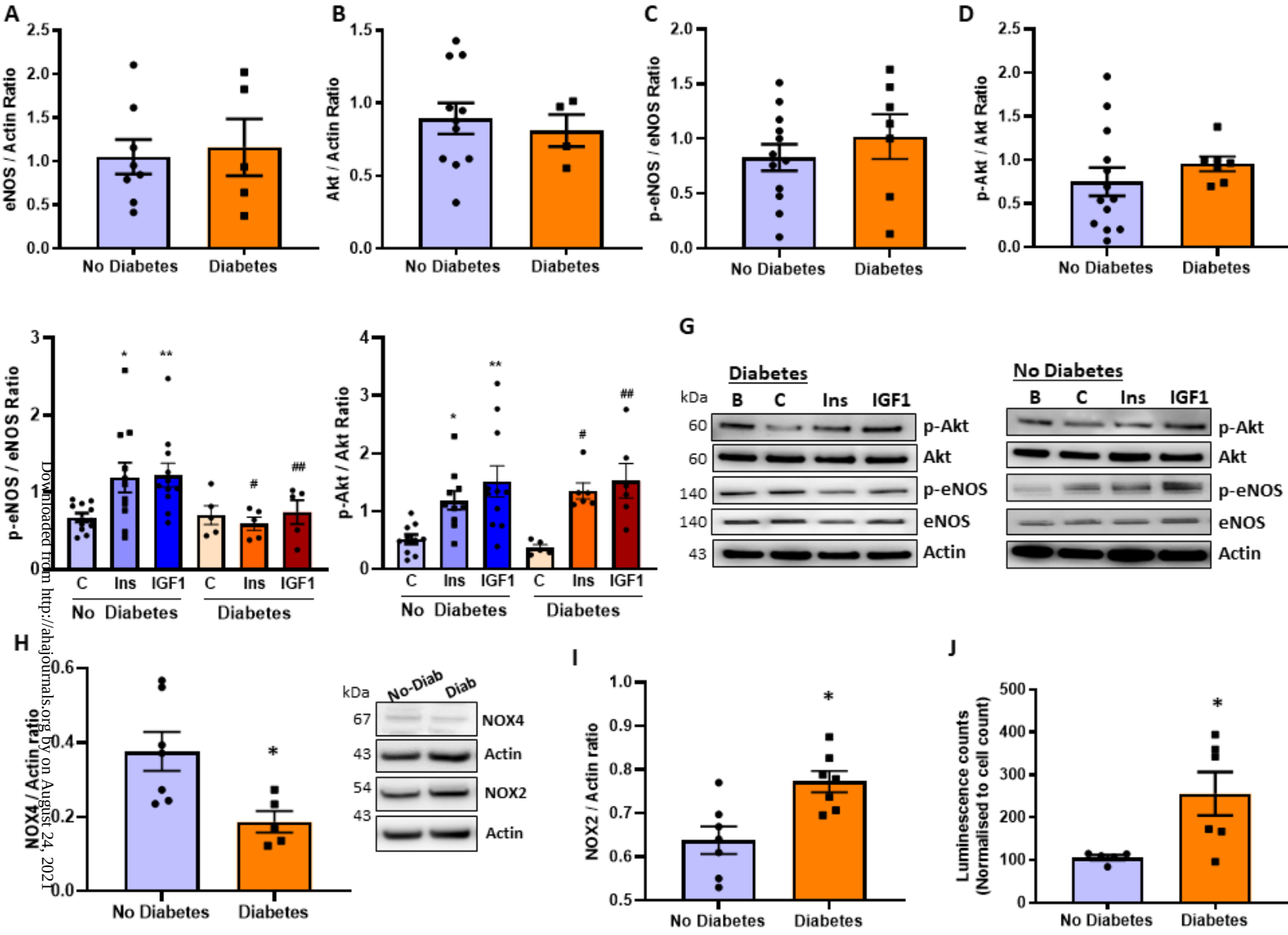

Figure 2

