NOX4 Pathway as a Source of Selective Insulin Resistance and Responsiveness

Xiangdong Wu, Kevin Jon Williams

Objective—Type 2 diabetes mellitus 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. Here, we undertook a comprehensive characterization of pathway-selective insulin resistance and responsiveness in liver and hepatocytes by examining 18 downstream targets of the insulin receptor, surveying the AKT, ERK, and NAD(P)H oxidase 4 pathways.

Methods/Results—Injection of insulin into hyperphagic, obese type 2 diabetes mellitus db/db mice failed to inactivate hepatic protein tyrosine phosphatase gene family members, a crucial action of NAD(P)H oxidase 4 previously thought to be required for all signaling through AKT and ERK. Insulin-stimulated type 2 diabetes mellitus livers unexpectedly produced an unusual form of AKT that was phosphorylated at Thr308 (pT308), with only weak insulin-stimulated phosphorylation at Ser473. Remarkably, knockdown or inhibition of NAD(P)H oxidase 4 in cultured hepatocytes recapitulated the entire complicated pattern of pathway-selective insulin resistance and responsiveness seen in vivo—namely, monophosphorylated pT308-AKT, impaired insulin-stimulated hypolipidemic and hypoglycemic pathways, but continued lipogenic pathways and robust ERK activation.

Conclusion—Functional disturbance of a single molecule, NAD(P)H oxidase 4, is sufficient to induce the key harmful features of deranged insulin signaling in type 2 diabetes mellitus, obesity, and other conditions associated with hyperinsulinemia and pathway-selective insulin resistance and responsiveness. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: diabetes mellitus ■ insulin resistance ■ lipids ■ obesity ■ signal transduction

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 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 on 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 lipids10,14,17 and it does not appropriately inhibit the secretion17–20 nor accelerate the clearance21–25 of atherogenic apolipoprotein (apo)-B-containing lipoproteins (Figure 1 and in the online-only Data Supplement).

Several explanations have been proposed for SEIRR,1,3,4,26–29 but all have focused on the 2 canonical limbs of the insulin signaling cascade, meaning protein kinase B (AKT) with its downstream targets and mitogen-activated protein 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 in extrahepatic tissues31 and AKT-mediated phosphorylations of forkhead box-1 (FOXO1) that suppress hepatic apoC-III expression20,32 and gluconeogenesis.33 Pathways downstream of AKT that stimulate lipid biosynthesis include alterations in 3 site-specific phosphorylations of ATP-citrate lyase, in part through GSK3β,34–36 and mTORC1-mediated transcription of lipidogenic genes37 via SREBP1c.28,29,38,39 Obesity and related conditions impair FOXO1 phosphorylation in vivo, resulting in abnormally high hepatic apolipoprotein C-III20,32 and phosphoenolpyruvate carboxykinase 1 (Pepck)40 mRNA lev-
els, whereas AKT-mediated phosphorylation of GSK3 is barely blunted (see the data in Figure 3 in Ref. 4141) and mTORC1 exhibits enhanced activity.42,43 In addition, SEIRR leaves the mitogen-activated protein kinase limb responsive to insulin, as assessed by ERK1,2 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 mitogen-activated protein kinases remains unclear.

There is, however, a noncanonical 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 ($O_2^{\cdot -}$) and its byproduct $H_2O_2$ that enhance signal propagation by disabling enzymes in the protein-tyrosine phosphatase 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; and second, the nonreceptor-type protein-tyrosine phosphatase -1B (PTP1B), which blunts insulin signaling by dephosphorylating tyrosyl residues within the insulin receptor and its ligands.

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 glucosegenes). Specific sites that become phosphorylated (p) on 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. ACC, indicates 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; PDPK1, 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.
substrates 1 and 2. Interfering with insulin-stimulated generation of superoxide and H2O2 was reported to cause abnormal persistence of PTEN and PTP1B 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 (December 10, 2011).

Materials 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/db mice (controls) and obese T2DM db/db (Lepr^med^/med^) littersmates on the C57BLKS background (Jackson Laboratory, Bar Harbor, ME) were fed ad libitum until age 14 weeks.23 We modified an insulin administration protocol38 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 (preinsulin sample). Because of the ligature, the cut surface on the remaining liver did not bleed. Next, 10 U of bovine insulin (#11882, 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 minutes, blood was obtained by heart puncture, and sections from other hepatic lobes were taken and snap-frozen (postinsulin sample). To verify diabetic status, hemoglobin A1c levels in blood were quantified by the ion-exchange method. For studies in vitro, we used 2 complementary systems. First, we prepared primary hepatocytes included 100 nmol/L dexamethasone to elevate the basal expression of this target, as described.28,62 Our computational analysis of the NOX4 sequence revealed 2 candidate caveolin-binding motifs (ΦΦΦΦΦΦΦΦΦΦ), where Φ represents an aromatic amino acid), in residues190/191WTHNLISIG/ and 209/210FENK/ES, similar to the single caveolin-binding motif in the insulin receptor (see Refs. 63–6563–65 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 Srebpl 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 Srebpl mRNA induction by insulin (not shown), even though phosphorylations upstream and downstream of mTORC1 remained responsive. Accordingly, primary cells also received 0.5 to 2 nmol/L T0901317, which is ≈10% of its IC50 for LXR activation.56 Primary hepatocytes replete or deficient in NOX4 were preincubated by a 30-minute pretreatment at 37°C with DMSO vehicle or 1.0 μmol/L diphenylenoiannotemone, respectively, followed by addition of 0 or 10 nmol/L insulin. These cells were then incubated for an additional 6 hours 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 postinsulin 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).8,67 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, which PTEN and PTP1B 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/db 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 Table I in the online-only Data Supplement, following the nomenclature in Figure 1. Close reading of product inserts from Cell Signaling Technologies (Beverly, MA) indicated that catalogue #4376 was

