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The NOX4 pathway as a source of selective insulin resistance and responsiveness

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Abstract

Objective—Type 2 diabetes mellitus (T2DM) and related syndromes exhibit a deadly triad of dyslipoproteinemia, which leads to atherosclerosis; hyperglycemia, which causes microvascular disease; and hypertension. These features share a common, but unexplained, origin – namely, pathway-selective insulin resistance and responsiveness (SEIRR). Here, we undertook a comprehensive characterization of SEIRR in liver and hepatocytes by examining 18 downstream targets of the insulin receptor, surveying the AKT, ERK, and NAD(P)H oxidase 4 (NOX4) pathways.

Methods/Results—Injection of insulin into hyperphagic, obese T2DM *db/db* mice failed to inactivate hepatic protein tyrosine phosphatase gene family members, a crucial action of NOX4 previously thought to be required for all signaling through AKT and ERK. Insulin-stimulated T2DM livers unexpectedly produced an unusual form of AKT that was phosphorylated at Thr308 (pT308), with only weak insulin-stimulated phosphorylation at Ser473. Remarkably, knock-down or inhibition of NOX4 in cultured hepatocytes recapitulated the entire complicated pattern of SEIRR seen in vivo – namely, monophosphorylated pT308-AKT, impaired insulin-stimulated hypolipidemic and hypoglycemic pathways, but continued lipogenic pathways and robust ERK activation.

Conclusions—Functional disturbance of a single molecule, NOX4, is sufficient to induce the key harmful features of deranged insulin signaling in T2DM, obesity, and other conditions associated with hyperinsulinemia and SEIRR.

Keywords

diabetes; insulin resistance; obesity; NOX4; lipogenesis

Introduction

Accelerated macrovascular and microvascular disease remain significant, and growing, causes of morbidity and mortality in type 2 diabetes mellitus (T2DM) and related syndromes. The hallmark of these conditions is a distinctive pattern of pathway-selective insulin resistance and responsiveness (SEIRR).^{1–4} The result is the deadly triad of dyslipoproteinemia, which leads to atherosclerotic macrovascular disease.^{5–8}

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hyperglycemia, which causes microvascular disease;^{9–11} and hypertension, which worsens both large- and small-vessel complications.^{6, 12, 13}

As an example of SEIRR, the T2DM liver upon stimulation with insulin fails to adequately take up glucose¹⁴ or suppress gluconeogenesis.^{15, 16} Yet the T2DM liver continues insulin-stimulated synthesis and storage of lipids;^{10, 14, 17} and it does not appropriately inhibit the secretion^{17–20} nor accelerate the clearance^{21–25} of atherogenic apolipoprotein (apo)-B-containing lipoproteins (Figure 1 and Supplemental Background).

Several explanations have been proposed for SEIRR,^{1, 3, 4, 26–29} but all have focused on the two canonical limbs of the insulin signaling cascade, meaning protein kinase B (AKT) with its downstream targets and mitogen-activated protein (MAP) kinases with their downstream targets (Figure 1; see reference³⁰ for a comprehensive, timely review). The effect of SEIRR on the AKT limb is particularly puzzling, because AKT regulates hypolipidemic and hypoglycemic pathways that become insulin-resistant, but also lipogenic pathways that remain insulin-responsive. As shown in Figure 1, hypolipidemic and hypoglycemic pathways downstream of AKT include translocation of facilitated glucose transporters (GLUT) in extrahepatic tissues³¹ and AKT-mediated phosphorylations of forkhead box-1 (FOXO1) that suppress hepatic apoC-III expression^{20, 32} and gluconeogenesis.³³ Pathways downstream of AKT that stimulate lipid biosynthesis include alterations in three site-specific phosphorylations of ATP-citrate lyase (ACL), in part through GSK3 β ,^{34–36} and mTORC1-mediated transcription of lipogenic genes³⁷ via SREBP1c.^{28, 29, 38, 39} Obesity and related conditions impair FOXO1 phosphorylation in vivo, resulting in abnormally high hepatic *Apoc*^{20, 32} and *Pepck*⁴⁰ mRNA levels, whereas AKT-mediated phosphorylation of GSK3 is barely blunted (see the data in figure 3 of reference⁴¹) and mTORC1 exhibits enhanced activity.^{42, 43} In addition, SEIRR leaves the MAP kinase limb responsive to insulin, as assessed by ERK^{1, 2} and JNK⁴⁴ phosphorylation, despite a marked impairment in overall insulin-stimulated tyrosine phosphorylation of the insulin receptor.^{1, 2} The basis for differential dysregulation of major AKT targets and MAP kinases remains unclear.

There is, however, a non-canonical third limb in insulin signaling. When insulin binds to the insulin receptor, it activates the NAD(P)H oxidase homologue NOX4 to generate a transient burst of superoxide ($O_2^{\bullet -}$) and its byproduct H_2O_2 that enhance signal propagation by disabling enzymes in the protein-tyrosine phosphatase (PTPase) gene family (Figure 1).^{45–50} Two such enzymes of note are, first, the phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which can terminate PI3K signaling by dephosphorylating its active lipid product, phosphatidylinositol-3,4,5-trisphosphate (PIP₃); and second, the nonreceptor-type PTPase-1B (PTP1B), which blunts insulin signaling by dephosphorylating tyrosyl residues within the insulin receptor and its substrates (IRS1,2). Interfering with insulin-stimulated generation of superoxide and H_2O_2 was reported to cause abnormal persistence of PTEN^{51, 52} and PTP1B^{48, 49} activities and hence a global decrease in all pathways downstream of the insulin receptor, including insulin-stimulated phosphorylation of AKT and ERK and cellular uptake of glucose. These studies found no role for impaired NOX4 activation in pathway selectivity of the responses to insulin.

