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Review Article

The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication

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Abstract: A surplus of food supply has evoked a worldwide increase in incidence of type 2 diabetes. This trend will have a significant impact on the life span of people living in modern societies. In contrast, reduced calorie intake has significant impact on preventing type 2 diabetes and increasing longevity. Increased production of reactive oxygen species (ROS), resulting in oxidative stress, has long been proposed as a unifying mechanism linking nutrient excess and diabetes. This review describes the updated mechanism by which oxidative stress provoked by nutrient excess contributes to the development of insulin resistance and pancreatic beta-cell failure. However, despite the promising results in cellular and animal models, major clinical trials have failed to demonstrate beneficial effect of antioxidants on the prevention of type 2 diabetes or the degree of glycemic control in individuals with diabetes. Emerging evidence shows that ROS also function as an insulin-signaling molecule in normal physiology and casts doubt on the potential beneficial effect of antioxidants. The gap between basic research and clinical outcomes heightens the importance for elucidating the precise molecular mechanisms by which cellular redox status affects insulin signaling.

Keywords: Reactive oxygen species, type 2 diabetes, insulin resistance, pancreatic beta-cell

Epidemiology

Type 2 diabetes mellitus has reached epidemic proportions with explosive increase in incidence worldwide over the past few decades. Although type 2 diabetes is more prevalent in developed countries, the increase in incidence seems to be more pronounced in non-European populations, especially those experiencing rapid Westernization [1]. For instance, type 2 diabetes was rare in the Pacific island of Nauru 50 years ago but now it affects almost half of Nauru residents [1]. Similar trends have also been observed in Native American, African Americans, and Asian Indians [1]. The driving force behind this epidemic has been attributed to environmental and behavioral factors, especially nutrient excess [1], as the genetic background remains largely stable over such a short period of time in human evolution. Various epidemiological studies also indicate that obesity is the major risk factor of type 2 diabetes [2]. In contrast, lifestyle modification including diet restriction and physical exercise in high-risk subjects has been shown to reduce progression to diabetes by about 60 % in several large independent prospective studies [3,4]. A recent long-term study in Rhesus monkeys found that animals fed on a calorie-restricted diet have fewer age-related diseases including diabetes, cancer, and cardiovascular diseases than those fed on a regular diet [5]. At the end of the study, 42% of monkeys on a regular diet developed diabetes or pre-diabetes. Strikingly, no monkeys on a calorie-restricted diet developed glucose homeostasis abnormalities [5]. Collectively, data from both epidemiological observation and interventional studies strongly indicate that nutrient excess

play a central role in the development of type 2 diabetes.

Metabolic staging of type 2 diabetes

Type 2 diabetes is a progressive disorder that begins with peripheral insulin resistance and ends with failure of pancreatic beta-cells. In most cases, peripheral insulin resistance, defined as the attenuated response to insulin in fat tissue, liver, and skeletal muscle, appears long before the development of hyperglycemia [6]. Resistance to insulin in skeletal muscle results in reduced glucose uptake. Resistance to the insulin-mediated suppression of hepatic gluconeogenesis and glycogenolysis increases glucose output from the liver. Resistance to the antilipolytic action of insulin in fat tissue causes increased lipolysis and increased free fatty acid (FFA) flux into circulation. Chronically elevated FFA can induce pancreatic beta-cell death ("lipotoxicity") [6]. To compensate for peripheral insulin resistance, pancreatic beta-cells increase in mass and secrete more insulin, leading to hyperinsulinemia. However, at some point, beta-cells can no longer compensate for peripheral insulin resistance and plasma glucose levels start to rise. Elevated glucose levels can further damage pancreatic beta-cells ("glucotoxicity"), leading to progressive loss of pancreatic islet beta cells and finally the development of frank hyperglycemia [6].