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 2 hours before supplementation with 0 or 10 nmol/L insulin. Exposure to 0 or 10 nmol/L insulin lasted 10 minutes for studies of phosphorylations of preexisting protein targets, 15 minutes to assess uptake of [3H]-deoxy-d-glucose, a nonmetabolizable glucose analog, or 18 hours for studies of SULF2 protein regulation. Serum-free medium was used only for short-term studies of phosphorylations or 2-deoxy-d-glucose uptake48,54, 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 Srebpl transcription,56 we relied on freshly isolated rat primary hepatocytes21 for our studies of mRNA regulation. Primary hepatocytes exhibit vigorous insulin-induced regulation of Pepck and Srebpl,28,38,56 but lose many physiological responses after prolonged culture, necessitating our use of a fast-acting chemical inhibitor of NOX4 (diphenylenoiannotemone).58,59 We applied diphenylenoiannotemone at a concentration, 1.0 μmol/L, that provides ≈90% inhibition.60 Owing to potential issues of selectivity, particularly at higher micromolar concentrations,61 we used diphenylenoiannotemone 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 100 nmol/L dexamethasone to elevate the basal expression of this target, as described.28,62 Our computational analysis of the NOX4 sequence revealed 2 candidate caveolin-binding motifs (ΦΦΦΦΦΦΦΦΦΦ), where Φ represents an aromatic amino acid), in residues190/191WTHNLISIG/ and 209/210FENK/ES, similar to the single caveolin-binding motif in the insulin receptor (see Refs. 63–6563–65 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 Srebpl 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 Srebpl mRNA induction by insulin (not shown), even though phosphorylations upstream and downstream of mTORC1 remained responsive. Accordingly, primary cells also received 0.5 to 2 nmol/L T0901317, which is ≈10% of its IC50 for LXR activation.23 Primary hepatocytes replete or deficient in NOX4 were preincubated by a 30-minute pretreatment at 37°C with DMSO vehicle or 1.0 μmol/L diphenylenoiannotemone, respectively, followed by addition of 0 or 10 nmol/L insulin. These cells were then incubated for an additional 6 hours to allow mRNA levels to change.

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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 Table I in the online-only Data Supplement, 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. 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 Table II in the online-only Data Supplement. In studies of labeled 2-deoxy-D-glucose uptake by cultured McArdle hepatocytes, this molecule was added during the final 4 minutes of incubation.

**Statistical Analyses**

Normally distributed data are reported as means±SEMs. For comparisons of enzymatic activities in preinsulin versus postinsulin 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, we found that insulin stimulation of T2DM livers in vivo failed to inhibit the activities of PTEN (Figure 2A) or PTP1B (Figure I in the online-only Data Supplement), 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. 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 protein-tyrosine phosphatase 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. Figure IIA and IIB in the online-only Data Supplement shows that T2DM impaired insulin-induced phosphorylation of hepatic FOXO1, consistent with prior reports of apoC-III overexpression and persistent gluconeogenesis, yet phosphorylations of GSK3ß, proline-rich AKT substrate of 40kDa, and ribosomal protein S6 kinase 1 (S6K1) remained robust, consistent with ongoing hepatic lipogenesis (see Figure I and Background in the online-only Data Supplement). 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 (Figure IIC).
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 knockdown abolished insulin-stimulated phosphorylation of FOXO1 at Thr24 (Figure 4A). Strikingly, the same NOX4-deficient hepatocytes showed unimpaired insulin-stimulated phosphorylations of GSK3β and ACC (Figure 4A). The 2 AKT-dependent inputs into mTORC1—namely, proline-rich AKT substrate of 40kDa and tuberin, 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 postinsulin phosphorylations (Figure 4B), exactly as in db/db livers (Figure IIB). The S6K1 is of particular interest, because it desensitizes insulin receptor substrate 1 in states of overnutrition and obesity (Ref. 71 and Figure 1). Taken together, Figure 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 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, proline-rich AKT substrate of 40kDa [PRAS40], tuberin [TSC2] and downstream (ribosomal protein S6 kinase 1 [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.
tein clearance. Under stimulation by insulin and other factors, the normal liver takes up one-third of each oral glucose load, but the process is defective in T2DM. 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 (reference 76 and Figure 4B). Regarding remnant lipoprotein clearance, we recently demonstrated that T2DM livers overexpress the heparan 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, whereas 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 nonesterified 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.
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 (PP5C), a novel binding partner we found for NOX4,\textsuperscript{70} may derange insulin-stimulated phosphorylations, while still allowing insulin-induction of a key target of mTORC1 and LXR—namely, \textit{Srebp-1c} mRNA. A regulatory role for PP5SC 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.\textsuperscript{48} We now speculate that these mutants would have sequestered PP5SC 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 PP5SC, ie, impaired insulin-stimulated formation of both pS473-AKT and pY204-ERK.\textsuperscript{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 PP5SC 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 2 sites on AKT are acted on by different kinases, PDPK1 and mTORC2 (references\textsuperscript{78,79} and Figure 1), from which we now infer differential dependence on NOX4, perhaps via PTEN (Refs. 50 and 52\textsuperscript{0.52} and Figure 2A). One model would be metabolic channelling of phosphatidylinositol-3,4,5-trisphosphate from PI3K directly to PDPK1, bypassing PTEN, whereas phosphatidylinositol-3,4,5-trisphosphate that is destined for mTORC2 might have to contact PTEN beforehand (Figure 1). Another possibility is that, despite its name, PDPK1 has been reported to be constitutively active,\textsuperscript{80,81} and so any process that brings it and its substrate together may be sufficient to produce pT308-AKT.\textsuperscript{82,83} In contrast, mTORC2 requires both intrinsic activation and physical contact with substrate.\textsuperscript{79} A third explanation could be that site-specific underphosphorylation of insulin receptor and its substrates Land 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 Ref. 26\textsuperscript{26} and the current Figure 1). Fourth, NOX4, possibly through PTEN and phosphatidylinositol-3,4,5-trisphosphate, might differentially regulate the enzymes that catalyze site-specific dephosphorylations of AKT (see references\textsuperscript{81,84}). Of note, monophosphorylation of AKT caused by artificial disruption of mTORC2 in nondiabetic 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 tuberin.\textsuperscript{85–89} These prior results add credence to the widely deranged substrate selectivity and other aberrant actions of pT308-AKT that we found in \textit{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.\textsuperscript{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 phos-
phorylation 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, hypercoagulability, destabilization of atheroma through overexpression of matrix metalloproteinases, and other vascular and nonvascular abnormalities. 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.