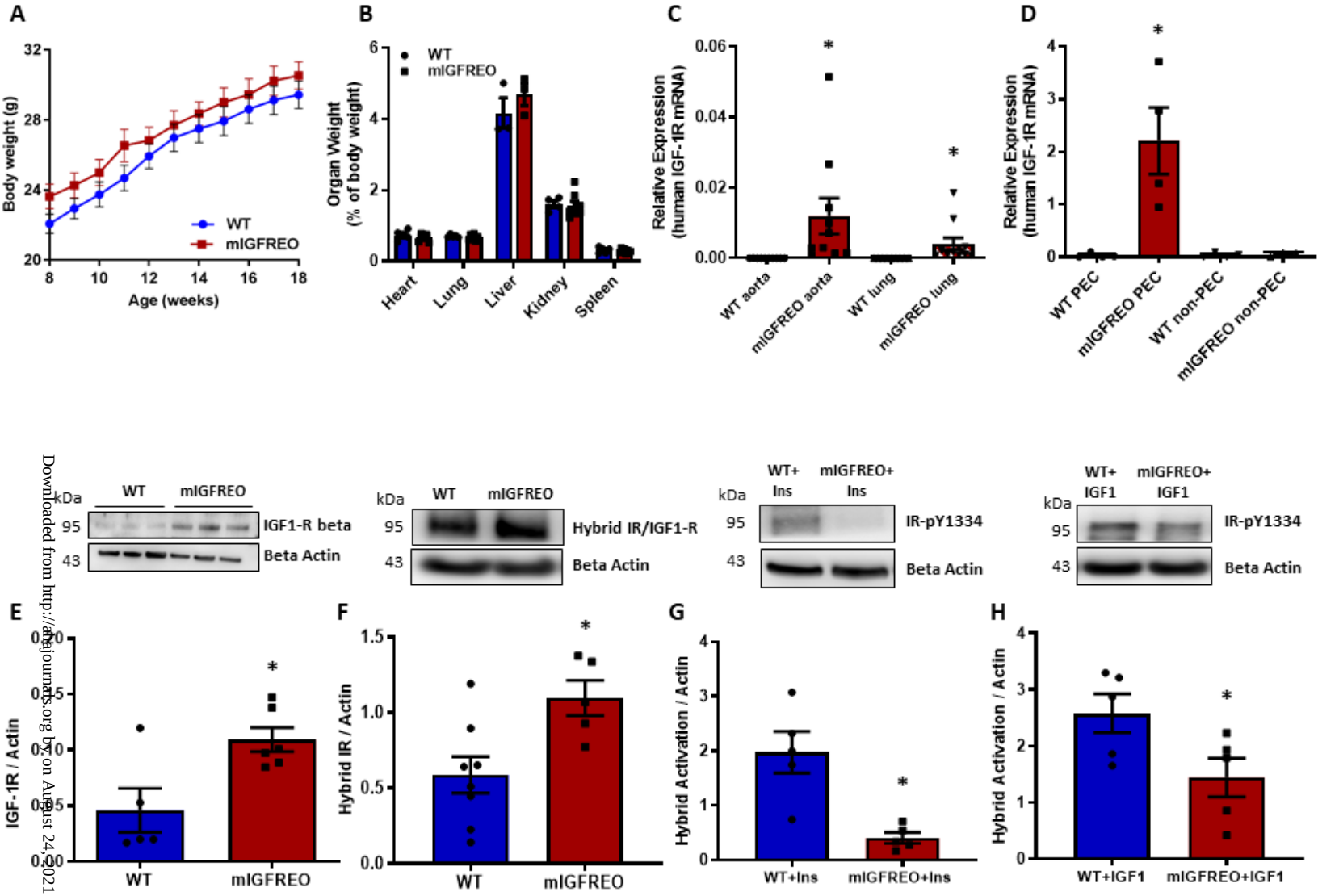

Figure 3

