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FORUM REVIEW ARTICLE

The Peroxide Dilemma: Opposing and Mediating Insulin Action

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Abstract

Recent compelling data show that reactive oxygen species (ROS) not only are a harmful by-product of aerobic metabolism, but also are used as signaling molecules to regulate various cellular processes. In mammalian cells, ROS are produced transiently in response to many extracellular stimuli, including insulin, and specific inhibition of the ROS suppresses insulin-dependent signaling. Initially, this finding rationalized the concept of ROS acting as insulin mimetics. However, it is becoming evident that ROS are also causal to diabetes, a metabolic disorder characterized by insufficiency of secretion of, or receptor insensitivity to, endogenous insulin. This notion underlines a dual role for ROS in insulin signaling as both deleterious and beneficiary. Moreover, it strongly suggests that a delicate redox balance is required for insulin signaling to remain "healthy" for an organism. *Antioxid. Redox Signal.* 15, 219–232.

Introduction

After the discovery of insulin in 1922 by Banting and Best and the establishment that insulin is the causative link in diabetes, it became essential to understand the cellular consequences of insulin action. By now, it is well documented how insulin, through activation of a complex signaling network, regulates glucose and lipid metabolism. In the presence of insulin, the insulin receptor (IR) triggers a series of downstream events leading to activation of two major signaling pathways: the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also called c-Akt) pathway and the Ras–mitogen-activated protein kinase (MAPK) pathway [reviewed in (6, 52, 79)]. In addition, other signaling events [*e.g.*, atypical protein kinase C (PKC) signaling] also contribute to the action of insulin (6).

The PI3K/PKB pathway is responsible for most of the metabolic actions of insulin, which is illustrated by the observation that an inactivating mutation in the PKBβ/AKT2 gene leads to the development of severe insulin resistance and diabetes mellitus (30). Whereas loss of function of PKB may confer insulin resistance, the opposite (*i.e.*. gain of function of PKB) contributes to tumorigenesis (1). Hence, PKB not only is involved in the regulation of glucose homeostasis, but it also mediates the proliferative effects of insulin (Fig. 1).

Besides insulin, many growth factors, especially those acting through tyrosine kinase receptors, use the PI3K/PKB pathway (43). Thus, a pertinent question is how does insulin or any other growth factor in that respect gain specificity?

Recent progress suggests that reactive oxygen species (ROS) may act as signaling molecules to integrate multiple signaling pathways by simultaneously affecting the activity of various signaling components of different pathways (77). Therefore, it creates a possibility that the differential impact of growth factors on cellular redox, and vice versa, may determine the strength and localization and thereby the specificity of signaling.

Cellular Locations of ROS Generation

For a long time, ROS were considered to be toxic by-products of certain metabolic systems, causing damage to the cellular content and eventually leading to cell death. However, now it is known that ROS can function as signaling molecules regulating diverse cellular processes and are even produced intentionally in response to specific stimuli, including hormones, growth factors, and proinflammatory cytokines (33). Under physiologic conditions, cellular ROS are produced primarily by several enzymatic systems (Fig. 2).

These include the mitochondrial electron-transport chain (ETC), NADPH oxidases (NOXs), oxidative protein folding in the endoplasmic reticulum (ER), and β-oxidation of fatty acids within peroxisomes (2, 50, 55, 90).

As ROS are diffusible and short-lived, it is important precisely to localize their production to guarantee specific oxidative regulation of certain proteins without imposing unwanted changes on others. NADPH oxidases are recognized as the major source of localized ROS production because of their specific localization within distinct cellular

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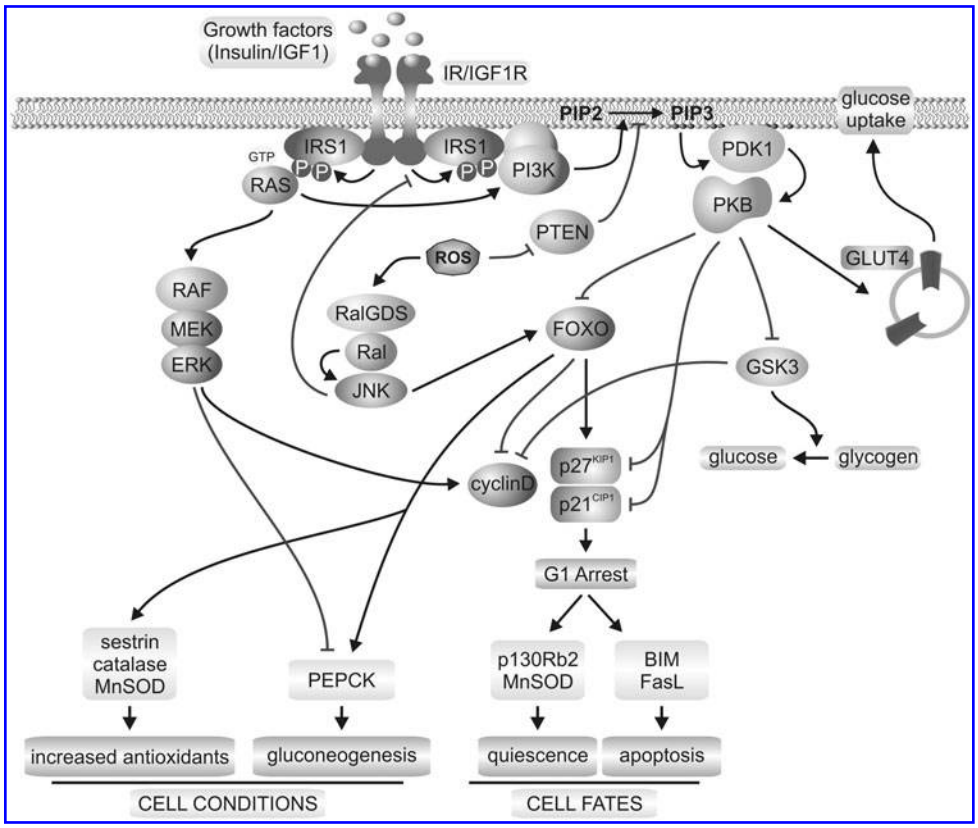

FIG. 1. Insulin signaling in regulation of the cell cycle and glucose homeostasis. Both the Ras and PKB arms of insulin-induced signaling regulate a multitude of events involved in cell-cycle progression. Proper cell-cycle progression requires sufficient energy resources, minimal cellular protein mass, and cell size. Thus the ability of insulin to regulate glucose metabolism fits into its broader role to regulate cell proliferation as it provides an energy resource for cell-cycle progression. Insulin influences cell-cycle progression via transcription (FOXO) and protein stability (PKB/GSK3). Important in this scheme is that through a regulated G_1 progression, cells can decide based on intra- and extracellular cues (most important, ROS and stress status) how to proceed (*e.g.*, apoptosis and quiescence), but also others like differentiation are a possibility. FOXO-mediated transcription of genes important for ROS protection and energy supply provides secondary requirements for these cell-fate decisions.

compartments [reviewed in (50, 81)]. They are multi-subunit enzymes composed of a core catalytic subunit (NOX1 to 5 and DUOX1 and DUOX2), regulatory subunit $p22^{phox}$ and cytosolic cofactors including $p47^{phox}$, $p40^{phox}$, $p67^{phox}$ and the small GTPase Rac. The requirement for $p22^{phox}$ and cytosolic subunits varies between different NOXs; however, it has been shown that $p22^{phox}$ is an essential regulatory component of NOX1 to 4 complexes (50).

In addition, phosphorylation of both core and regulatory subunits of various NADPH oxidases may play an important role in positive and negative regulation of NOX activity (8). A large number of kinases, including PKC, extracellular signal-regulated kinase1/2 (ERK1/2), p38MAPK, p21-activated protein kinase 1 (PAK1), and PKB have been reported to mediate phosphorylation of the NOX2 complex. Direct phosphorylation of NOX2 by PKC in response to neutrophil stimulation enhances the catalytic activity of NOX2 and its association with cytosolic cofactors. Moreover, in the same system, it has also been shown that PKC-mediated phosphorylation of $p47^{phox}$ is essential for release of the auto-inhibitory conformation of $p47^{phox}$ and therefore activation of the NOX2 complex. In addition, PKB directly binds and phosphorylates $p47^{phox}$ on neutrophil stimulation (8). Whether insulin triggers phosphorylation of NOX complexes and thereby contributes to their regulation is currently unknown.