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Disclosures
None.

References
35. Potapova IA, El-Maghrabi MR, Doronin SV, Benjamin WB. Phosphor- ylation of recombiant human ATP-citrate lyase by CAMP-dependent


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

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Supplemental Background

Prior literature has documented major site-specific phosphorylations and functions in the AKT limb of insulin receptor signaling (see Figure 1). Insulin-stimulated phosphorylation of FOXO1 has been reported at Thr24, a key site normally phosphorylated by activated AKT, leading to exclusion of FOXO1 from the nucleus and arresting its activity as a transcription factor for apoC-III and gluconeogenic genes.

The AKT limb also affects several molecules involved in lipogenesis. The AKT target site on glycogen synthase kinase-3β (GSK3β) is Ser9. Phosphorylation by AKT inactivates GSK3, and may thereby block this factor from inhibiting ACL, a key enzyme in fatty acid, cholesterol, and new glucose biosynthesis, as well as glycogen synthase. Insulin induces activation and site-specific serine-phosphorylation of a second enzyme in fatty acid biosynthesis, acetyl-CoA carboxylase-1 (ACC). The effect of phosphorylation of ACC at Ser79 on its activity in vitro remains an open question, whereas in vivo, T2DM increases hepatic ACC activity and Ser79-phosphorylation together. We infer that overphosphorylation at Ser79 in T2DM liver in vivo could be driven by hyperinsulinemia in combination with continued responsiveness of this portion of the insulin receptor-AKT signaling cascade.

Another lipogenic target of AKT is the mammalian target of rapamycin complex-1 (mTORC1), which is activated in the tissues of obese mice. This complex has two AKT-dependent inputs: PRAS40 and TSC2. Insulin stimulates AKT to phosphorylate PRAS40 at Thr246. Insulin-stimulated phosphorylation of TSC2 occurs at a key site acted on by AKT, Thr1462. Insulin-induced mTORC1 activity can be assess by the phosphorylation of one of its substrates – namely, the ribosomal protein S6 kinase 1 (S6K1), an enzyme that desensitizes IRS1 in states of overnutrition and obesity (reference and Figure 1). Activated mTORC1 induces Srebp1c mRNA, which encodes a major insulin-responsive transcription factor in lipogenesis. Activated mTORC1 might also increase lipogenesis through induction of ER stress and hence
cleavage and activation of the SREBP1c protein.\textsuperscript{27, 28} All of these effects are displayed schematically in Figure 1.

Pathways of interest that are not in Figure 1 include suppression of \textit{Irs2} mRNA by insulin;\textsuperscript{29} insulin-induced activation of mitogen-activated protein kinases in addition to ERK1,2;\textsuperscript{30} our sequence analysis of NOX4 and subsequent inference that NOX4 in cholesterol-rich caveolae could contribute to the putative generation of oxysterol ligands for LXR after insulin stimulation (Methods); inhibition of GLUT translocation by activated mTORC1;\textsuperscript{31} insulin-stimulated production of the vasodilator NO via activated AKT and of the vasoconstrictor endothelin-1 via activated ERK;\textsuperscript{32} direct effects of activated PI3K on ER stress;\textsuperscript{33, 34} insulin-stimulated phosphorylation and inhibition of FOXA2, a transcriptional factor that otherwise drives the expression of genes encoding enzymes of fatty acid oxidation, ketogenesis, and glycolysis;\textsuperscript{35} insulin-induced cleavage and activation of SREBP1c protein via PI3K;\textsuperscript{36} ERK-mediated phosphorylation of SREBP1c at Ser93, which enhances transactivation of its target genes;\textsuperscript{37-39} the ability of activated MEK to bind, inhibit, and provoke the expulsion of PPAR\textsubscript{\gamma} from the nucleus;\textsuperscript{40, 41} and effects of insulin on sympathetic activity,\textsuperscript{42, 43} renal sodium excretion,\textsuperscript{42, 44-46} ERK-mediated phosphorylation and activation of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase,\textsuperscript{47} coagulation,\textsuperscript{48-50} expression of matrix metalloproteinases,\textsuperscript{30} and secretion of apoB-containing lipoproteins.\textsuperscript{5, 51-54}
Supplemental References


44. Atchley DW, Loeb RF, Richards DW, Benedict EM, Driscoll ME. On diabetic acidosis: A detailed study of electrolyte balances following the


Supplemental figure legends

**Supplemental Figure I**: Type 2 diabetes renders the liver unable to inactivate PTP1B in response to insulin. Displayed are PTP1B activities from the same liver samples as in Figure 2, which were obtained just before (Pre) and 10min after (Post) an intravenous injection of insulin into lean db/m mice (controls) and their hyperphagic, obese T2DM db/db littersmates, as indicated. PTP1B activities in liver homogenates were assayed under strictly anaerobic conditions (mean±SEM, n=3). Statistical comparisons by the paired t-test are indicated.