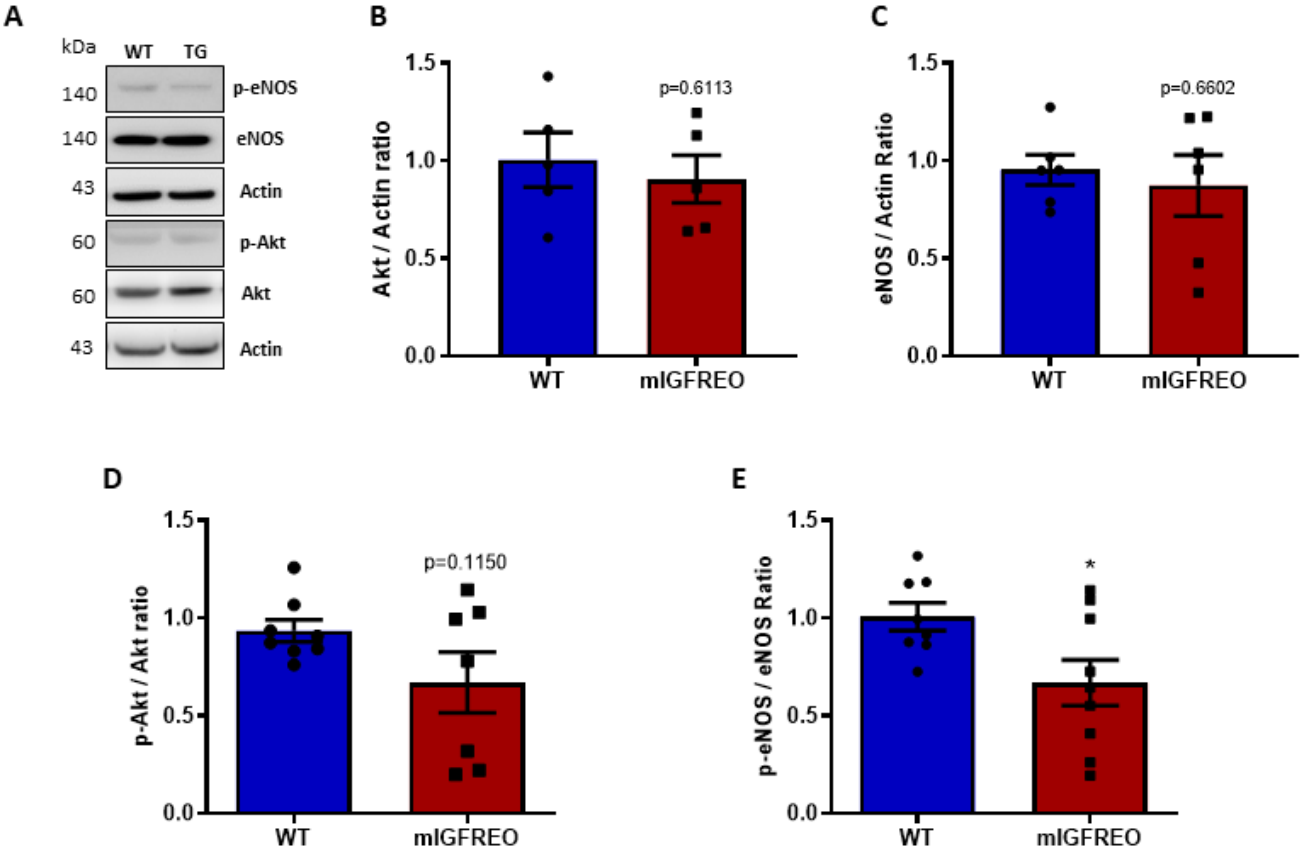

Figure 4