In the current study, we undertook a comprehensive examination of SEIRR in T2DM livers in vivo and in cultured hepatocytes by examining 18 downstream targets of the insulin receptor, including key members of the AKT, ERK, and NOX4 pathways (Figure 1). Unexpectedly, we found that functional derangement of a single molecule, NOX4, is sufficient to induce the key harmful features of SEIRR seen in vivo. This work was presented at the XVII Lipid Meeting Leipzig (10 December 2011).⁵³

Material and Methods

Insulin stimulation of the livers of control and T2DM mice in vivo

To characterize SEIRR in vivo, we used hyperphagic mice, which mimic humans who overeat and thereby develop the same complications, including obesity, fatty liver, dyslipoproteinemia, hyperglycemia, hyperinsulinemia, and disturbed insulin signaling. Lean *db/m* mice (controls) and obese, T2DM *db/db* (*Lepr^{db/db}*) littermates on the C57BLKS background (Jackson Laboratory, Bar Harbor, ME) were fed ad-libitum until age 14 weeks.²³ We modified an insulin administration protocol⁴¹ to allow sampling of their livers in vivo. Animals were fasted overnight, weighed, and then anesthetized. The abdominal cavity was opened, a single lobe of liver was gently tied off, and a sample distal to the ligature was surgically removed and promptly snap-frozen (pre-insulin sample). Because of the ligature, the cut surface on the remaining liver did not bleed. Next, 10 units of bovine insulin (#I1882, Sigma Chemical Company, St. Louis, MO) per kg of body weight was administered into the vena cava, followed by staunching of the puncture site with the tip of a moistened cotton swab. To avoid acute hypoglycemia, we bathed the exposed peritoneum in a warm 10% dextrose solution. After 10 min, blood was obtained by heart puncture, and sections from other hepatic lobes were taken and snap-frozen (post-insulin sample). To verify diabetic status, hemoglobin A1c levels in blood were quantified by the Vanderbilt Mouse Metabolic Phenotyping Center (Nashville, TN), giving values of 3.9%±0.05% in *db/m* and 7.4%±0.56% in *db/db* (mean±SEM, n=3, P<0.005 by the unpaired Student's *t*-test). All animal protocols were approved by the Institutional Animal Care and Use Committee.

Insulin stimulation of cultured liver cells, with and without active NOX4

For studies in vitro, we used two complementary systems. First, we previously reported that, upon exposure to physiologic concentrations of insulin, McArdle 7777 rat hepatoma cells exhibit robust insulin-induced suppression of SULF2, an inhibitor of remnant lipoprotein uptake,²³ and our studies here indicated that these cells phosphorylate every downstream target of the insulin receptor that we examined (Figure 1 and Results). Moreover, McArdle cells can be maintained in culture for the three days typically required for siRNA knockdown.²³ Thus, cells replete or deficient in NOX4 were prepared by transfection with 50 nM nontarget siRNA or rat *Nox4* siRNA (#D-001810-01-05 and # L-080114-01-0010, respectively; Dharmacon, Lafayette, CO), using the siIMPORTER transfection reagent (#64-101, Millipore Corporation, Billerica, MA), for 4h in serum-free medium and then overnight in DMEM with 10% FBS, followed by an additional 48h at 37°C in DMEM/10% FBS without siRNA. In accordance with prior literature,^{1, 23, 48, 54} cells were switched to serum-free (DMEM/1% BSA) or low-serum (DMEM/2% FBS) medium for 2h before supplementation with 0 or 10nM insulin. Exposure to 0 or 10nM insulin lasted 10 min for studies of phosphorylations of pre-existing protein targets, 15 min to assess uptake of [³H]2-deoxy-D-glucose, a non-metabolizable glucose analogue, or 18 h for studies of SULF2 protein regulation. Serum-free medium was used only for short-term studies of phosphorylations or 2-deoxy-D-glucose uptake;^{1, 48, 54} the longer studies used low-serum medium to maintain cell health.²³

Second, because McArdle cells appear to lack sufficient endogenous expression of LXR,⁵⁵ a required factor for insulin to induce *Srebp1c* transcription,⁵⁶ we relied on freshly isolated rat primary hepatocytes⁵⁷ for our studies of mRNA regulation. Primary hepatocytes exhibit vigorous insulin-induced regulation of *Pepck* and *Srebp1c*,^{28, 38, 56} but lose many physiologic responses after prolonged culture, necessitating our use of a fast-acting chemical inhibitor of NOX4 (diphenyleneiodonium, DPI).^{58, 59} We applied DPI at a concentration, 1.0 μM, that provides ~90% inhibition.⁶⁰ Owing to potential issues of selectivity, particularly at higher micromolar concentrations,⁶¹ we used DPI in only a limited fashion.

To allow studies of the suppressive effect of insulin on *Pepck* mRNA levels, all culture media for primary hepatocytes included 100nM dexamethasone to elevate the basal expression of this target, as described.^{28, 62} Our computational analysis of the NOX4 sequence revealed two candidate caveolin-binding motifs ($\Phi X \Phi X X X X \Phi$, where Φ represents an aromatic amino acid), in residues ¹⁹⁰FWYTHNLF¹⁹⁷ and ⁵⁷⁰FEYNKESF⁵⁷⁷, similar to the single caveolin-binding motif in the insulin receptor (see references^{63–65} and Figure 1). Thus, we inferred that NOX4 would reside in cholesterol-rich caveolae, and that its superoxide burst could thereby contribute to the putative generation of oxysterol ligands for LXR after insulin stimulation. Activation of LXR appears to be an essential step in *Srebp1c* induction,⁵⁶ in addition to the phosphorylation cascades leading to mTORC1 (reference²⁸ and Figure 1). Consistent with this model, we found that global inhibition of NOX4 in primary hepatocytes blocked *Srebp1c* mRNA induction by insulin (not shown), even though phosphorylations upstream and downstream of mTORC1 remained responsive. Accordingly, primary cells also received 0.5-2nM TO901317, which is 10% of its IC₅₀ for LXR activation.⁶⁶ Primary hepatocytes replete or deficient in NOX4 were prepared by a 30-min pre-incubation at 37°C with DMSO vehicle or 1.0 μM DPI, respectively, followed by addition of 0 or 10nM insulin. These cells were then incubated for an additional 6h to allow mRNA levels to change.

Assays of 18 downstream targets of the insulin receptor, including key members of the AKT, ERK, and NOX4 pathways

Pre- and post-insulin liver samples in which we assayed phosphatase activities were handled under strictly anaerobic conditions in an enclosed work station (model #1025, Thermo Fisher Scientific, Marietta, OH).^{48, 67} The liver samples were used to prepare homogenates, from which we immunoprecipitated PTEN and PTP1B in separate aliquots and then quantified their enzymatic activities. The substrate was *para*-nitrophenyl phosphate (*p*NPP), which PTEN⁶⁸ and PTP1B^{48, 67} cleave into a product that absorbs 410-nm light. Activities were normalized to the amounts of recovered enzyme, which were assessed by densitometry of immunoblots by ImageJ software (<http://rsbweb.nih.gov/ij/download.html>) and expressed as the sum of gray values above baseline. Enzyme recovery did not significantly differ between *db/m* and *db/db* livers.