**Supplemental Figure II**: Type 2 diabetes impairs a key insulin-stimulated hypolipidemic and hypoglycemic pathway in liver, yet preserves lipogenic pathways and robust ERK activation. Displayed are immunoblots from the same liver samples as in Figure 2, which were obtained just before (Pre) and 10min after (Post) an intravenous injection of insulin into lean db/m mice (controls) and their hyperphagic, obese T2DM db/db littersmates, as indicated. Panel A: Resistance of FOXO1 to insulin-stimulated phosphorylation in T2DM db/db livers compared to control db/m livers (TG-rich lipoprotein clearance and Gluconeogenesis pathways from Figure 1), yet continued responsiveness of GSK3β (Lipogenesis-I pathway from Figure 1). Panel B: Continued activation of molecules upstream (PRAS40) and downstream (S6K1) of mTORC1 in T2DM db/db livers (Lipogenesis-D pathway from Figure 1). The 70-kDa isoform of S6K1 is indicated. Panel C: Continued responsiveness of ERK to insulin-stimulated phosphorylations in T2DM db/db livers (pT202-ERK, pY204-ERK). Immunoblots for total (t-, meaning phosphorylated plus unphosphorylated) amounts of each target protein are shown for each sample. Numbers over the lanes refer to individual animals.
Supplemental Figure III: Inhibition of NOX4 in primary rat hepatocytes impairs the ability of insulin to suppress *Irs2* mRNA levels. Displayed are mRNA quantifications from the same set of cultured hepatocytes as in Figure 5A-C. As indicated, primary rat hepatocytes were pretreated with 0 (vehicle) or 1.0 µM DPI (an inhibitor of NOX4), exposed to 0 or 10nM insulin for 6 h, and then harvested. Displayed are *Irs2* mRNA levels normalized to *β-actin* mRNA levels (ΔCt) and then expressed relative to the mean value from the cells that had been incubated with neither DPI nor insulin (2^{ΔΔCt}; mean±SEM, n=4). 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).
**Supplemental Table I: Antibodies against target proteins.**

<table>
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<th>Target Protein</th>
<th>Epitope</th>
<th>Supplier/ Catalog Number</th>
<th>Description</th>
<th>Molecular weight of target protein</th>
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<td>ACC</td>
<td>p-ACC</td>
<td>Cell Signaling #3661</td>
<td>Rabbit polyclonal antibody (Ab) against acetyl-CoA carboxylase that is phosphorylated at Ser79</td>
<td>280 kDa</td>
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<tr>
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<td>t-ACC</td>
<td>Cell Signaling #3676</td>
<td>Rabbit monoclonal antibody (mAb) against total acetyl-CoA carboxylase (phosphorylated and non-phosphorylated)</td>
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<td>Rabbit polyclonal Ab against total FOXO1 (used here for immunoprecipitation)</td>
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<td>Cell Signaling #9464</td>
<td>Rabbit polyclonal Ab against FOXO1 phosphorylated at Thr24 or FOXO3A phosphorylated at Thr32</td>
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<td>t-FOXO1</td>
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<td>Rabbit mAb against total FOXO1 phosphorylated at Thr24 (used here for immunoblotting)</td>
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**Supplemental Table II**: Primer and probe sequences for quantitative real-time PCR.

<table>
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<th>Target mRNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
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<tr>
<td>Apoc3</td>
<td>5'-atg cag ggc tac atg gaa ca-3'</td>
<td>5'-cac agc tat atc aga ctc ct-3'</td>
<td>F-5'-tcc aag acg gtc cag gat gca ct-3'-Q</td>
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<td>Irs2</td>
<td>5'-atg aac ctg gac ttc agt tct-3'</td>
<td>5'-atc cat gga gcc tac tgt gt-3'</td>
<td>F-5'-tcc ccc aag cct agc acc cgc-3'-Q</td>
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<td>Pepck</td>
<td>5'-agt cac cat cac ttc ctg ga-3'</td>
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<td>Srebp1c</td>
<td>5'-gga gcc atg gat tgc aca tt-3'</td>
<td>5'-cat caa ata ggc cag gga ag-3'</td>
<td>F-5'-tg ctt cag ctc atc aac aac caa gac a-3'-Q</td>
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<td>13-actin</td>
<td>5'-tgc ctg acg gtc agg tca-3'</td>
<td>5'-cag gaa gga agg ctg gaa g-3'</td>
<td>F-5'-ca cta tcg gca atg agc ggt tcc g-3'-Q</td>
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In the probe sequences, F and Q denote the positions of the FAM fluorophore and TAMRA quencher, respectively.
Supplemental Figure I

PTP1B Activity (A$_{410}$ of product per recovered enzyme)