Besides mitochondria- and NADPH oxidases–derived ROS, it has been estimated that about 25% of cellular ROS may be generated in the ER. This is associated with the activity of two enzymes: protein disulfide isomerase (PDI1) and ER oxidoreductin (ERO1), which are involved in oxidative protein folding (55). Overload of the ER folding capacity is inevitably associated with increased ROS generation and leads to induction of ER stress and secondary activation of an adaptive signaling cascade known as the unfolded protein response (UPR). The UPR coordinates many biologic processes to restore ER homeostasis. One of the UPR effectors is double-stranded RNA-activated protein kinase–like ER kinase (PERK). Active PERK transiently attenuates mRNA translation, thereby preventing further influx of newly synthesized polypeptides into the stressed ER lumen. PERK also can phosphorylate and activate nuclear respiratory factor 2 (NRF2). Cells depleted of NRF2 are highly sensitive to ER stress–induced apoptosis. NRF1 and NRF2 are transcription factors that regulate transcription of genes encoding ROS-detoxifying enzymes. Therefore, activation of PERK protects cells from oxidative stress, which is supported by the fact that $PERK^{-/-}$ cells accumulate ROS when exposed to ER stress (18).

Another branch of the UPR, consisting of inositol-requiring kinase 1 (IRE1) and X-box–binding protein (XBP1), leads to the activation of ER-associated degradation (ERAD) of malfolded proteins (69). During ERAD, improperly folded proteins are redirected to the cytoplasm and degraded, which leads to reduction in ER-protein load, thereby preventing

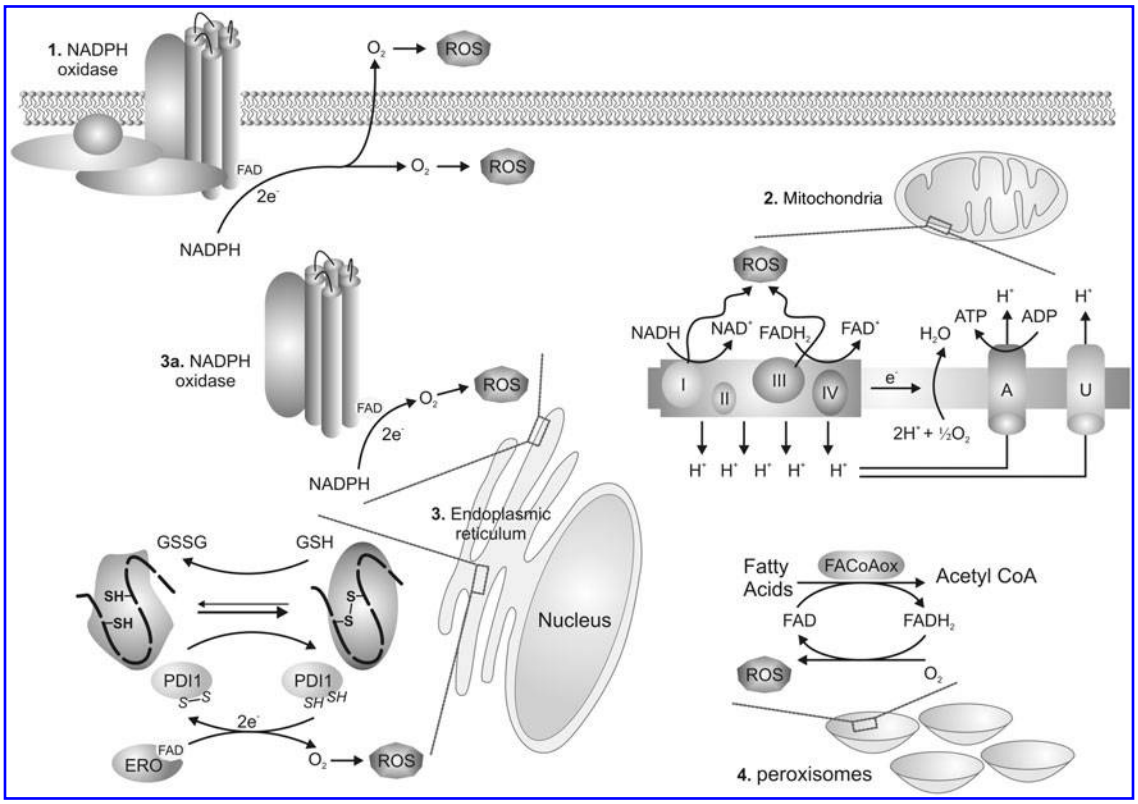

FIG. 2. Major cellular sources of ROS under physiologic conditions. Generation of ROS occurs at numerous cellular locations including the plasma membrane, mitochondria, peroxisomes, and endoplasmic reticulum. ROS are primarily produced intracellularly by four metabolic sources: the mitochondrial ETC **(1)** and NADPH oxidases **(2, 3a)**, oxidative protein folding **(3)** and β-oxidation of fatty acids **(4)**.

unnecessary consumption of glutathione (GSH) that is indispensible for counterbalancing elevated ROS levels (69).

ER stress also increases the concentration of Ca^{2+} in the cytosol because of leakage of Ca^{2+} from the ER lumen. This released Ca^{2+} may subsequently enter mitochondria, where the increased Ca^{2+} concentration can induce generation of ROS. This occurs because of inhibition of complex III (by release of cytochrome *c*) or complex IV (by stimulation of nitric oxide synthase and generation of NO•). In addition, ROS are generated because of Ca^{2+}-mediated stimulation of the tricarboxylic acid (TCA) cycle, thereby increasing O_2 consumption, and finally by Ca^{2+}-induced permeability transition pore opening, causing GSH leakage (17, 69).

Furthermore, all ER processes require a vast amount of energy supplied by mitochondria. Thus, any increase in ER activity increases mitochondrial oxidative phosphorylation and consequently elevates ROS. In addition, elevated Ca^{2+} levels in the cytosol might activate PKC and, by that, promote the activity of NADPH oxidase and p66SHC even further, thereby enhancing ROS production. Therefore, increased ROS levels in the ER can stimulate the production of ROS within other cellular compartments, as depicted in Fig. 3.

The Friend: ROS as Signaling Molecule for Insulin

Insulin stimulates ROS production

Insulin is one of numerous extracellular stimuli that increase intracellular H_2O_2 concentration. Increased H_2O_2 is essential for efficient insulin signaling, as blocking H_2O_2 production reduces insulin signaling (34). At the plasma membrane, NOX4 and NOX3 have been implicated in insulin-induced H_2O_2 generation and signaling. Ectopic expression of dominant-negative NOX4 in differentiated 3T3-L1 adipocytes attenuated insulin-stimulated H_2O_2 generation, tyrosine phosphorylation of IR and insulin-receptor substrate-1 (IRS-1), activation of downstream kinases, and glucose uptake. In accordance, siRNA-mediated knockdown of NOX4 inhibited insulin signaling (53). Conversely, in HepG2 cells, ablation of NOX3 expression abrogated H_2O_2 production after insulin stimulation and modulated insulin-induced MAPK/ERK phosphorylation (11). Thus, NOX3 or NOX4 or both may mediate H_2O_2 involvement in insulin signaling, or alternatively, the experimental conditions affect the role of $p22^{phox}$, which is a regulatory component of NOX4, but also interacts with NOX1, NOX2, and NOX3. The requirements for $p22^{phox}$ to function in NOX4 versus NOX2 and 3 regulation apparently differ (84). Thus, manipulating either NOX isoform may have consequences for the ability of $p22^{phox}$ to regulate other isoforms.