All other tissue and cellular extractions, immunoprecipitations, immunoblots, and qRT-PCR reactions were performed as we previously described.²³ Antibodies against target proteins (total target as well as forms with site-specific phosphorylations) are listed in Supplemental Table I, following the nomenclature in Figure 1. Close reading of product inserts from Cell Signaling Technologies (Beverly, MA) indicated that catalogue #4376 was specific for pT202-ERK and catalogue #4377 was specific for pY204-ERK, instead of recognizing solely the doubly phosphorylated forms. Clean immunoblots of pT24-FOXO1 from liver homogenates required prior immunoprecipitation of total FOXO1.⁶⁹ Primers and probes for qRT-PCR were synthesized by the Gene Expression Facility at the University of North Carolina (Chapel Hill, NC, Dr. Hyung Suk Kim, director), and the sequences are given in Supplemental Table II. In studies of labeled 2-deoxy-D-glucose uptake by cultured McArdle hepatocytes, this molecule was added during the final 4 min of incubation.^{48, 54}

Statistical analyses

Normally distributed data are reported as means±SEMs. For comparisons of enzymatic activities in pre-insulin versus post-insulin liver samples, the paired *t*-test was used. For comparisons involving several groups of cultured hepatocytes, ANOVA was initially used, followed by pairwise comparisons using the Student-Newman-Keuls *q* statistic.

Results

Characterization of pathway-selective insulin resistance and responsiveness in the livers of hyperphagic T2DM mice

We began with a detailed characterization of SEIRR in T2DM *db/db* livers in vivo. Consistent with our preliminary report showing dysregulation of the hepatic NOX4 pathway in *db/db* mice,⁷⁰ we found that insulin stimulation of T2DM livers in vivo failed to inhibit the activities of PTEN (Figure 2A) or PTP1B (Supplemental Figure I), in contrast to the responses of control *db/m* livers. Surprisingly, despite the inability to suppress PTEN or PTP1B activities, acute insulin administration in vivo provoked completely normal phosphorylation of AKT at Thr308 (pT308) in the livers of obese T2DM *db/db* mice compared to lean *db/m* controls (Figure 2B, upper immunoblots). The original report of SEIRR documented a significant defect in insulin-stimulated phosphorylation of AKT at Ser473 (pS473) in isolated microvessels from obese, hyperphagic rats, but did not examine Thr308.¹ In line with that report, we found only weak insulin-stimulated pS473-AKT in *db/db* livers compared to *db/m* controls (Figure 2B, lower immunoblots). Thus, acute insulin stimulation of T2DM livers in vivo failed to inactivate PTPase gene family members, yet unexpectedly produced an unusual, monophosphorylated form of AKT, pT308-AKT.

To determine the consequences of these disturbances in insulin signaling in vivo, we next examined a set of downstream targets of AKT in the same insulin-stimulated *db/m* and *db/db* livers. Supplemental Figure II-A–B shows that T2DM impaired insulin-induced phosphorylation of hepatic FOXO1, consistent with prior reports of apoC-III overexpression and persistent gluconeogenesis, yet phosphorylations of GSK3 β , PRAS40, and S6K1 remained robust, consistent with ongoing hepatic lipogenesis (see Figure 1 and Supplemental Background). We also found that insulin-stimulation of T2DM *db/db* livers in vivo provoked vigorous phosphorylation of both the Thr202 and Tyr204 sites on ERK1 (Supplemental Figure II-C).

Cell-culture models of SEIRR from deficiency or inhibition of NOX4

To explain the complicated pattern of insulin resistance and responsiveness in T2DM *db/db* livers in vivo, we hypothesized that disturbed NOX4 function might play a causal role. As our initial model, we used siRNA to knock-down this enzyme in cultured rat McArdle hepatoma cells (Figure 3A). Remarkably, knock-down of NOX4 preserved insulin-stimulated phosphorylation of AKT at Thr308, but blocked insulin-stimulated phosphorylation at Ser473 (Figure 3B), thereby recapitulating the production of monophosphorylated pT308-AKT that we saw in T2DM livers in vivo. To examine the other canonical limb of insulin signaling, we found that NOX4 knock-down still permitted vigorous insulin-stimulated phosphorylations of ERK1 at Thr204 and Tyr204 (Figure 3C), replicating the ERK response that we saw in T2DM livers in vivo. Thus, impairment of NOX4 does not produce the global decrease in insulin-stimulated signaling that had been previously reported, but instead causes an unexpected pattern of site-specific blockages or continued phosphorylations of AKT and ERK that mimics the upstream elements of SEIRR that we saw in the livers of hyperphagic T2DM *db/db* mice in vivo.

We next examined the effect of NOX4 deficiency on key insulin-stimulated phosphorylations downstream of AKT. We found that NOX4 knock-down abolished insulin-stimulated phosphorylation of FOXO1 at Thr24 (Figure 4A). Strikingly, the same NOX4-deficient hepatocytes showed unimpaired insulin-stimulated phosphorylations of GSK3 β and ACC (Figure 4A). The two AKT-dependent inputs into mTORC1 – namely, PRAS40 and TSC2, showed continued responsiveness to insulin in these cells (Figure 4B). Likewise, in NOX4-deficient hepatocytes, insulin still stimulated brisk phosphorylation of the

mTORC1 substrate S6K1 (85-kDa isoform), indicating unimpaired activation of mTORC1 (Figure 4B). The 70-kDa S6K1 isoform exhibited high basal and post-insulin phosphorylations (Figure 4B), exactly as in *db/db* livers (Supplemental Figure II-B). The S6K1 is of particular interest, because it desensitizes IRS1 in states of overnutrition and obesity (reference⁷¹ and Figure 1). Taken together, Figures 3 and 4 indicate that interference with just a single molecule, NOX4, causes the same complicated pattern of disturbed insulin-stimulated phosphorylations that we saw in T2DM livers in vivo in Figure 2 and Supplemental Figure II.