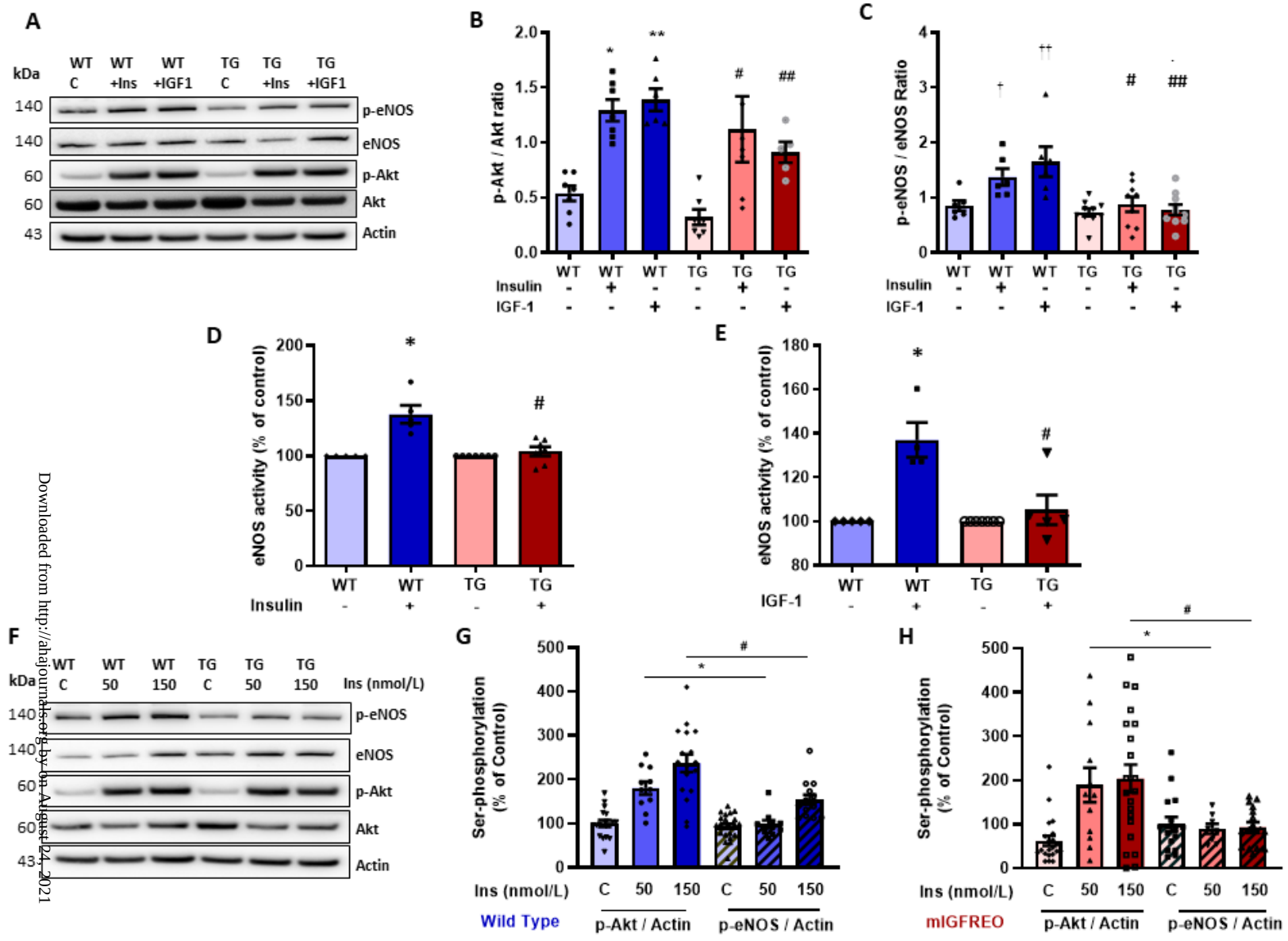

Figure 5