Insulin-mediated regulation of p66SHC may also directly affect the cellular ROS status. The SHC protein family consists of three members (*i.e.*, p46, p52, and p66SHC). All three SHC isoforms become rapidly tyrosine phosphorylated after insulin treatment of cells. However, p46/52SHC is involved in mediating MAPK/ERK activation, whereas p66SHC appears to inhibit this role of p46/p52SHC (65). In addition, p66SHC may function as a redox enzyme through oxidation of cytochrome *c* (64). This function requires its unique N-terminus and, interestingly, may be redox dependent. It has been

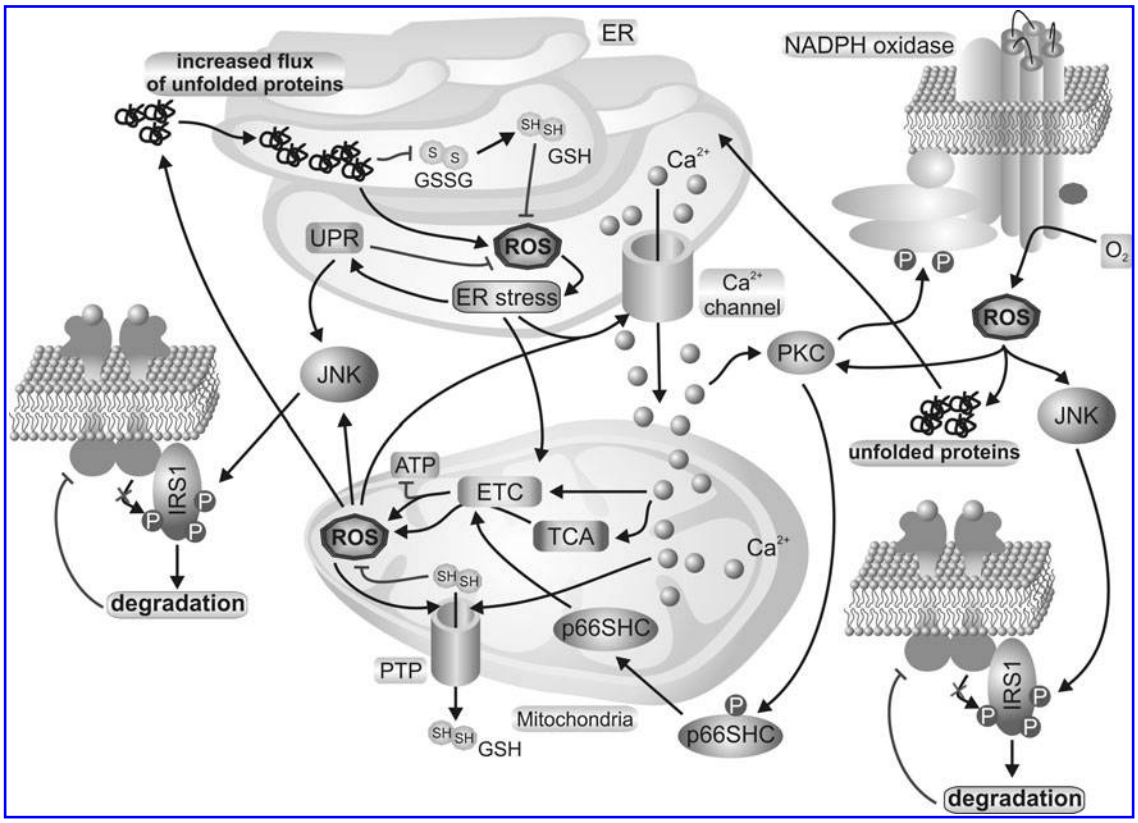

FIG. 3. The vicious cycle of ER stress and overall ROS production. Simplified model illustrating how ER stress induces mitochondria-mediated ROS production and enhances the activity of NADPH oxidase and p66SHC. This is mediated mainly by ER stress–induced release of Ca^{2+} from the ER lumen. The unresolved ER stress induces production of ROS in other cellular compartments, leading to further oxidative-stress induction. This results in an increased degree of misfolded proteins and additional release of Ca^{2+} from the ER, which sustains ER stress. Oxidative stress and ER stress both inhibit insulin signaling through activation of the protein kinase JNK.

shown that activation of p66SHC requires its tetramerization via formation of disulfide bonds between its N-terminal parts. In turn, GSH and thioredoxin (TRX) can reduce and inactivate p66SHC, which results in a thiol-based redox-sensor system (31). Furthermore, insulin via redox-sensitive PKCβ was shown to phosphorylate p66SHC. However, this phosphorylation enhances apoptosis through mitochondrial dysfunction. This suggests a role for p66SHC under pathologic rather than normal conditions, and loss of PKCβ protects mice against high-fat diet (HFD)–induced diabetes (41). Based on these observations, it is likely that insulin, through p66SHC, increases mitochondria-derived H_2O_2. However, it is unclear what the relative contribution (quantitative and qualitative) of p66SHC-mediated ROS changes is compared with NOX-mediated changes in insulin signaling.

The Forkhead Box O (FOXO) transcription factors regulate expression of numerous genes encoding antioxidants, including superoxide dismutase (MnSOD) enzymes, catalase, sestrins, and selenoprotein P (22). Consequently, insulin-induced inhibition of FOXOs will reduce the expression of these antioxidants. The consequence of this is currently unknown. However, cell-cycle progression is known to depend in part on ROS (70), and insulin-induced FOXO inhibition may thus result in downregulation of those antioxidants that otherwise would impair cell-cycle progression. Thus, FOXOs regulate antioxidants in the context of additional functions.

Insulin controls mitochondrial function through numerous pathways. Insulin controls mitochondrial biogenesis by controlling transcription of the PPAR-γ coactivator 1α (PGC1α), in part through direct PGC1α/FOXO interaction (15). Furthermore, by regulating glucose availability and by other means, it potently stimulates mitochondrial oxidative capacity and ATP production (15). Thus, insulin treatment of cells will enhance mitochondrial O_2 consumption and hence ROS production. In conclusion, although the paradigm of ROS involvement in insulin signaling was set by H_2O_2 generation through NOX activation, it appears that insulin can lead to ROS generation at multiple cellular locations, all of which may directly or indirectly affect insulin signaling.

Targets of ROS in Insulin Signaling

Phosphatases

The ability of H_2O_2 to oxidize thiols of cysteine residues provides a mechanism for the regulation of protein activity (Fig. 4). The sensitivity of cysteine residues to oxidation depends on the pK_a value of their thiol groups, which is usually around 8.5 at neutral pH. However, the electrostatic interaction of the thiol group with adjacent polar or positively charged amino acids reducs its pK_a and enhances reactivity with H_2O_2. In addition, cysteine oxidation depends on the accessibility of H_2O_2 to possible reactive cysteine residues (64).

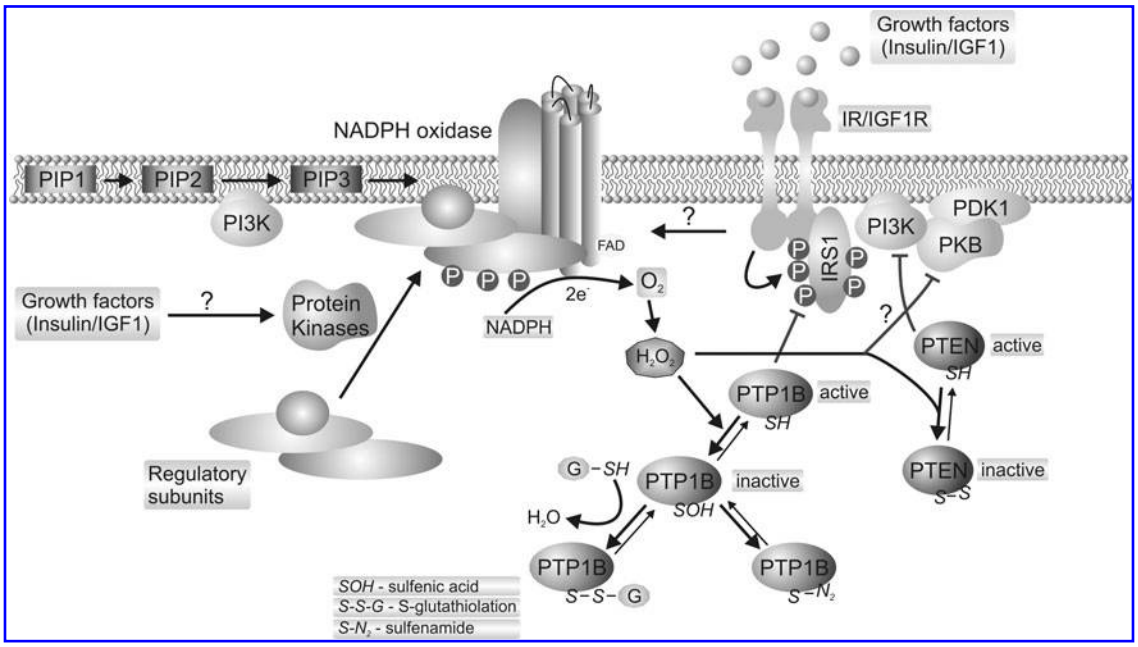

FIG. 4. Insulin stimulates oxidative inactivation of PTPs. On activation of insulin signaling, the NADPH oxidase–mediated generation of ROS increases. The recruitment of PTPs to the plasma membrane stimulates oxidation of their catalytically active cysteine residues, resulting in inactivation of PTP. This promotes Tyr phosphorylation of IRS or activation of PKB and augments insulin action (see text for further details).