To assess metabolic effects caused by disturbances in NOX4, we focused on several functional endpoints, beginning with mRNA regulation. Consistent with Figure 4A, chemical inhibition of NOX4 blunted major functional effects of AKT-mediated phosphorylation of FOXO1 – namely, downstream suppression of the mRNAs encoding *Apoc3* (Figure 5A) and *Pepck* (Figure 5B). These results indicate that deficiency or inhibition of NOX4 in hepatocytes renders these cells resistant to key hypolipidemic and hypoglycemic actions of insulin that are downstream of AKT, similar to SEIRR in vivo. Likewise, *Irs2* mRNA, a factor regulated similarly to *Apoc3* and *Pepck*,⁷² became resistant to suppression by insulin in the absence of functional NOX4 (Supplemental Figure III). Consistent with our finding of continued mTORC1 activation (Figure 4B), NOX4 inhibition still allowed high insulin-induced levels of *Srebp1c* mRNA, which encodes a major transcription factor in lipogenesis (Figure 5C).^{28, 38, 39, 73, 74}

Lastly, we examined the effects of disturbed NOX4 function on glucose handling and regulation of remnant lipoprotein clearance. Under stimulation by insulin and other factors, the normal liver takes up one-third of each oral glucose load,⁷⁵ but the process is defective in T2DM.¹⁴ Figure 6A shows that NOX4 deficiency nearly abolished the ability of insulin to stimulate glucose uptake into McArdle hepatocytes. Our current results show impairments in both basal and insulin-stimulated glucose uptake by hepatocytes, consistent with the finding that activated mTORC1 impairs glucose uptake by the liver (reference⁷⁶ and Figure 4B). Regarding remnant lipoprotein clearance, we recently demonstrated that T2DM livers overexpress the heparan sulfate glucosamine-6-*O*-endosulfatase-2 (SULF2), a novel factor that complexes with the syndecan-1 heparan sulfate proteoglycan and impairs hepatocyte uptake of atherogenic remnant lipoproteins (references^{23, 24} and Figure 1). Overexpression of SULF2 is mediated in part through an impaired response to insulin.²³ Figure 6B shows that NOX4 deficiency abolishes the ability of insulin to suppress SULF2 protein expression in cultured hepatocytes, again mimicking T2DM liver.

Discussion

Our results indicate that T2DM liver exhibits defects in specific NOX4 functions, and that impairment of NOX4 in cultured hepatocytes produces a distinctive pattern of selective insulin resistance/responsiveness that recapitulates all known features of hepatic SEIRR in vivo. These include inhibition of key hypolipidemic and hypoglycemic effects of insulin, but continued activation of several pathways mediating lipogenesis, ongoing responsiveness of ERK, and loss of the ability of insulin to suppress SULF2 expression. Strikingly, in Figure 1, all pathways shown in blue become resistant, while all pathways in red remain responsive. From the standpoint of human health, it is the worst possible combination of effects.

Several other abnormalities have been proposed as underlying causes for impaired insulin signaling, such as an increased flux of non-esterified fatty acids, insulin-independent activation of mTOR by nutrient excess, infiltration of key tissues by immune cells, and ER stress. Unfortunately, many studies of these factors have focused largely or exclusively on their effects on glucose metabolism. Given our current understanding of SEIRR, however, it

is essential to examine all key insulin signaling pathways, to determine if a proposed cause can faithfully produce the entire pattern of SEIRR that arises from chronic, flagrantly positive caloric balance.

Fragmentary information in the literature hints at problems with these other hypotheses. For example, an animal model of obesity induced by a high-fat, high-sucrose diet failed to show robust insulin-stimulated phosphorylation of hepatic AKT at Thr308.⁴³ In another study, rapidly elevated plasma concentrations of non-esterified fatty acids in vivo blocked insulin-stimulated phosphorylation of aortic ERK.⁴⁴ These disturbances in insulin-stimulated phosphorylations differ significantly from what we (Figure 2) and others¹ have described in simple chronic hyperphagia. Doubts about a dominant role for fatty acid flux were reinforced by work showing that drastic lowering of plasma concentrations of non-esterified fatty acids in obese, glucose-intolerant individuals only partially corrected their ability to handle carbohydrates in response to insulin.⁷⁷ Regarding other proposed causes of selective insulin resistance, our finding of continued insulin-stimulated phosphorylation of a subset of the downstream targets of AKT in vivo (Supplemental Figure II-A–B) contradicts both the mTOR hypothesis and the ER stress hypothesis, as depicted in figure 3c,d of reference³⁰. More detailed investigations will be needed to fully evaluate the roles of these well-known prior models.

The mechanism for NOX4 dysfunction in T2DM liver remains unknown, but we are particularly interested in the possibility that striking overexpression of protein phosphatase 5 (PPP5C), a novel binding partner we found for NOX4,⁷⁰ may derange insulin-stimulated phosphorylations, while still allowing insulin-induction of a key target of mTORC1 and LXR – namely, *Srebp-1c* mRNA. A regulatory role for PPP5C might also reconcile our current results with earlier work showing that overexpression of enzymatically inactive NOX4 mutants in adipocytes blunts the canonical AKT and ERK limbs of insulin signaling.⁴⁸ We now speculate that these mutants would have sequestered PPP5C so that it was no longer available to interact with the endogenous, active NOX4, thereby mimicking an unusual phenotype that we had reported from knocking down PPP5C, i.e., impaired insulin-stimulated formation of both pS473-AKT and pY204-ERK.⁷⁰ Cell type-specific effects might also have played a role. If confirmed by additional studies, derangements in insulin signaling from either too much or too little PPP5C will imply a need for caution in manipulating this molecule therapeutically.