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<th>Insulin:</th>
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<th>Post $db/m$</th>
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<th>Post $db/db$</th>
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P<0.01
NS
Supplemental Figure II-A,B

II-A

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<th>db/m Post</th>
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Insulin: Pre vs. Post

- pT24-FOXO1
- t-FOXO1
- pS9-GSK3β
- t-GSK3β

II-B

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Insulin: Pre vs. Post

- pT246-PRAS40
- t-PRAS40
- pT389-S6K1
- t-S6K1
Supplemental Figure II-C

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</table>

- pT202-ERK
- t-ERK
- pY204-ERK
- t-ERK
Supplemental Figure III

![Graph showing Irs2 mRNA expression under different insulin and DPI conditions.](image-url)

- Insulin (nM): 0, 10
- Vehicle
- DPI

Irs2 mRNA (normalized %)
배경
제2형 당뇨병 및 이와 관련된 증후군은 죽상경화증을 초래하는 이상지질혈증, 미세혈관합병증을 초래하는 고혈당, 그리고 고혈압의 이른바 죽음의 삼중주를 나타낸다. 이러한특징적 증상들은 공통된 경로-선택적 인슐린저항성과 반응성에서 유래하나 그 근원은 잘 설명되지 않고 있다. 이 연구에서는 간조직과간세포에서 경로-선택적 인슐린저항성과 반응성에 관여하는 AKT, ERK, NAD(P)H oxidase 4 pathway에 포함되는 인슐린 수용체의 18개 하위 표적의 특성을 포괄적으로 평가하였다.

방법 및 결과
과식하는 비만한 제2형 당뇨병 모델인 db/db mice에인슐린을 주사하면 간조직의 protein tyrosine phosphatase gene family member가 비활성화되지 않는다. Protein tyrosine phosphatase gene family member의 비활성화는 NAD(P)H oxidase 4에 의한 결정적인 작용으로 AKT와 ERK를통한 모든 신호 전달에 필요한 것으로 생각되어왔다. 인슐린으로 자극한 제2형 당뇨병 생쥐의간에서는 예상과 달리 Thr308 (pT308)이 주로인산화되고, Ser473에는 미약한 인산화를 보이는특이한 형태의 AKT를 생성하였다. 주목할만한것은, 배양한 간세포에서 NAD(P)H oxidase 4의완전 결핍 또는 억제가 생체에서 나타난 경로-선택적 인슐린 저항성과 반응성의 여러 복잡한형태를 개괄적으로 보여주었다는 것이다. 즉, NAD(P)H oxidase 4의 완전 결핍 또는 억제로단인산화된(monophosphorylated) pT308-AKT,인슐린에 의한 혈장의 지질과 혈당을 낮추는경로의 이상, 지속적인 지질 생성 경로와 ERK활성화가 나타났다.

결론
NAD(P)H oxidase 4 한 분자의 기능적 이상은제2형 당뇨병, 비만 및 고인슐린혈증과 경로-선택적 인슐린 저항성과 반응성에 관련된 다른여러 상황에서 나타나는 인슐린 신호전달체계의주요 유해한 특징들을 유도하였다.
Commentary

인슐린 신호전달체계를 살펴 보면, 인슐린 수용체는 자체가 tyrosine kinase의 활성을 갖고 있어 인슐린이 인슐린 수용체에 결합하면 수용체의 자가인산화가 일어나고, insulin receptor substrate (IRS)를 tyrosine phosphorylation을 통해 활성화한다. IRS의 활성화는 연이어 PI3K, AKT의 활성화를 유도하고 GLUT의 세포막으로의 이동이 일어나며, 포도당 섭취가 증가하게 된다. 한편, 이렇게 활성화된 인슐린 신호를 탈인산화를 통해 종료시키는 경로로 phosphatase and tensin homolog (PTEN)과 protein-tyrosine phosphatase-1B (PTP1B)가 존재한다. 인슐린 자극에 의해 정상적으로 생성되는 superoxide와 H$_2$O$_2$의 생산은 PTEN과 PTP1B의 비정상적인 활성을 초래하고 인슐린 경로의 주요 단백들의 인산화 및 포도당 섭취를 감소시키게 되어 인슐린 저항성이 나타나게 된다. 인슐린에 의한 superoxide와 H$_2$O$_2$의 생산은 NOX4의 역할이다.

NOX4는 세포 내 ROS 생성에 관여하는 효소인 NADPH oxidase의 7개의 isoform 중 하나로 거의 모든 세포에 존재하며, endothelial cell, vascular smooth muscle cell, cardiac fibroblast, cardiomyocyte, heart, macrophage 등 심혈관계 세포에도 풍부히 존재한다. NOX4는 다른 NOX1 또는 NOX2와는 달리 superoxide 대신 과산화수소를 주로 생성하며 지속적으로 발현되는 특성이 있다. 또한 세포 내에서의 분포가 다양하여 세포질그룹(endoplasmic reticulum), 세포막, 혈, 미토콘드리아 등에 존재한다. 다양한 심혈관질환 모델 즉, 고혈압, 폐장식화증, 당뇨병, 재협착, 허혈 재판류 손상, 섬유화, 혈관 노화, 폐동맥 고혈압 등에서 혈관 NOX4의 발현이 증가되어 있다고 보고하고 있다. 최근까지 여러 연구에서 NOX4의 역할에 대한 결과들을 많이 발표하였는데, 그 예를 살펴보자면

1) NOX4를 십수세포에서 과발현시키면 미토콘드리아 기능 이상으로 인한 산화 스트레스의 증가, 세포자멸사 증가 및 손상의 기능 이상을 초래하였다.