Oxidation of the cysteine residue results in formation of sulfenic acid (SOH), which can further react with a second cysteine, either in the same or another protein, to yield a disulfide. Alternatively, sulfenic acid can form a disulfide bond with GSH that results in S-glutathionylation or can be targeted by amide nitrogen of the neighboring residue and form a sulfenamide. All these modifications are reversible and can be reduced by reductase systems to regenerate the thiols (64). It has been postulated that formation of sulfenamide functions as a protective intermediate preventing irreversible oxidation of protein tyrosine phosphatases (PTPs) and might facilitate reactivation of PTPs once their activity is required (83). Nevertheless, when levels of H_2O_2 are greatly elevated, the labile sulfenic acid may be further oxidized by H_2O_2, which results in irreversible modification and, in most cases, inactivation of the proteins. Thus, cysteine oxidation can be used to modulate protein functions, and in insulin signaling, H_2O_2 is used to signal through the cysteine oxidation of pathway components.

PTPs limit the rate and duration of insulin signaling. The signature motif of the family of PTPs, [I/V]HCxxGxxR[S/T], contains an invariant cysteine that functions as a nucleophile in catalysis. The catalytic pocket of the PTPs reduces the normally high pK_a of the cysteine residue, thereby enhancing its nucleophilic property, but at the cost of becoming more sensitive to oxidation. Initial work from several laboratories showed that PTPs are oxidized after treatment of cells with H_2O_2. This reduces PTP activity, resulting in increased tyrosine phosphorylation of IR (57). Importantly, this relies on the activity of NOX but also most probably requires local inactivation of antioxidant defenses. It has been shown recently that on growth-factor stimulation, peroxiredoxin 1 (PRX1) is inactivated, allowing transient high concentrations of H_2O_2 to accumulate locally. This inhibition is restricted to the plasma membrane–associated pool of PRX1 and depends on the activity of c-Src protein tyrosine kinase and NOX1. In summary, stimulation with growth factors triggers the generation of H_2O_2 by activating membrane-associated NOX1. Simultaneously, activation of c-Src results in phosphorylation and inactivation of PRX1, therefore preventing newly synthesized H_2O_2 from being converted into nonreactive species. The increase in H_2O_2 may further promote phosphorylation and subsequent inactivation of PRX1 because of ROS-dependent stimulation of c-Src activity and inactivation of PTPs (87).

The predisposition of PRXs to transient inactivation has been proposed as a "floodgate" that permits H_2O_2 to accumulate and to act as a signaling molecule (88). Although this is a possible explanation, it should be noted that PRXs may also function as prooxidants (i.e., whereas normally PRX removes H_2O_2 by coupling the oxidation to TRX, it may also couple oxidation to other proteins involved in signaling and, as such, "transmit" the H_2O_2 signal to target proteins to oxidize cysteines present in these proteins (26).

Several phosphatases use a catalytic cysteine to regulate insulin signaling. These include phosphatase and tensin homologue (PTEN) and PTPs, among which protein tyrosine phosphatases 1B (PTP1B) is the major one (79). PTP1B is expressed in all insulin-responsive tissues. Overexpression studies have shown that on insulin stimulation, PTP1B interacts directly with IR and mediates its dephosphorylation, which results in attenuation of insulin signaling and impaired glucose incorporation into glycogen (44, 72). Accordingly, $PTP1B^{-/-}$ mice exhibited increased IR phosphorylation in liver and muscle tissue after insulin injection, enhanced insulin sensitivity, and were resistant to obesity (24). Several studies in rodents and humans with insulin resistance, diabetes, and obesity showed increased expression of PTP1B in these conditions (93). PTP1B becomes reversibly oxidized and

inactivated on stimulation with epidermal growth factor (EGF) and insulin (12, 47, 54). It has been shown in endothelial cells that oxidation of PTP1B in response to EGF depends on its colocalization with NOX4 in the ER. Moreover, targeting of PTP1B to the cytosol increases its activity and abolishes the ability of NOX4 to oxidize PTP1B and to stimulate EGF signaling (12). Thus, colocalization of NOX complexes with redox-sensitive targets provides a means to determine the selectivity of intracellular ROS signaling. In addition to PTP1B, oxidative regulation was reported also for other members of the PTP superfamily involved in insulin signaling, including PTEN and T-cell PTP (TCPTP).

Two isoforms have been described for human TCPTP, an endoplasmic reticulum–targeted 48-kDa form (TC48) and a nuclear 45-kDa form (TC45). TCPTP can interact with IR and dephosphorylates its *β*-subunit, both *in vitro* and in cellular context, and therefore augments insulin signaling. It has been shown that insulin stimulates nuclear exit of TC45, colocalization with the IR, and rapid oxidation of TC45, resulting in inactivation of TC45. (56). Accordingly, cells lacking TCPTP show enhanced insulin-induced activation of PKB (28). This indicates that TCPTP, similar to PTP1B, acts to inhibit insulin signaling and that insulin-induced oxidation relieves this inhibition. However, PTP1B and TCPTP have been also shown to display substrate specificity. For example, it has been reported for JAK/STAT signaling that JAK2 is dephosphorylated by PTP1B, whereas TCPTP shows preference for JAK1/3; thus, these two phosphatases may have both a redundant and a complementary role in regulating insulin signaling.

Two other PTPs [*i.e.*, low-molecular-weight PTP (LMWPTP) and an Src homology 2 (SH2) domain–containing phosphatase (SHP2)], have also been suggested as negative regulators of insulin signaling. However, $SHP2^{-/-}$ mice do not display obvious deregulation of insulin signaling (5), and knowledge about LMWPTP is lacking in this respect. Also, LMWPTP and SHP2 are oxidized in response to platelet-derived growth factor (PDGF) stimulation (16). Considering that PTP1B and TCPTP are oxidized in response to insulin and EGF and this oxidation has no impact on PDGF signaling, it is equally possible that LMWPTP is specific to PDGF rather than insulin signaling.

Studies from the groups of Rhee and Downes (46, 49) showed that treatment of cells with EGF, PDGF, or insulin all result in inactivation of PTEN because of the formation of an intramolecular disulfide between the catalytically active cysteine and a proximal "backdoor" cysteine. Subsequently, studies using glutathione peroxidase (GPX1)-knockout mice revealed that these mice exhibit increased insulin sensitivity and were protected from high-fat-diet–induced insulin resistance because of enhanced glucose uptake in muscles (51). Prolonged and elevated oxidation of PTEN is at least partly responsible for this phenotype. At the cellular level, GPX1 deficiency enhances insulin-induced ROS levels, which promotes oxidation of PTEN, resulting in its inactivation. This in turn elevates PI3K/Akt signaling and improves insulin sensitivity. In agreement, the phenotype exhibited by $GPX1^{-/-}$ mice can be reversed by treatment with the antioxidant *N*-acetyl cysteine (51).

Taken together, these studies further support the promoting role of ROS in insulin signaling due to reversible oxidation and inhibition of phosphatases. Thus, activation of insulin signaling leads to elevation in H_2O_2 levels that potentiates signal transduction due to oxidative inactivation of PTPs, which otherwise counteracts insulin action.

Kinases

Besides phosphatases, ROS can also alter the activity of other proteins involved in insulin action and consequently, suppress or stimulate transmission of insulin signaling (Fig.