There are several possibilities for how disturbances in NOX4 function lead to the extensive manifestations of SEIRR that we report here. For example, the two sites on AKT are acted on by different kinases, PDPK1 and mTORC2 (references^{78, 79} and Figure 1), from which we now infer differential dependence on NOX4, perhaps via PTEN (references^{50, 52} and Figure 2A). One model would be metabolic channeling of PIP₃ from PI3K directly to PDPK1, bypassing PTEN, whereas PIP₃ that is destined for mTORC2 might have to contact PTEN beforehand (see Figure 1). Another possibility is that, despite its name, PDPK1 has been reported to be constitutively active,^{80, 81} and so any process that brings it and its substrate together may be sufficient to produce pT308-AKT.^{82, 83} In contrast, mTORC2 requires both intrinsic activation and physical contact with substrate.⁷⁹ A third explanation could be that site-specific underphosphorylation of IRS1,2 in SEIRR causes the recruitment or activation of an unusual set of PI3K isoforms that trigger PDPK1 to act on AKT, but fail to signal to mTORC2 (compare with figure 7 in reference²⁶ and the current Figure 1). Fourth, NOX4, possibly through PTEN and PIP₃, might differentially regulate the enzymes that catalyze site-specific dephosphorylations of AKT (see references^{81, 84}). Of note, monophosphorylation of AKT caused by artificial disruption of mTORC2 in non-diabetic animals impairs insulin-stimulated glucose uptake and the formation of pT24-FOXO1, yet still allows insulin, IGF-1, and PDGF to provoke phosphorylations of GSK3β and

TSC2.^{85–89} These prior results add credence to the widely deranged substrate selectivity and other aberrant actions of pT308-AKT that we found in *db/db* livers and in our hepatocyte models of SEIRR. Robust activation of the ERK pathway in T2DM despite low total tyrosine phosphorylation of the insulin receptor has been a long-standing mystery.^{1, 2} The phenomenon may indicate reliance on a small but key subset of tyrosyl targets that we speculate would continue to undergo insulin-stimulated phosphorylation despite NOX4 dysfunction, thereby allowing activation of SHC and its downstream targets (Figure 1).

Overall, our findings suggest a unified molecular basis for fatty liver, atherogenic dyslipoproteinemia, hyperglycemia, and hence accelerated atherosclerosis and microvascular disease in T2DM, obesity, and related syndromes. Dysregulation of the NOX4 pathway in other insulin-responsive cell types and organs may further contribute to lipoprotein and glucose abnormalities, hypertension,^{4, 90–95} hypercoagulability,^{6, 96–98} destabilization of atheromata through overexpression of matrix metalloproteinases,⁴⁴ and other vascular and non-vascular complications. Although by no means imminent, either NOX4 or its binding partner may become suitable targets for manipulation *in vivo*, to correct SEIRR and its devastating sequelae. In the meantime, improvements in lifestyle are likely to remain the best therapeutic approach, whenever they can be achieved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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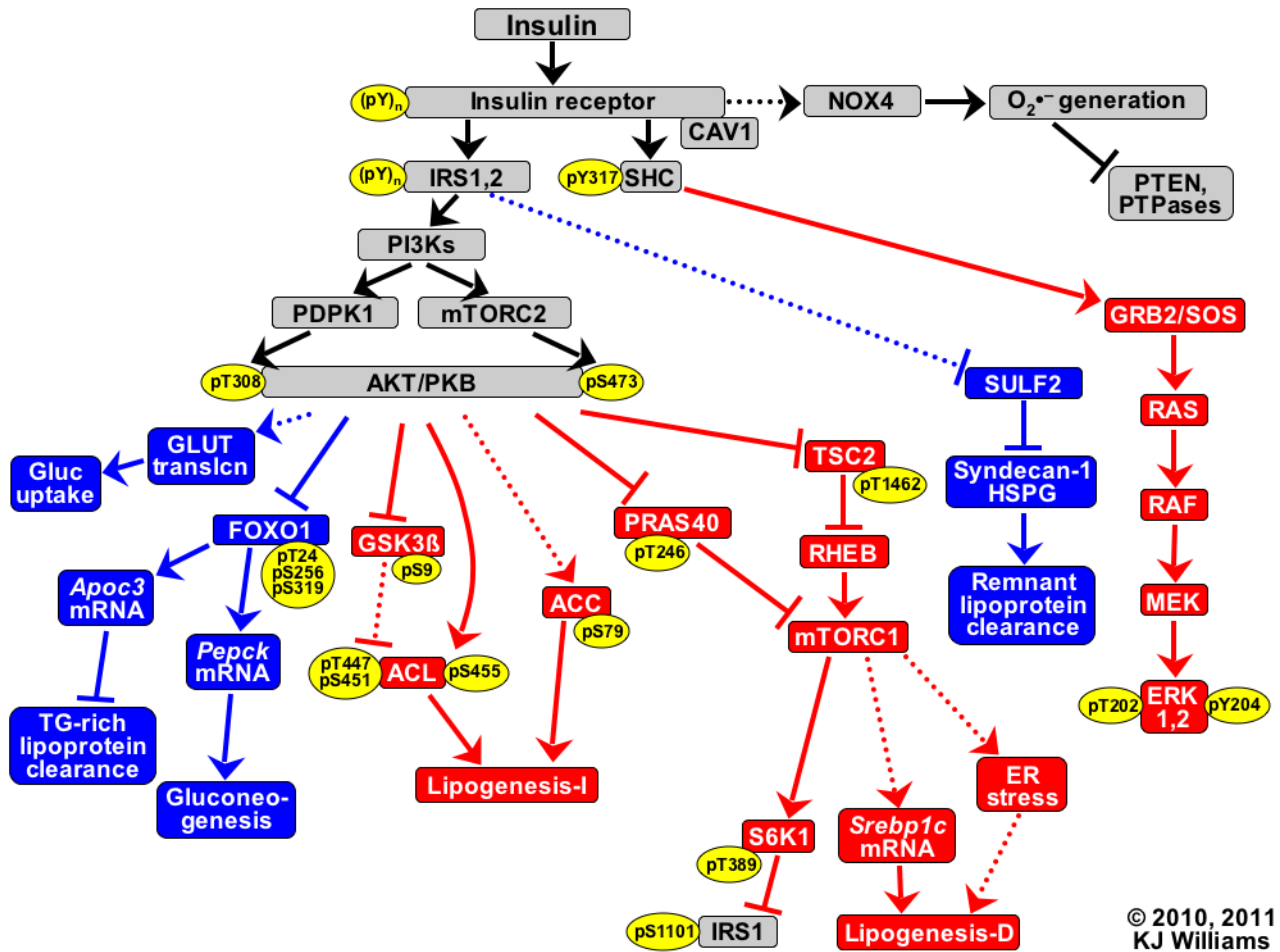
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Figure 1.