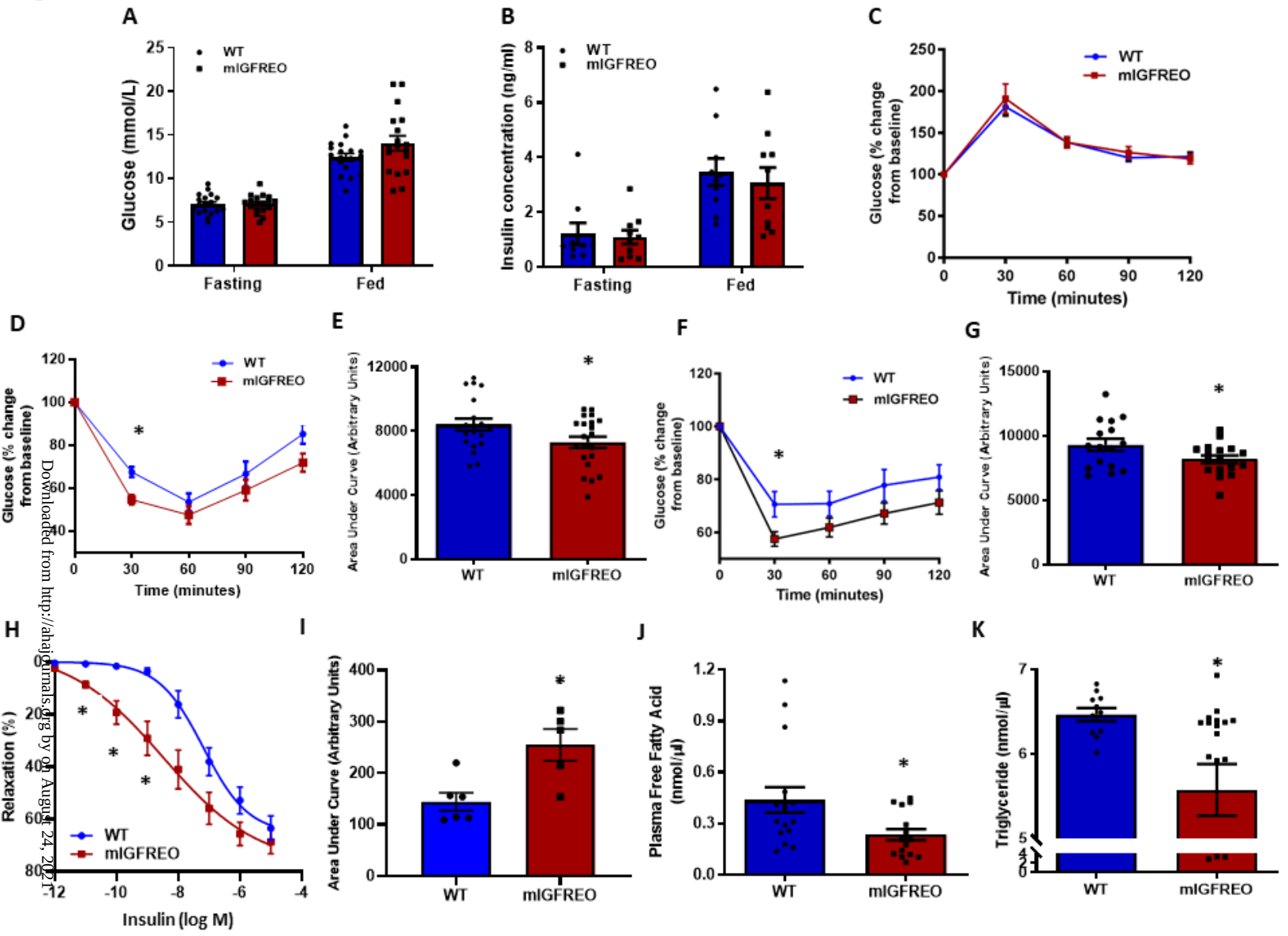

Figure 6