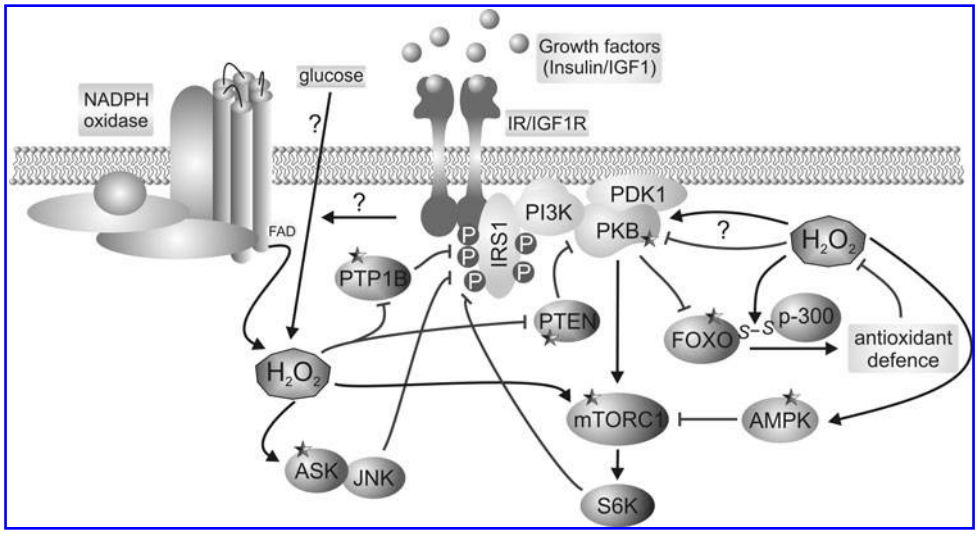

FIG. 5. ROS-mediated feed-forward and feed-back signaling regulating insulin action. Cysteine oxidation is a major way in which H_2O_2 can modulate protein function. The known mediators of insulin signaling that are directly sensitive to cysteine oxidation are indicated by *stars*. ROS can have stimulatory effects on insulin signaling due to inactivation of PTPs (*e.g.*, PTP1B and PTEN). In turn, ROS promote feedback signaling via, for example, JNK and mTOR, which attenuate insulin action. In addition, ROS-mediated activation of FOXO is important for antioxidant defenses decreasing cellular ROS levels and therefore diminishes negative-feedback signaling. Whether ROS promote or demote insulin signaling possibly depends on ROS levels and duration.

5). The crystal structure of PKB revealed an intermolecular disulfide bond between two cysteine residues within the kinase domain, making PKB a possible target of redox regulation. An increase in ROS may result in cysteine oxidation within PKB to form an internal disulfide bond. This promotes the binding of protein phosphatase 2 (PP2A) to PKB and inhibition of its activity. However, PP2A itself contains redox-sensitive cysteine residue and is potentially susceptible to inhibition by H_2O_2. Moreover, oxidized PKB can be reduced by glutaredoxins (GRX); therefore, overexpression of GRX sustains PKB activity (59). Thus, ROS can activate or inhibit PKB activity (or both). This raises the possibility that during insulin signaling, PKB is differentially regulated, depending on the duration and strength of ROS signaling.

Besides PKB, also other kinases directly or indirectly involved in insulin signaling can be subjected to regulation by cysteine oxidation. H_2O_2 treatment of cells results in activation of AMP-dependent protein kinase (AMPK), and this can occur independent of changes in the AMP/ATP ratio. In agreement with this, both *in vitro* and in cells, ROS induced *S*-glutathionylation of both α and β subunits of AMPK and mutation of Cys^{299} of AMPK resulted in loss of AMPK responsiveness to ROS (94). Insulin activates numerous PKC isoforms, including PKCβ, and this isoform has also been shown to become active after an increase in ROS (32). However, the mechanism underlying PKCβ activation by ROS is still unresolved.

Finally, structural analysis of mammalian target of rapamycin (mTOR) revealed a disulfide bond apparently involved in the regulation of protein stability (20). This may be a mechanism through which the cellular redox state would regulate the abundance and thus the activity of mTOR.

Importantly, cellular ROS can also have an impact on insulin signaling via stress-responsive pathways. For example, it has been shown that tumor necrosis factor α (TNF-α) treatment of Huh7 cells increases mitochondrial ROS levels and activates apoptosis signal-regulating kinase 1 (ASK1), which resulted in impaired insulin signaling (42). In unstimulated cells, ASK1 is in complex with TRX1, which prevents it from becoming active. H_2O_2 induces dissociation of TRX1 and formation of an intermolecular disulfide bond between ASK1 monomers, resulting in di/multimerization and activation of ASK1 (60). The ASK1-dependent negative regulation of insulin signaling is most probably mediated by its downstream target cJun NH_2-terminal kinase 1 (JNK1), which can target IRS for degradation. ASK1-mediated activation of JNK1 can be reversed by MAPK phosphatases (MKPs), which, similar to other PTPs, can be negatively regulated because of oxidation of their catalytic cysteine. Thus, elevated ROS levels promote the phosphorylation of JNK1 that is necessary for its enzymatic activity by activating upstream kinases and inhibiting phosphatases.

In addition to targeting IRS, JNK can potentially regulate the activity of several other insulin signaling intermediates, such as PKB and FOXO [for a recent review, see (82)]. In the case of FOXOs, it has been shown that in response to H_2O_2 treatment, JNK directly phosphorylates FOXO4 in a Ral-dependent manner (25). This phosphorylation counteracts the PKB-mediated export of FOXO4 from the nucleus, promoting FOXO4 activation under oxidative stress conditions [reviewed in (22, 78, 82]. Besides being a target of upstream redox-sensitive pathways, FOXO4 itself functions as a redox sensor. Increased ROS trigger disulfide-bond formation between FOXO4 and regulatory proteins [*i.e.*, E1A-binding protein p300 (p300)/CREB-binding protein (CBP) acetyl transferases], and this is indispensable for FOXO4 acetylation (21). As FOXOs also direct transcription of antioxidant genes, this makes them true redox sensors.

In summary, it is apparent that ROS regulates multiple transducers of insulin signaling and thus can inhibit or sustain signaling. This suggests that ROS concentrations and localization are important determinants for the outcome of the interplay between ROS and insulin signaling.

The Enemy: ROS Deregulating Insulin Signaling

As discussed earlier, ROS play an important role in proper tuning of insulin signal transduction, and the importance of ROS is further emphasized, albeit paradoxically, by substantial evidence that implicates ROS in the development of diabetes, but also in the complications accompanying diabetes, such as cardiovascular problems, kidney disease, stroke, and so on (7). Aberrant ROS signaling plays a role in both type 1 diabetes, characterized by loss of insulin production due to loss of pancreatic β-cells, and type 2 diabetes, characterized by progressive loss of insulin responsiveness combined with β-cell dysfunction during disease progression (7). A variety of conditions can cause diabetes, including hyperinsulinemia, hyperglycemia, obesity, inflammatory signaling, and so on, and although the mechanism(s) used by these conditions to induce diabetes is in part unknown, the possibility emerges that ROS play a unifying causal role under all these conditions.

Initially, it was shown that in TNF-α– and dexamethasone-induced insulin resistance, cellular redox changes, and ROS levels increase (40). Reversal of ROS increase, by MnSOD, and catalase expression or administration of SOD mimetics, all relieved insulin resistance to some extent (38, 40). In obese mice as well as insulin-resistant mice, reducing ROS levels increased insulin sensitivity and glucose homeostasis (27, 40).

In agreement, TNF-α–mediated insulin resistance results from TNF-α–induced activation of the stress kinase JNK (37); TNF-α induction of JNK requires ROS; and JNK activity is controlled by cellular redox in general. Numerous studies showed that JNK1 mediates phosphorylation of IRS-1 on Ser^{307} (corresponding to Ser^{312} in humans) (67). This residue is adjacent to the phosphotyrosine-binding domain, and its phosphorylation disrupts the interaction between IRS-1 and IR, and thereby hinders Tyr phosphorylation and promotes the degradation of IRS-1 (9). Moreover, JNK can phosphorylate IRS-2 on Thr^{347}, which likely is an inhibitory residue as well (74). The significance of JNK in the regulation of insulin signaling has been confirmed in $JNK^{-/-}$ mice, in which genetic disruption of JNK1 (but not JNK2) results in resistance to obesity and reduction in insulin resistance, accompanied by a decrease in IRS-1 serine phosphorylation (37, 67). In addition, total JNK activity is often significantly elevated in various tissues in type 2 diabetes patients and in animal models of obesity and diabetes (37).