Key branches of the insulin signaling cascade. Hypolipidemic and hypoglycemic pathways that are normally triggered by insulin are indicated in blue, whereas insulin-stimulated pathways that activate lipogenesis and MAP kinases are shown in red. Solid lines represent recognized pathways; dotted lines are less well-characterized or putative. Pointed arrowheads indicate stimulation of the immediately downstream molecule or process; flat arrowheads indicate inhibition. Thus, endpoints that receive signals from the insulin receptor via pathways with an even number of flat arrowheads (0 or 2) are activated by insulin (*Gluc uptake*, *TG-rich lipoprotein clearance*, both mechanisms for *Lipogenesis*, *Remnant lipoprotein clearance*, and phosphorylations of *ERK*). Pathways with an odd number of flat arrowheads are inhibited by insulin (*PTEN*, *PTPases*, and *Gluconeogenesis*). Specific sites that become phosphorylated (*p*) upon insulin stimulation are indicated within yellow ovals, and numbers indicate residues in human sequences. *Lipogenesis-I* refers to lipogenesis that becomes activated independently from new protein synthesis; *Lipogenesis-D* depends on new protein synthesis.

Abbreviations: ACC, acetyl-CoA carboxylase-1; ACL, ATP citrate lyase; AKT/PKB, protein kinase B; *Apoc3*, apolipoprotein C-III; CAV1, caveolin-1; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FOXO1, forkhead box O1A; Gluc, glucose; GLUT transcn, translocation of facilitated glucose transporters; GRB2, growth factor receptor-binding protein 2; GSK3β, glycogen synthase kinase 3β; HSPG, heparan sulfate proteoglycan; IRS, insulin receptor substrate; MEK, MAP/ERK kinase; mTORC, mammalian target of rapamycin complex; NOX4, NAD(P)H oxidase 4; PDK1, 3'-

phosphoinositide-dependent protein kinase 1; *Pepck*, phosphoenolpyruvate carboxykinase 1; PI3Ks, isoforms of phosphatidylinositol 3'-kinase; PRAS40, proline-rich AKT substrate of 40kDa; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PTPases, protein-tyrosine phosphatases such as PTP1B; RAF, *v-raf-1* murine leukemia viral oncogene homolog 1; RAS, rat sarcoma guanosine-nucleotide-binding protein; RHEB, RAS homolog enriched in brain; S6K1, ribosomal protein S6 kinase 1; SHC, Src homologous and collagen-like protein; SOS1, son of sevenless (*Drosophila*) homolog 1; *Srebp1c*, sterol regulatory element-binding protein-1c, which its discoverers originally named adipocyte determination- and differentiation-dependent factor 1 (ADD1); SULF2, heparan sulfate glucosamine-6-O-endosulfatase-2; TG, triglyceride; TSC2, tuberin.

Figure 2A

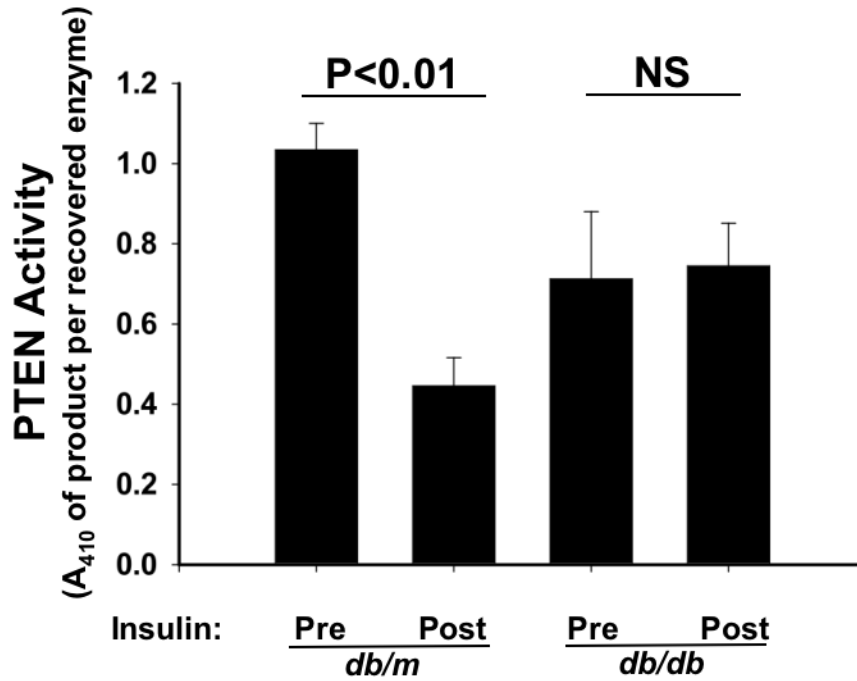


Figure 2B

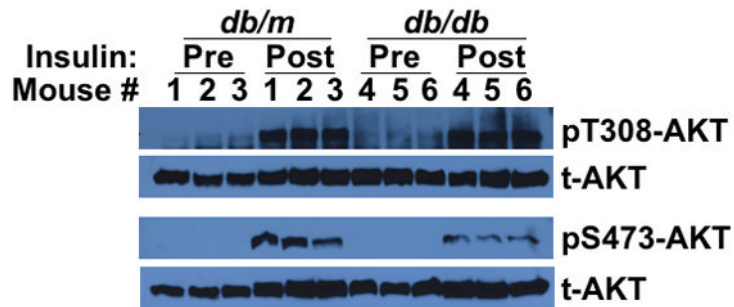


Figure 2. Type 2 diabetes renders the liver unable to inactivate PTEN in response to insulin, yet still able to phosphorylate AKT at Thr308. Liver samples were obtained just before (*Pre*) and 10min after (*Post*) an intravenous injection of insulin into 14-week-old lean *db/m* mice (controls) and their hyperphagic, obese T2DM *db/db* littermates, as indicated. Other nomenclature follows Figure 1. Panel A: PTEN activities in liver homogenates, assayed under strictly anaerobic conditions (mean±SEM, n=3). Statistical comparisons by the paired *t*-test are indicated. Panel B: Phosphorylation of AKT at Thr308 (*pT308-AKT*, upper immunoblots) and Ser473 (*pS473-AKT*, lower immunoblots). Immunoblots for total AKT (*t-AKT*, meaning phosphorylated plus unphosphorylated forms) is also shown for each sample. Numbers over the lanes refer to individual animals.