2) 비만과 인슐린 저항성이 동반된 동물 모델인 Zucker rat에서 NOX4의 knockdown이 촉진동맥의 손상을 억제하였다.

3) NOX4의 억제제 투여가 세포주에서 산화 스트레스와 염증 반응을 예방하였다.

이러한 연구 결과들은 본 논문의 결과가 반대되는 결과로 보일 수 있으나 이는 NOX4가 혈관 질환에 관여할 가능성을 제시할 뿐이기 때문에 원인과 결과를 설명하고 있는 데이다.

일반적으로 세포 내 reactive oxygen species (ROS; superoxide, H$_2$O$_2$)의 증가는 당뇨병과 합병증 발생의 병태생리에 주요한 역할을 수행한다. 그러나 다양한 성장 인자, cytokine 또는 호르몬의 자극에 대하여 발생하는 일시적인 소량의 ROS는 이들 리간드에 대한 세포의 반응을 증가시킬 수도 있다. 같은 맥락에서 NOX4에 의한 ROS의 증가가 유해한 산화 스트레스의 원인이 아니라 체내 신호전달에 기여할 여지가 있다.

NOX4의 인슐린 저항성 발생 및 심혈관질환에서의 역할에 대한 논란들은 최근까지 NOX4가 결핍되거나 과발현된 적절한 동물 모델이 없었던 것에 기인한다. 최근 NOX4 결핍 모델을 이용한 몇개의 논문이 발표되어 흥미로운 결과들을 보여주었다. NOX4 결핍 생쥐에서는 지방 조직의 축적, 당뇨병과 조기 인슐린 저항성, 미토콘드리아에서의 fatty acid β-oxidation 결핍으로 인한 심한 지방간 소견을 보였다. 한편 NOX4 결
핍 생쥐를 이용한 또 다른 연구에서는 허혈에 의한 혈관 생성이 억제되고 안지오텐신-II 매개 혈관염증, 혈관 재형성, 내피세포 기능이상이 악화되었는데, NOX4를 내피세포에서 과발현시키면 aortic capillary sprouting이 증가하면서 뒷다리의 허혈이 회복되었다. 이런 결과는 NOX4 결핍 시 나타나는 eNOS의 발현 감소 및 NO 발생 감소와 연관될 것으로 생각된다.

본 연구에서는 NOX4의 결핍이 이른바 대사증후군의 모든 표현형의 발생을 유도할 수 있어, 치료의 주요 표적이 될 수 있다고 주장하고 있다. 그러나 NOX4 결핍 뇌졸중 생쥐와 NOX4 저해제로 치료한 생쥐는 산화 스트레스 발생, blood-brain-barrier leakage, neuronal apoptosis로부터 보호되는 효과가 있었다는 보고가 있어 NOX4의 역할에 대한 논란은 완전히 해결된 것은 아닌 것으로 생각된다. NOX4에 의해 주로 H₂O₂가 발생되느냐 혹은 superoxide가 발생되느냐에 따라 그 효과가 달라질 가능성이 높다. 인슐린 저항성 상태에서 관찰되는 잘 알려진 인슐린 신호 전달계인 IR, IRS, PI3K, AKT와 함께 NOX4도 주요한 경로 이상이 될 것으로 생각되며, NOX4의 발현을 조절하는 자극에 대해서는 TGF-β 이외에는 잘 알려지지 않고 있어 이에 대한 연구들이 기대된다.

REFERENCES
NOX4 Pathway as a Source of Selective Insulin Resistance and Responsiveness

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Objective—Type 2 diabetes mellitus 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. Here, we undertook a comprehensive characterization of pathway-selective insulin resistance and responsiveness in liver and hepatocytes by examining 18 downstream targets of the insulin receptor, surveying the AKT, ERK, and NAD(P)H oxidase 4 pathways.

Methods and Results—Injection of insulin into hyperphagic, obese type 2 diabetic db/db mice failed to inactivate hepatic protein tyrosine phosphatase gene family members, a crucial action of NAD(P)H oxidase 4 previously thought to be required for all signaling through AKT and ERK. Insulin-stimulated type 2 diabetic livers unexpectedly produced an unusual form of AKT that was phosphorylated at Thr308 (pT308), with only weak insulin-stimulated phosphorylation at Ser473. Remarkably, knockdown or inhibition of NAD(P)H oxidase 4 in cultured hepatocytes recapitulated the entire complicated pattern of pathway-selective insulin resistance and responsiveness seen in vivo—namely, monophosphorylated pT308-AKT, impaired insulin-stimulated pathways for lowering plasma lipids and glucose, but continued lipogenic pathways and robust ERK activation.

Conclusion—Functional disturbance of a single molecule, NAD(P)H oxidase 4, is sufficient to induce the key harmful features of deranged insulin signaling in type 2 diabetes mellitus, obesity, and other conditions associated with hyperinsulinemia and pathway-selective insulin resistance and responsiveness. (Arterioscler Thromb Vasc Biol. 2012;32:1236-1245.)

Key Words: diabetes mellitus ■ insulin resistance ■ lipids ■ obesity ■ signal transduction

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 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 on 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 secretion17-20 nor accelerate the clearance21-25 of atherogenic apolipoprotein B–containing lipoproteins (Figure 1; for additional background, see the online-only Data Supplement).