Finally, inhibition of JNK signaling by using competitive inhibitors disrupting the interaction between JNK and the scaffolding protein JNK-interacting protein-1 (JIP1) restores insulin sensitivity in db/db mice (13, 76). Similarly, mice expressing a mutant form of JIP1, unable to activate JNK, are protected against obesity-induced insulin resistance (58).

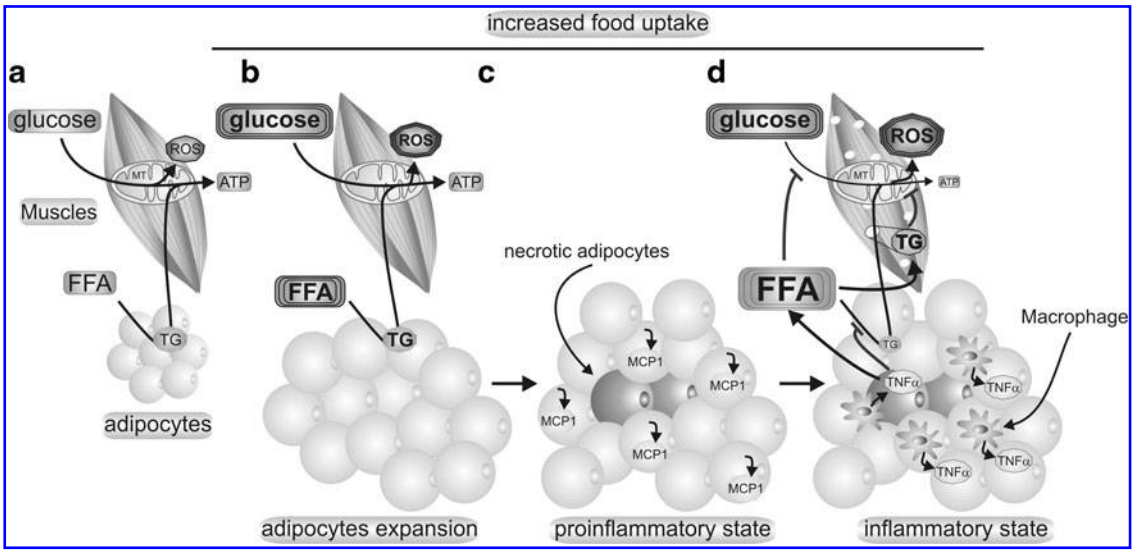

FIG. 6. Obesity-induced inflammation as an underlying mechanism of insulin resistance. In the lean state **(a)**, FFAs are stored primarily by adipocytes as triglycerides (TGs), which provide energy supply for muscles. **(b)** Overnutrition (elevated levels of circulating FFAs) leads to increased TG levels and adipocyte enlargement. **(c)** Further influx of FFAs promotes necrosis of adipocytes and secretion of chemoattractants (*e.g.*, MCP1), which together result in the infiltration of adipose tissue by macrophages. **(d)** The macrophage-mediated secretion of proinflammatory cytokines (*e.g.*, TNF-α) impairs storage of TGs and enhances lipolysis, thereby further increasing levels of circulating FFAs, which eventually accumulate in muscles. This hinders mitochondrial respiration and insulin-stimulated glucose transport. In addition, TNF-α induces systemic inflammation, which leads to insulin resistance in peripheral tissues (*e.g.*, muscle). The figure was adapted from (36).

Besides regulating JNK activity, TNF-α is critical in the induction of inflammatory responses, and chronic low-level inflammation is a key pathogenic mechanism underlying type 2 diabetes (39). The so-called metabolic inflammation is induced by accumulation of fat tissue and exhaustion of the lipid storage capacity of adipocytes, as depicted in Fig. 6. This triggers infiltration of fat tissue by immune cells (*e.g.*, neutrophils, eosinophils, and macrophages). Once recruited, macrophages express TNF-α and other inflammatory cytokines, which sustains the inflammatory state interfering with insulin signaling. Interestingly, adipocytes can also initiate inflammatory responses, which is most probably primary to the recruitment of macrophages (39). Adipocyte-induced inflammation is possibly mediated by ER and oxidative stress, both associated with obesity (see also Fig. 7). In addition, TNF-α–mediated inhibition of peroxisome proliferator-activated receptor γ (PPAR-γ) activity, resulting in increased levels of circulating free fatty acids (FFAs) and deposition of triglycerides (TGs) in nonfat tissues, is also crucial to the induction of systemic inflammation (36).

Another important mediator of the inflammatory functions of TNF-α is IκB kinase β (IKKβ). Importantly, IKKβ can interact with IRS-1 and phosphorylate the inhibitory Ser^{307}(9), and reduced signaling through the IKKβ pathway, either by salicylate inhibition or decreased IKKβ expression, is accompanied by improved insulin sensitivity *in vivo* (89). Furthermore,

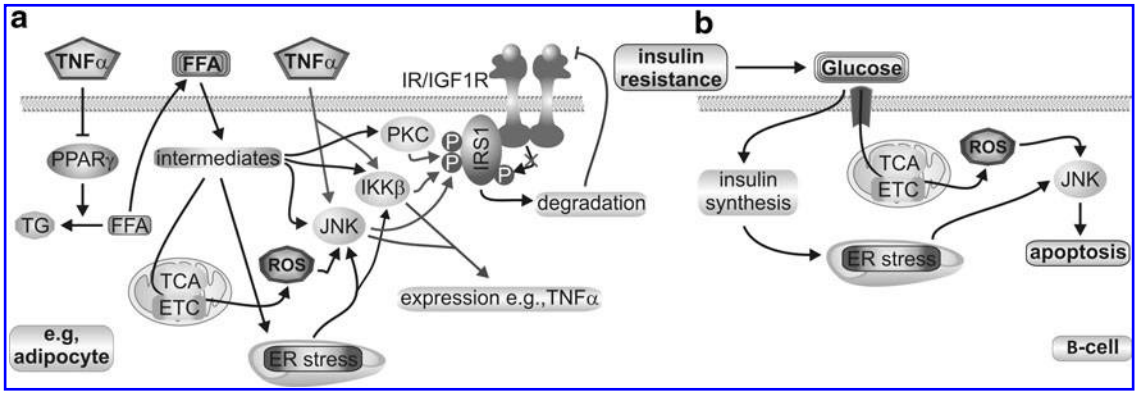

FIG. 7. Molecular mechanism of obesity-induced insulin resistance and β-cell failure. (a) At the molecular level, TNF signaling and accumulation of intracellular lipid metabolites induce the activity of various serine kinases that phosphorylate IRS, thereby triggering its degradation. This leads to a decrease in glucose uptake and insulin resistance. **(b)** Elevated glucose levels, due to overnutrition or insulin resistance, increase the demand for insulin production. This induces ER stress in β-cells. Simultaneously, overload of mitochondria enhances ROS production and triggers mitochondrial stress, thereby decreasing ATP production. Together this may cause depletion of β-cells due to induction of apoptosis.

selective deletion of IKKβ in myeloid cells preserves whole-body insulin sensitivity and protects against HFD-induced resistance to insulin. In contrast, liver-specific IKKβ deficiency leads to the development of insulin resistance in muscle and fat in response to HFD, obesity, or aging (3). These contradictory results may indicate that phosphorylation on IRS-1 has tissue-specific consequences. Additionally, the notion that multiple serine residues in IRS-1 are phosphorylated suggests the possibility that the co-occurrence of a specific set of multiple phosphorylations may differentially regulate signal transduction (9). Moreover, insulin itself stimulates serine phosphorylation on IRS-1 through multiple kinases, including PKB, PKCζ, MAPK, glycogen synthase kinase 3 (GSK), and p70 S6 kinase (S6K) (9). It remains to be established whether phosphorylation of IRS-1 by these kinases has an inhibitory effect on insulin signaling.