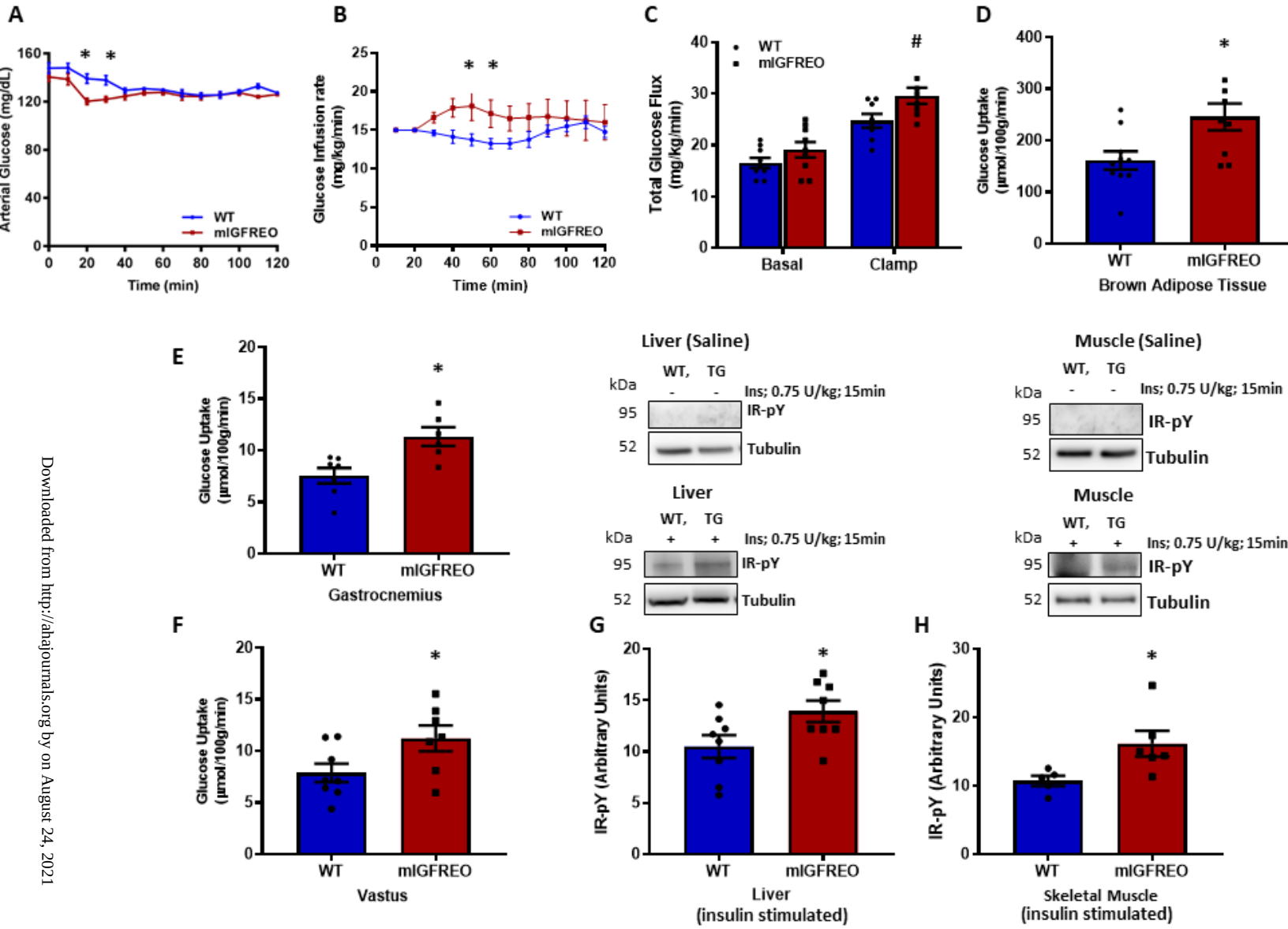

Figure 7