Several explanations have been proposed for SEIRR,1,3,4,26-29 but all have focused on the 2 canonical limbs of the insulin signaling cascade, meaning protein kinase B (AKT) with its downstream targets and mitogen-activated protein kinases with their downstream targets30 (Figure 1). The effect of SEIRR on the AKT limb is particularly puzzling, because AKT regulates lipid-lowering and glucose-lowering pathways that become insulin-resistant, but also lipogenic pathways that remain insulin-responsive. As shown in Figure 1, lipid- and glucose-lowering pathways downstream of AKT include translocation of facilitated glucose transporters in extrahepatic tissues31 and AKT-mediated phosphorylations of forkhead box-O1 (FOXO1) that suppress hepatic apolipoprotein C-III (apoC-III) expression20,32 and gluconeogenesis.33 Pathways downstream of AKT that stimulate lipid biosynthesis include alterations in 3 site-specific phosphorylations of ATP-citrate lyase, in part through GSK3ß,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 ApoC3,30,32 and phosphoenolpyruvate carboxykinase 1 (Pepck)40 mRNA levels, whereas AKT-mediated

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phosphorylation of GSK3 is barely blunted and mTORC1 exhibits enhanced activity. In addition, SEIRR leaves the mitogen-activated protein kinase limb responsive to insulin, as assessed by ERK and JNK phosphorylation, despite a marked impairment in overall insulin-stimulated tyrosine phosphorylation of the insulin receptor. The basis for differential dysregulation of major AKT targets and mitogen-activated protein kinases remains unclear.

There is, however, a noncanonical 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 ($O_2^{•−}$) and its byproduct $H_2O_2$. This signal propagates by disabling enzymes in the protein-tyrosine phosphatase gene family and by terminating PI3K signaling by dephosphorylating tyrosyl residues within the insulin receptor and its substrates. Interfering with insulin-stimulated generation of superoxide.
superoxide and H$_2$O$_2$ was reported to cause abnormal persistence of PTEN and PTP1B 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 (December 10, 2011).

Materials 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/db 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 11 weeks. We modified an insulin administration protocol 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 (preinsulin sample). Because of the ligature, the cut surface on the remaining liver did not bleed. Next, 10 U of bovine insulin (#1882, 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 minutes, blood was obtained by heart puncture, and sections from other hepatic lobes were taken and snap-frozen (postinsulin sample). To verify diabetic status, hemoglobin A1c levels in blood were quantified by the Vanderbilt Metabolic Phenotyping Center (Nashville, TN). We determined serum-free (DMEM/1% BSA) or low-serum (DMEM/2% FBS) medium for 2 hours before supplementation with 0 or 10 nmol/L insulin. Exposure to 0 or 10 nmol/L insulin lasted 10 minutes for studies of phosphorylations of preexisting protein targets, 15 minutes to assess uptake of [H]2-deoxy-D-glucose, a nonmetabolizable glucose analog, or 18 hours for studies of SULF2 protein regulation. Serum-free medium was used only for short-term studies of phosphorylations or 2-deoxy-D-glucose uptake; the longer studies used low-serum medium to maintain cell health. Second, because McArdle cells appear to lack sufficient endogenous expression of LXR, a required factor for insulin to induce Srebp1c transcription,26 we relied on freshly isolated rat primary hepatocytes for our studies of mRNA regulation. Primary hepatocytes exhibit vigorous insulin-induced regulation of Srebp and Srebp1c,28,38,36 but lose many physiological responses after prolonged culture, necessitating our use of a fast-acting chemical inhibitor of NOX4 (diphenyleneiodonium).56 We applied diphenyleneiodonium at a concentration, 1.0 μmol/L, that provides ~90% inhibition.66 Owing to potential issues of selectivity, particularly at higher micromolar concentrations,56,66 we used diphenyleneiodonium in a limited fashion. To allow studies of the suppressive effect of insulin on Pepck mRNA levels, all culture media for primary hepatocytes included 100 nmol/L dexamethasone to elevate the basal expression of this target, as described.66,67 Our computational analysis of the NOX4 sequence revealed 2 candidate caveolin-binding motifs (ΦXYΦXXXΦ, where Φ represents an aromatic amino acid), in residues 197-219 and 290-311, similar to the single caveolin-binding motif in the insulin receptor.56 We therefore 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,66 in addition to the phosphorylation cascades leading to mTORC128 (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 to 2 mmol/L TO901317, which is ≤10% of its IC$_{50}$ for LXR activation.66 Primary hepatocytes replete or deficient in functional NOX4 were prepared by a 30-minute preincubation at 37°C with DMSO vehicle or 1.0 μmol/L diphenyleneiodonium, respectively, followed by addition of 0 or 10 nmol/L insulin. These cells were then incubated for an additional 6 hours 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 postinsulin liver samples in which we assayed phosphorylation activities were handled under strictly anaerobic conditions in an enclosed work station (model #1025, Thermo Fisher Scientific, Marietta, OH).68 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, which PTEN and PTP1B 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 with 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/db and d/db livers. All other tissue and cellular extractions, immunoprecipitations, immunoblots, and quantitative real-time reverse-transcription polymerase chain 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 Table I in the online-only Data Supplement, 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 FOXO1.60 Primers and probes for quantitative real-time reverse-transcription polymerase chain reactions 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 Table II in the online-only Data Supplement. In studies of labeled 2-deoxy-D-glucose uptake by cultured McArdle hepatocytes, this molecule was added during the final 4 minutes of incubation.48,54

Statistical Analyses
Normally distributed data are reported as means±SEMs. For comparisons of enzymatic activities in preinsulin versus postinsulin 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 (Figure I in the online-only Data Supplement), 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 Thr308.7 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 protein-tyrosine phosphatase 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. Figure IIA and IIB in the online-only Data Supplement shows that T2DM impaired insulin-stimulated phosphorylation of hepatic FOXO1, consistent with prior reports of apoC-III overexpression and persistent gluconeogenesis, yet phosphorylations of GSK3β, the proline-rich AKT substrate of 40kDa, and ribosomal protein S6 kinase 1 (S6K1) remained robust, consistent with ongoing hepatic lipogenesis (see Figure 1 and the additional background in the online-only Data Supplement). 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 (Figure IIC).