However, similar to JNK, S6K deletion in mice protects against age- and diet-induced obesity while enhancing insulin sensitivity (9), suggesting negative feedback by S6K. In agreement with their role in regulating S6K activity, pharmacologic inhibition of mTOR (rapamycin) or activation of AMPK [metformin or aminoimidazole carboxamide ribonucleotide (AICAR)] provides similar protection against development of insulin resistance in response to HFD, obesity, or aging (9). As discussed earlier, besides JNK, AMPK and mTOR also are potential direct targets of cellular ROS, and as such, ROS may also signal through these proteins to mediate insulin resistance.

This illustrates the importance of serine phosphorylation of IRS proteins in determining the outcome of insulin signaling, and clearly ROS are important but not unique regulators of IRS phosphorylation. However, many studies confirmed a role for ROS in inducing insulin resistance or β-cell failure or both, which we briefly summarize.

Hyperglycemia and ROS

Diabetes is characterized by hyperglycemia. Hyperglycemia induces an increase in cellular ROS and cellular damage (66), and this involves at least four metabolic pathways [*i.e.*, increased polyol pathway flux, increased advanced glycation end product (AGE) formation, activation of PKC isoforms, and increased hexosamine pathway flux (66)].

Hyperglycemia results in enhanced shunting of glucose into the hexosamine pathway, generating excess NADH and $FADH_2$, and a subsequent increase in mitochondrial membrane potential. This inhibits electron transfer at complex III, resulting in a marked increase in $O_2^{\bullet-}$ production (66). Increased $O_2^{\bullet-}$ levels inhibit glucose-6-phosphate dehydrogenase, which is essential for providing reducing equivalents (NADPH) to the antioxidant defense system. Hyperglycemia also elevates sorbitol, which is metabolized to fructose, thereby increasing the ratio of NADH to NAD^+ (10). This results in *de novo* synthesis of diacylglycerol, followed by activation of PKC, IKKβ, and NADPH oxidases. PKC-β activation by hyperglycemia may be important in this respect, because it has been shown that PKC-$\beta^{-/-}$ mice are protected against diet-induced obesity and insulin resistance (41).

In agreement with this, decreasing $O_2^{\bullet-}$ production by depolarization of the mitochondrial membrane was sufficient to abolish the activation of pathways downstream of hyperglycemia. Activation of uncoupling proteins (UCPs) reduces the mitochondrial membrane potential because of their ability to facilitate proton leakage across the membrane, thereby inducing partial depolarization and decrease in membrane potential. Consequently, this leads to reduced electron flow via the ETC and a decline in superoxide production (66). It has been shown that endogenously produced mitochondrial $O_2^{\bullet-}$ enhances expression of UCP2 and activates UCP2-mediated proton leakage, followed by a reduction in ATP levels and an impairment in glucose-stimulated insulin secretion (66). $UCP2^{-/-}$ mice exhibit a higher level of ROS production, and ectopic expression of UCPs in cultured neurons blocks glucose-induced apoptosis by preventing mitochondrial hyperpolarization and formation of ROS (4, 66). Similarly, it has been shown that UCP2 is important for protection of the β-cell against oxidative stress (66). Thus, UCPs play an essential role in the regulation of ROS levels generated by mitochondria, and deregulation of their activity might be a key factor involved in glucotoxicity.

Obesity and ROS

Obesity caused by excessive intake of nutrients, including glucose and fat, is rapidly becoming the major cause of diabetes (Fig. 6). Several mechanisms by which nutrient excess contributes to diabetes onset have been proposed. For example, enhanced fat intake may account for increased FFA flux into the circulation with ectopic accumulation of fat in the skeletal muscle, and this is associated with insulin resistance in humans (36). This is supported by the observation that both excessive fat tissue, as seen in common obesity, and the inability of store fat, as seen in congenital or acquired lipodystrophy, are associated with insulin resistance (36, 85). FFA and intermediates have been shown to activate the immune responses (see earlier) but also PKC-θ, a serine/threonine kinase that can phosphorylate serine residues of IRS and thus attenuate insulin signaling (9). Recent data also connect prolonged increased nutrient intake to ROS production. Excessive nutrients, through metabolic pathways, generate a surplus of reducing equivalents and augment the rate of electron flux through the mitochondrial ETC. Electron leakage from complex I and III of the ETC will increase accordingly, leading to increased production of $O_2^{\bullet-}$, and subsequently, H_2O_2 (36). As discussed earlier, this activates JNK, which in turn phosphorylates IRS to inhibit insulin signaling.

Substantial evidence exists for ER stress as an underlying mechanism for obesity-mediated deterioration in insulin signaling and the development of diabetes. With cell culture and mouse models, it has been shown that obesity causes ER stress in liver and adipose tissue, which subsequently leads to suppression of insulin signaling via hyperactivation of JNK (62). In addition, mice haploinsufficient for XBP1 are prone to ER stress and eventually develop diet-induced peripheral insulin resistance and type 2 diabetes (62). In accordance, treatment of obese and diabetic mice with chemical or pharmaceutical chaperones results in normalization of hyperglycemia, restoration of systemic insulin sensitivity, resolution of fatty liver disease, and enhancement of insulin function (63). In addition, a direct link between PI3K signaling and the regulation of the cellular response to ER stress has been proposed. The p85α regulatory subunit of PI3K was shown to interact with XBP1 in an ER stress–dependent manner. This

interaction is abolished in ob/ob mice, resulting in a severe defect in XBP-1 translocation to the nucleus. Similarly, liver-specific p85α deletion inhibits nuclear translocation of XBP1, leading to attenuation of UPR (86). These studies directly link UPR to the PI3K pathway, suggesting that insulin itself may support UPR activity.

β-cell failure due to ROS

Pancreatic β-cells are highly specialized to produce and secrete insulin in response to elevated glucose levels (Fig. 7). This imposes a high oxidative protein-folding demand on β-cells. Therefore, β-cells contain a relative low level of antioxidants, the downside being vulnerability to changes in cellular redox. In agreement, mice deficient for PERK, a UPR transducer responsible for attenuating global protein translation, display reduced β-cell mass (92). This phenotype is reminiscent of the Wolcott-Rallison syndrome in humans, an autosomal recessive disorder resulting from PERK mutation and characterized by severe infantile diabetes (23). Conversely, the transcription factor C/EBP homologous protein (CHOP) also involved in UPR, can mediate β-cell apoptosis under ER stress. Therefore, mice lacking CHOP were protected from ER stress–induced β-cell apoptosis (75). Furthermore, overexpression of the ER chaperone, BiP/GRP78, reverses hyperglycemia-induced inhibition of insulin synthesis and secretion (91). Thus, ER stress is an important parameter of β-cell function.

Whereas in type 1 diabetes, β-cells are depleted because of autoimmune reactions, defective proliferation is a major cause of β-cell dysfunction in type 2 diabetes. This is illustrated by the diabetic phenotype of numerous transgenic mice with alterations in cell-cycle–regulatory genes. For example, $p27^{kip1-/-}$ mice show increased β-cell proliferation and suppressed hyperglycemia, whereas $p27^{kip1}$ overexpression resulted in severe diabetes (80). In addition, cyclin $D2^{-/-}$ mice develop severe early-onset diabetes because of defective β-cell replication (45). A common feature of these perturbations is that these are all under the control of PKB/FOXO signaling (Fig. 1). In agreement, FOXOs regulate β-cell proliferation in addition to gluconeogenesis, and gain-of-function mutation of FOXO1 in mice causes a diabetic phenotype arising from increased hepatic glucose output and reduced β-cell mass (35).