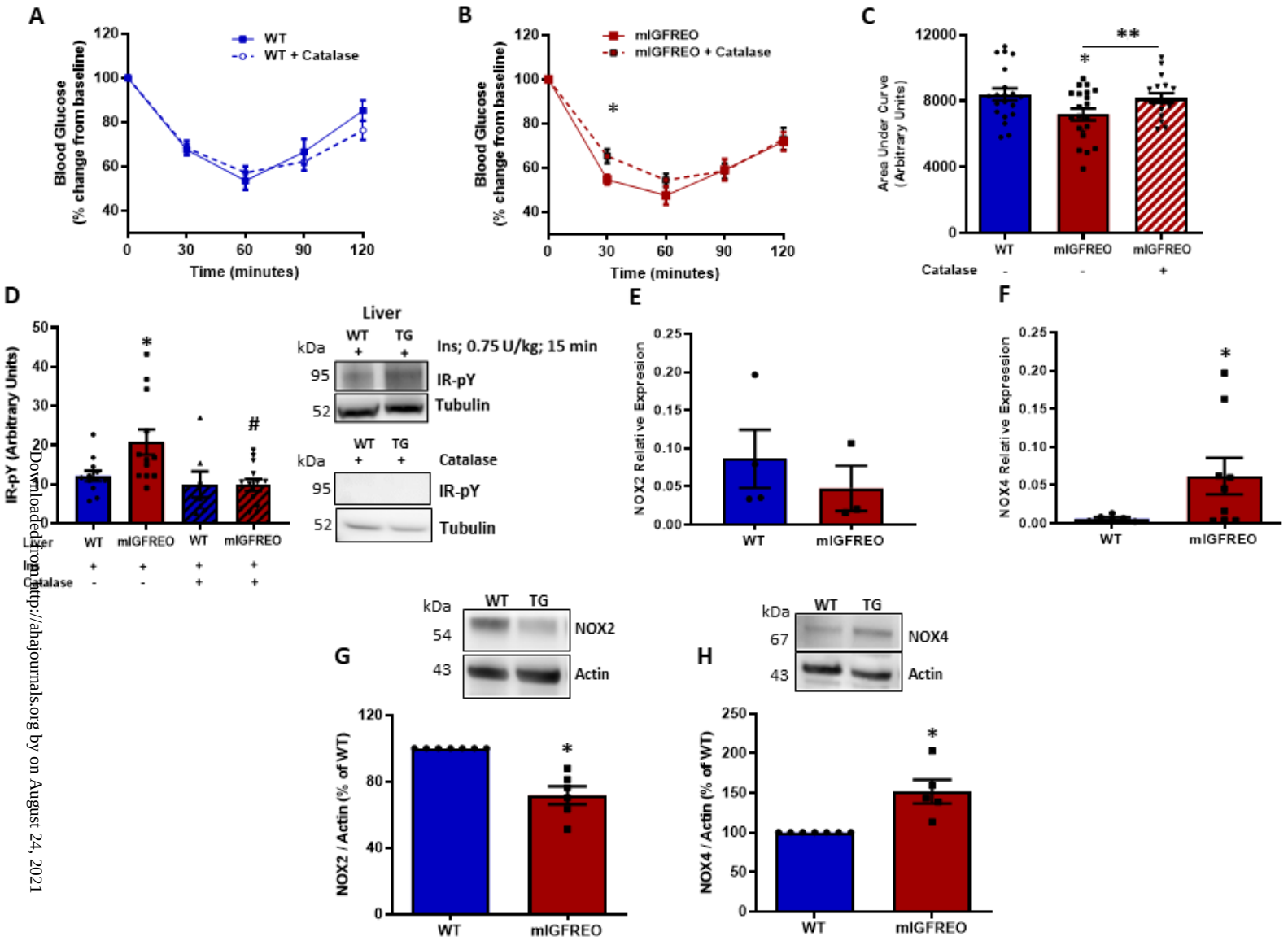

Figure 8