The insulin-signaling pathway

The insulin receptors carry intrinsic tyrosine kinase activities. Insulin binding to insulin receptor triggers auto-phosphorylation of the receptor. The phosphorylated receptor next phosphorylates the insulin receptor substrate proteins (IRS) on tyrosine residues [7]. The tyrosine phosphorylation activity is a distinguished feature of insulin receptor since tyrosine phosphorylation is infrequent (< 0.03% of total amino acid phosphorylation) in mammalian cells [8]. Phosphorylated IRS recruits a variety of SH2 domain-containing proteins to initiate a complex signaling cascade [7]. One of these proteins, the phosphotidylinisitol-3'-OH kinase (PI3K) mediates most of the metabolic actions of insulin through activating a number of serine/threonine kinases including the Akt /protein kinase B (PKB), atypical protein kinase C (aPKC) -λ/ζ, and the mammalian target of rapamycin (mTOR). Akt/PKB stimulates glucose uptake into the cell, promotes glycogen synthesis, suppresses hepatic gluconeogenesis, increase fatty acid and triglycerides synthesis and suppress lipolysis in adipose tissue [7].

Regulation of insulin-signaling pathway

Insulin signaling is regulated at several nodes. The protein tyrosine phosphatases (PTP), especially PTP-1B, dephosphorylate insulin receptor and IRS-1 at tyrosine residues [9]. The tyrosine dephosphorylation of the insulin receptor by PTP -1B is a constitutive process that exerts a negative regulatory tone to the insulin-signaling pathway. The most compelling evidence for the negative regulatory activity of PTP-1B is based on studies showing increased insulin sensitivity in the mice lacking PTP-1B [10,11]. Lipid phosphatases, including the phosphatase and tensin homologue (PTEN) and the SH2 domain-containing inositol-5-phosphatase (SHIP2), also negatively regulate insulin signaling by tyrosine dephosphorylation of PIP_3 to PIP_2 [7]. The negative regulatory role of lipid phosphatases in insulin signaling is demonstrated by studies showing that mice lacking PTEN or SHIP2 display increased insulin sensitivity [12-15].

On the other hand, the serine/threonine phosphorylation of the insulin receptor and IRS proteins provides another key negative regulatory mechanism of insulin signaling [16]. Serine/threonine phosphorylation of the insulin receptor or IRS blocks insulin signaling by opposing tyrosine phosphorylation [16]. Insulin receptors or IRS protein harbors several potential serine/threonine phosphorylation sites. Under physiological condition, several "intrinsic" downstream serine/threonine kinases in the insulin pathway such as Akt/PKB, mTOR, and ERK1 phosphorylate serine/threonine residues of the IRS proteins and inhibit insulin signaling as a negative feedback regulation [16].

The origins of reactive oxygen species

Molecular oxygen (O_2) was generally absent until the appearance of photosynthetic organisms (blue-green algae) about 2.5 billion years ago [17]. Photosynthetic organisms convert carbon dioxide (CO_2) and water to glucose and O_2. In this process, solar energy was transduced into chemical energy of carbon bonds. Water was oxidized to O_2 to generate reducing power (hydrogen atoms) required for photosynthesis