Figure 3A

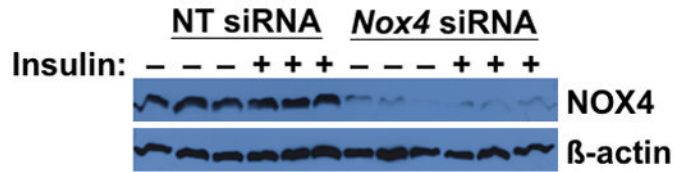


Figure 3B

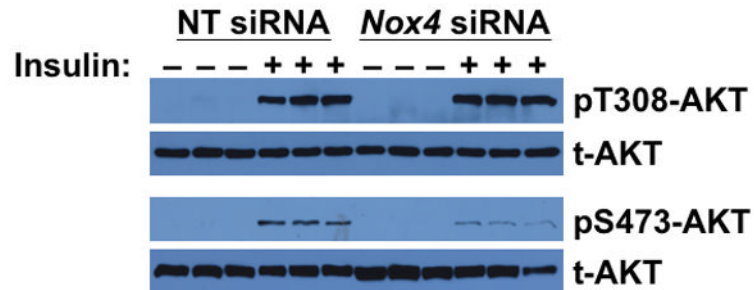


Figure 3C

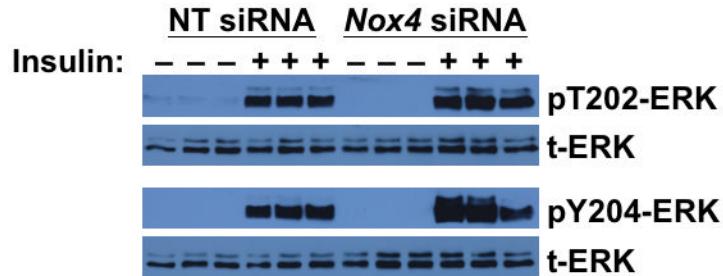


Figure 3.

Deficiency of NOX4 in cultured liver cells mimics the effects of type 2 diabetes on the two canonical mediators of insulin signaling, AKT and ERK. As indicated, McArdle hepatocytes were pretreated with nontarget (*NT*) control siRNA or *Nox4* siRNA, exposed to 0 or 10nM insulin for 10 min, and then harvested. This figure displays immunoblots in triplicate from a single set of cultured cells. Each lane represents a separate culture well. Panel A: Anti-NOX4 immunoblot. Panel B: Insulin-stimulated phosphorylations of AKT (*pT308-AKT*, *pS473-AKT*, and total AKT). Panel C: Insulin-stimulated phosphorylations of ERK (*pT202-ERK*, *pY204-ERK*, and total ERK). The data from these cultured cells are representative of a total of three independent insulin signaling experiments.

Figure 4A

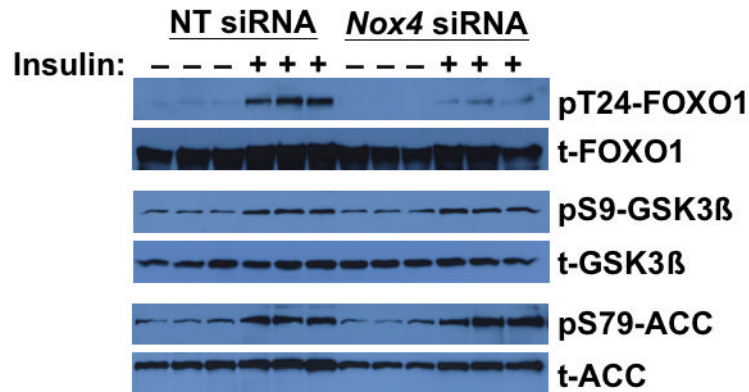


Figure 4B

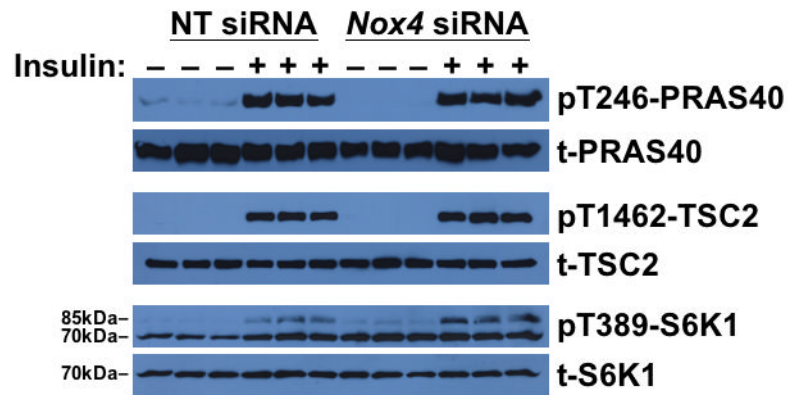


Figure 4. Deficiency of NOX4 in cultured liver cells mimics the effects of type 2 diabetes on phosphorylations downstream of AKT, thereby rendering hypolipidemic and hypoglycemic pathways resistant to insulin, while leaving lipogenic pathways insulin-responsive. Displayed are immunoblots from the same single set of cultured McArdle hepatocytes as in Figure 3. Panel A: Resistance of FOXO1 to insulin-stimulated phosphorylation in NOX4-deficient hepatocytes (*TG-rich lipoprotein clearance* and *Gluconeogenesis* pathways from Figure 1), yet continued responsiveness of GSK3β and ACC (*Lipogenesis-I* pathway from Figure 1). Panel B: Continued activation of molecules upstream (PRAS40, TSC2) and downstream (S6K1) of mTORC1 in NOX4-deficient hepatocytes (*Lipogenesis-D* pathway from Figure 1). Both the 70- and 85-kDa isoforms of S6K1 can be seen on the immunoblot for pT389-S6K1, as indicated. The data from these cultured cells are representative of a total of three independent experiments.