Figure 2. Type 2 diabetes renders the liver unable to inactivate the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in response to insulin, yet still able to phosphorylate AKT at Thr308. Liver samples were obtained just before (Pre) and 10 minutes after (Post) an intravenous injection of insulin into 11-week-old lean db/m mice (controls) and their hyperphagic, obese T2DM db/db littermates, as indicated. Other nomenclature follows Figure 1. A, PTEN activities in liver homogenates, assayed under strictly anaerobic conditions (mean±SEM, n=3). Statistical comparisons by the paired t test are indicated. 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) are also shown for each sample. Numbers over the lanes refer to individual animals.

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 knockdown this enzyme in cultured rat McArdle hepatoma cells (Figure 3A). Remarkably, knockdown 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 knockdown still permitted vigorous insulin-stimulated phosphorylations of ERK1 at Thr204 and Tyr204 (Figure 3C), replicating the ERK response that we observed 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 that consists of blockage of pS473-AKT, a finding consistent with ongoing hepatic lipogenesis (see Figure 1 and the additional background in the online-only Data Supplement).
but with continued phosphorylations of the other AKT and ERK sites, thereby mimicking the upstream elements of SEIRR that we documented 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 knockdown abolished insulin-stimulated phosphorylation of FOXO1 at Thr24 (Figure 4A). Strikingly, the same NOX4-deficient hepatocytes showed unimpaired insulin-stimulated phosphorylations of GSK3\(\beta\) and ACC (Figure 4A). The 2 AKT-dependent inputs into mTORC1—namely, the proline-rich AKT substrate of 40kDa and tuberin, 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 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.

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 lipid-lowering and glucose-lowering actions of insulin that are downstream of AKT, similar to SEIRR in vivo. Likewise, insulin receptor substrate-2 mRNA, a factor regulated similarly to Apoc3 and Pepck,\(^72\) became resistant to suppression by insulin in the absence of functional NOX4 (Figure III in the online-only Data Supplement). 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, but the process is defective in T2DM. Figure 6A shows that NOX4 deficiency impaired glucose uptake by McArdle hepatocytes. Our results indicate defects in both basal and insulin-stimulated glucose uptake by NOX4-deficient hepatocytes, consistent with the finding that activated mTORC1 impairs glucose uptake by the liver (Figure 4B). Regarding remnant lipoprotein clearance, we recently demonstrated that T2DM livers overexpress SULF2, a novel factor that complexes with the syndecan-1 heparan sulfate proteoglycan and impairs hepatic uptake of atherogenic remnant lipoproteins (Figure 1). Overexpression of SULF2 is mediated in part through an impaired response to insulin. 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 lipid- and glucose-lowering 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, whereas 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 nonesterified 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 imbalance.
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. In another study, rapidly elevated plasma concentrations of nonesterified fatty acids in vivo blocked insulin-stimulated phosphorylation of aortic ERK. These disturbances in insulin-stimulated phosphorylations differ significantly from what we (Figure 2) and others have described in simple chronic hyperphagia. Doubts about a dominant role for fatty acid flux were reenforced by work showing that drastic lowering of plasma concentrations of nonesterified fatty acids in obese, glucose-intolerant individuals only partially corrected their ability to handle carbohydrates in response to insulin. Regarding other proposed causes of selective insulin resistance, our hepatocyte models of SEIRR that we report here. For example, the 2 sites on AKT are acted on by different kinases, PDK1 and mTORC2, which lead to the extensive manifestations of pathway-selective insulin resistance. 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, 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. 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, ie, impaired insulin-stimulated formation of both pS473-AKT and pY204-ERK. 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 2 sites on AKT are acted on by different kinases, PDK1 and mTORC2, which lead to the extensive manifestations of pathway-selective insulin resistance. 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, 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. 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, ie, impaired insulin-stimulated formation of both pS473-AKT and pY204-ERK. 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.
would suffice to activate SHC, the adaptor protein that triggers the cascade leading to ERK (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, hypercoagulability, destabilization of atheromata through overexpression of matrix metalloproteinases, and other vascular and nonvascular 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.

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Disclosures
None.

References
3. Shimomura I, Matsuda M, Hammer RE, Bashmakov Y, Brown MS, Goldstein JL. Decreased IRS-2 and increased SREBP-1c lead to mixed lipoprotein and glucose overproduction in insulin resistance. Evidence for enhanced lipoprotein assembly, reduced intracellular apolipoprotein degradation, and increased microsomal triglyceride transfer protein in a fructose-fed hamster model. J Biol Chem. 2000;275:8416–8425.


55. Wu X, Li M, Chen K, Liu M-L, Williams KJ. Protein phosphatase 5, a novel member of the insulin signaling cascade, promotes insulin-stimulated phosphorylation of a highly specific subset of activation sites on ERK1 and Akt. Diabetes. 2010;59(Suppl. 1):A13.