Diabetes and Mitochondrial Dysfunction

Insulin regulates mitochondrial metabolism in numerous ways [for a recent review, see (15)], and insulin resistance is associated with mitochondrial dysfunction. Recently, it was found that FOXO1 integrates insulin signaling with mitochondrial functions (14). Liver-specific deletion of IRS-1 and IRS-2 causes insulin resistance, but also severe mitochondrial abnormalities followed by disruption in the integrity of mitochondrial ETC. As a consequence, ATP generation and fatty acid oxidation is impaired. IRS-1/2 deletion, as well as obesity-induced hepatic insulin resistance, was shown to correlate with hyperactive FOXO1, resulting in deregulated expression of many hepatic genes, including heme oxygenase (decycling) 1 (*Hmox1)*. HMOX1 can disrupt ETC, which attenuates the concentration of NAD^+, leading to inhibition of the NAD-dependent deacetylase sirtuin-1 (SIRT1). SIRT1-mediated acetylation of PGC1α abolishes its ability to promote mitochondrial biogenesis (14). Thus, hyperactivated FOXO1, because of decreased insulin signaling, is central in deregulating mitochondrial function during hepatic insulin resistance. Importantly, dysfunctions in mitochondrial ETC may also result in elevated ROS production, leading to oxidative stress and progression to diabetes by JNK and other stress kinases, as discussed earlier. Importantly, under these conditions, ROS contributes to FOXO activation, whereas SIRT1 supports deacetylation of FOXO1, yet this increases FOXO1 transcriptional activity. Hence, inhibition of SIRT1 increases the acetylation not only of PGC1α but also of FOXO1, raising the question why increased acetylation of FOXO1 cannot counteract the increased activity of FOXO observed with aberrant insulin signaling. One possible explanation may be that acetylation of FOXO rather regulates substrate specificity, as previously suggested (21).

Recent studies have also indicated an important role for mTOR in regulating mitochondrial function. Pharmacologic inhibition of mTOR results in decreased expression of the PGC1, followed by a reduction in mitochondrial gene expression and oxygen consumption (19). In agreement, mice specifically lacking RAPTOR in muscle and therefore a functional mTOR complex 1 (TORC1), exhibit severe attenuation of the number of mitochondria, paralleled by an increase in glycogen content (73). This results in impaired oxidative capacity of muscles and a compensatory increase in glycolytic activity. Interestingly, targeted deletion of raptor in adipose tissue does not affect the number of mitochondria, but it increases the expression of UCP1 (73). This is most probably the underlying mechanism for the elevated energy expenditure observed in these mice. Probably, therefore, adipose-knockout mice are resistant to diet-induced obesity and exhibit insulin hypersensitivity with enhanced glucose tolerance. The insulin sensitivity is due to increased insulin signaling via PKB in both adipose tissue and skeletal muscle (73). Taken together, these studies delineate a role of mTOR in metabolic and mitochondrial control in adipose and muscle tissues. Nevertheless, the details hereof are not yet fully understood and at least appear tissue specific.

ROS, Aging, and the Insulin Axis

In the nematode *Caenorhabditis elegans*, an insulin-like signaling pathway controls the FOXO homologue abnormal dauer formation protein 16 (DAF-16). Remarkably, insulin signaling to DAF-16 has been shown to be a key component in controlling the adult *C. elegans* life span (48). One of the prevailing theories in aging is the free-radical theory of aging, which states that the lifetime accumulation of cellular damage, caused mostly by ROS, drives the process of aging. Along this line, it has been suggested that the ability of the insulin/DAF-16 pathway to reduce oxidative stress rationalizes this concept. In contrast, recent evidence opposes this concept or at least argues it not to be as straightforward. For example, glucose restriction alters mitochondrial metabolism and elevates ROS levels, yet increasing the life span of *C. elegans* (71). However, the primary biologic role of insulin/DAF-16(FOXO) signaling is to allow adaptation to hostile conditions, and apparently adaptation can also be triggered by mild stress. The phenomenon of stress tolerance induced by a mild stress condition is generally referred to as "hormesis" [for a balanced review, see (29)]. Hormesis can be

induced by various forms of stress, including ER stress (68), and in oxidative tolerance due to improved mitochondrial function, this is also called mitohormesis. Hormesis or mitohormesis may result in an increased life span (29, 71). Thus, the common denominator for aging remains increased stress resistance, whether it is induced by loss of insulin signaling or mild preconditioning by stress. Interestingly, we have shown FOXO to be activated by ROS because of JNK-mediated phosphorylation (25), and JNK extends the life span in a DAF-16/FOXO–dependent manner (61). Consequently, DAF-16/FOXO can be activated by loss of insulin signaling or by increased ROS. Indeed, DAF-16 has been implied in mediating some forms of hormesis (29).

This reinforces the central role of ROS in tailoring insulin signaling in aging and disease, but also indicates that our understanding of this interplay is still far from complete.

Concluding Remarks

The insulin-dependent production of ROS is indispensable for the proper propagation of insulin signaling. However, excessive ROS generation also poses a threat to insulin signaling and may cause disruption leading to diabetes. The physiologic function of ROS in the regulation of insulin signaling can be simplified to the generation of $O^{2\bullet-}$ and H_2O_2 by NADPH oxidases and cell organelles (Fig. 4). H_2O_2 promotes signal transduction predominantly because of oxidative inactivation of inhibitory phosphatases. Remarkably, ROS through other targets (*e.g.*, JNK also potently downregulates insulin signaling). Thus, for this dual activity of ROS to result in sensible signaling location of ROS production, level of ROS concentration, ROS lifetime, and so on, will be important determinants. Low levels of ROS existing over a short time most likely will have a stimulatory effect on insulin signaling and promote proliferation. Conversely, high and sustained ROS levels due to possible induction of oxidative stress would inhibit insulin activity to stop proliferation and to allow activation of stress responses. Further understanding of this intricate interplay between ROS and insulin not only may result in the development of effective treatments for diabetes, but also may expand our understanding of how ROS and insulin cooperatively affect the life span and possibly also other age-related disease. As such, this will remain an important paradigm for further studies.

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Abbreviations Used

Acyl-CoA = acetyl coenzyme A
AGE = advanced glycation end product
AICAR = aminoimidazole carboxamide ribonucleotide
AMP = adenosine monophosphate
AMPK = AMP-dependent protein kinase
ASK1 = apoptosis signal-regulating kinase 1
CHOP = C/EBP homologous protein
DAF-16 = abnormal dauer formation 16
DUOX = dual oxidase
EGF = epidermal growth factor
ER = endoplasmic reticulum
ERAD = ER-associated degradation
ERK1/2 = extracellular signal-regulated kinase 1/2
ERO1 = ER oxidoreductin 1
ETC = electron-transport chain
FFAs = free fatty acids
FOXO = forkhead box O
HFD = high-fat diet
GPX = glutathione peroxidase
GRX = glutaredoxin
GSH = glutathione
GSK3 = glycogen synthase kinase 3
IKKβ = IκB kinase β
IR = insulin receptor
IRE1 = inositol-requiring kinase 1
IRS = insulin receptor substrate
JAK/STAT = Janus kinase/signal transducers and activators of transcription
JIP1 = JNK-interacting protein 1
JNK1 = cJun NH2-terminal kinase 1
LMWPTP = low-molecular-weight PTP
MKPs = mitogen-activated protein kinase (MAPK) phosphatases
MnSOD = superoxide dismutase enzyme
mTOR = mammalian target of rapamycin
NOX = NADPH oxidase
NRF2 = nuclear respiratory factor 2
p38MAPK = p38 mitogen-activated protein kinase
P300/CBP = E1A binding protein p300/.CREB-binding protein
PAK1 = p21-activated protein kinase 1
PDGF = platelet-derived growth factor
PDI1 = protein disulfide isomerase
PDK1 = 3-phosphoinositide-dependent protein kinase-1
PEPCK = phosphoenolpyruvate carboxykinase
PERK = protein kinase RNA-like endoplasmic reticulum kinase
PGC1 = PPAR-γ coactivator 1
PI3K = phosphoinositide 3-kinase
PKB = protein kinase B (also c-Akt)
PKC = protein kinase C
PP2A = protein phosphatase 2
PPAR = peroxisome proliferator-activated receptor
PRX1 = peroxiredoxin 1
PTEN = phosphatase and tensin homologue
PTP = permeability transition pore
PTP1B = protein tyrosine phosphatase 1B
PTPs = protein tyrosine phosphatases
ROS = reactive oxygen species
S6K = p70 S6 kinase
SHP2 = Src homology 2 (SH2) domain–containing phosphatase
SIRT1 = sirtuin 1
SOH = sulfenic acid
TCA = tricarboxylic acid cycle
TCPTP = T-cell protein tyrosine phosphatase
TG = triglyceride
TNF-α = tumor necrosis factor α
TRX = thioredoxin
UCP = uncoupling protein
UPR = unfolded protein response
XBP1 = X-box binding protein 1

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