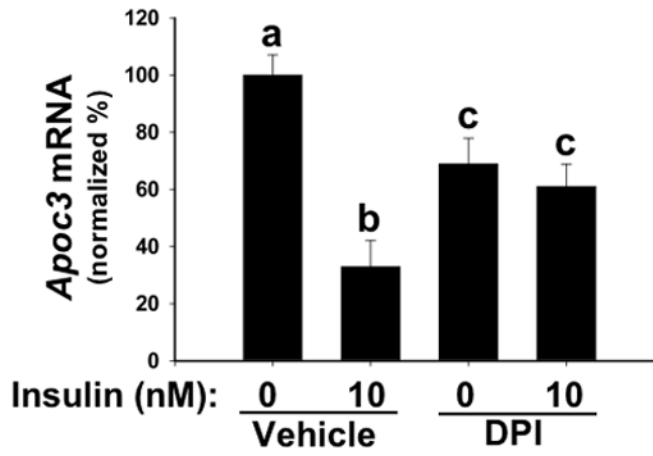
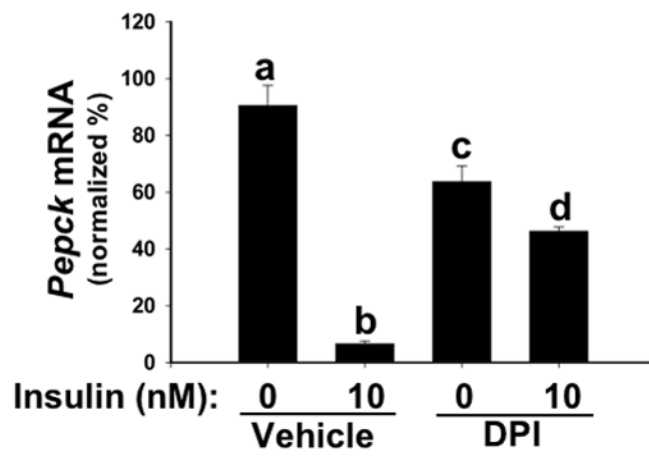
Figure 5A**Figure 5B**

Figure 5C

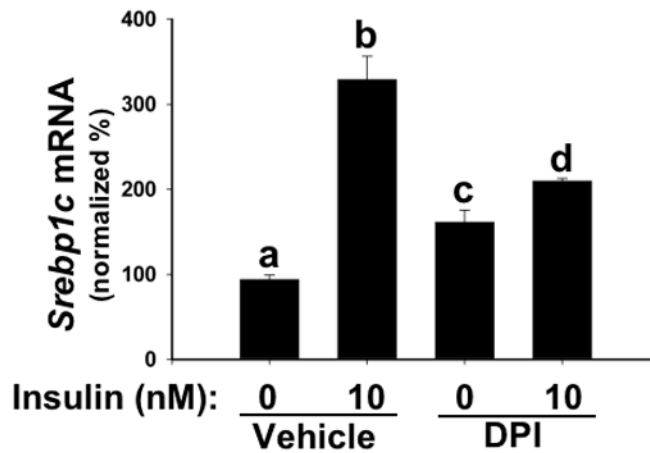


Figure 5.

Inhibition of NOX4 in primary hepatocytes dysregulates key mRNAs in lipid and glucose control, thereby recapitulating harmful features of hepatic SEIRR.

As indicated, rat primary hepatocytes were placed in media supplemented with 0 (*Vehicle*) or 1.0 μM *DPI* (an inhibitor of NOX4) and then incubated for 30 min at 37°C, after which the media were further supplemented to achieve insulin concentrations of 0 or 10nM. The cells were incubated for an additional 6h, to allow mRNA levels to change, and then harvested. Levels of *Apoc3* (Panel *A*), *Pepck* (Panel *B*), and *Srebp1c* (Panel *C*) mRNA were assessed by way of qRT-PCR, normalized to β -*actin* mRNA levels (Ct), and then expressed relative to the mean value from the cells that had been incubated with neither DPI nor insulin ($2^{-\Delta\Delta\text{Ct}}$; mean \pm SEM, n=4). Displayed are quantifications of mRNA levels in a single set of cells. P<0.005 by ANOVA; columns labeled with different lowercase letters (a, b, c, d) are statistically different by the Student-Newman-Keuls test (P<0.01).

Figure 6A

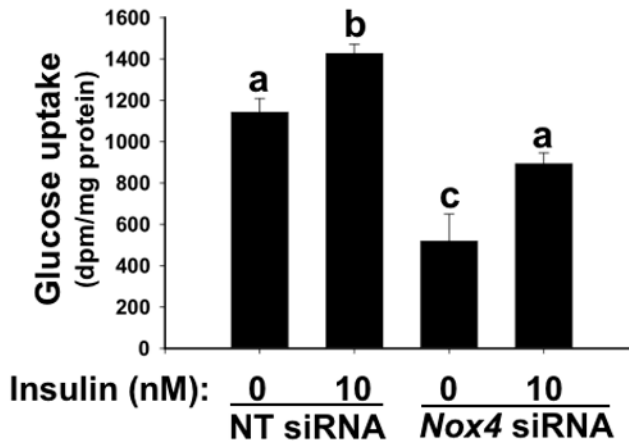


Figure 6B

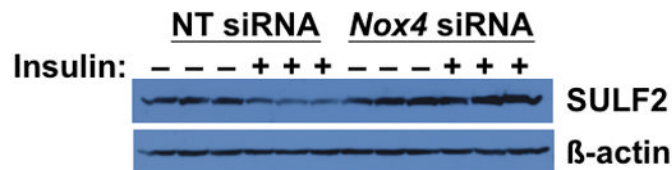


Figure 6.

Deficiency of NOX4 in cultured liver cells impairs hypolipidemic and hypoglycemic functions, thereby recapitulating key harmful features of SEIRR in vivo.

Panel A: NOX4 deficiency impairs glucose uptake by McArdle hepatocytes. Cells were pre-treated with non-target (*NT*) or *Nox4* siRNA, supplemented with 0 or 10nM insulin, as indicated, and then exposed to [³H]2-deoxy-D-glucose for the final 4 min. Displayed are cellular tritium dpms per mg of cellular protein (mean±SEM, n=3). P<0.001 by ANOVA; columns labeled with different lowercase letters (a, b, c) are statistically different by the Student-Newman-Keuls test (P<0.01). Panel B: NOX4 deficiency impairs the ability of insulin to suppress SULF2 expression (*Remnant lipoprotein clearance* from Figure 1).

McArdle cells were treated with non-target (*NT*) or *Nox4* siRNA, without or with insulin, as indicated. Cells were harvested 18h after the addition of insulin, to allow time to degrade pre-existing SULF2 protein. Displayed are immunoblots in triplicate for SULF2 and, as a loading control, β-actin.