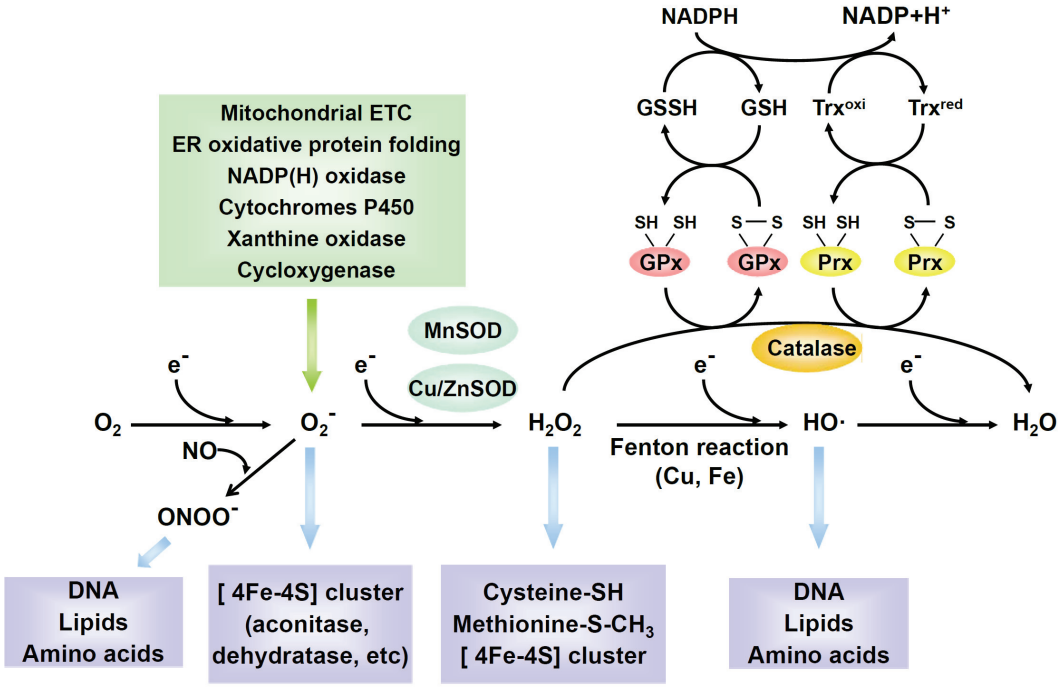

Figure 1. The cellular origins of reactive oxygen species, their targets, and antioxidant systems. ETC, electron transfer chain; ER, endoplasmic reticulum; NADP(H), nicotinamide adenine dinucleotide phosphate; MnSOD, manganese superoxide dismutase; Cu/ZnSOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; Prx, peroxiredoxin; Trx, thioredoxin.

and O_2 was released into the atmosphere as a by-product [17]. The gradual build-up of O_2 in the atmosphere drives the evolution of aerobic organisms. About 1.5 billion years ago, eukaryotes containing mitochondria appeared. In mitochondria, glucose is oxidized to CO_2 and water. Reducing equivalents generated from oxidation of glucose pass electrons through the mitochondrial electron transfer chain (ETC), building up a proton gradient to drive the phosphorylation of adenosine 5'-diphosphate (ADP) to adenosine 5'-triphosphate (ATP), a process called "oxidative phosphorylation" [17]. The electrons ultimately react with O_2 to form H_2O, a fully reduced form of O_2. In this process, O_2 serves as a terminal oxidant (receiver of electrons). This process is highly energy-efficient and is superior to fermentation or respiratory pathways that rely on other terminal oxidants. However, the utilization of O_2 as a terminal substrate in oxidative phsophorylation is risky. When electrons pass through mitochondrial ETC, a fraction of them (0.1-0.5%) escape the ETC and combines with O_2 prematurely, resulting in the generation of partially reduced product- superoxide (O_2^-) [18,19]. Superoxides and other partially reduced form of O_2 are reactive oxygen species (ROS) that can react with biomolecules and interfere with biological processes (**Figure 1**).

The mitochondrial ETC is the major endogenous source of superoxide. Other sources of endogenous superoxide include protein folding and disulfide bond formation in endoplasmic reticulum (ER) [20], nicotinamide adenine dinucleotide phosphate (NADP[H]) oxidase in the phagosome membrane of phagocytes or in the plasma membrane of non-phagocytes [21] and metabolism of drugs or xenobiotics by cytochrome P450 enzymes.

Antioxidant mechanism

Superoxide is converted spontaneously or by manganese superoxide dismutase (MnSOD) in mitochondria or copper-zinc superoxide dismutase (Cu/ZnSOD) in cytosol to hydrogen peroxide (H_2O_2) [22]. H_2O_2 can be removed by either catalase or peroxidase. Catalase is mainly expressed in peroxisomes and catalyzes direct

decomposition of H_2O_2 to O_2 and water. In contrast, glutathione peroxidase removes H_2O_2 peroxide by coupling the oxidation of glutathione (GSH), an abundant thiol-containing tripeptide. Thioredoxin peroxidase (peroxiredoxin) removes H_2O_2 by coupling the oxidation of thioredoxin, a widely distributed polypeptide containing 2 thiol groups. Thioredoxin peroxidase is slower at catalyzing H_2O_2 but is more abundant in amount than glutathione peroxidase in most tissues [22]. Thioredoxin peroxidase has been shown to be efficient in eliminating low concentration of H_2O_2 because of their low *Km* for H_2O_2. However, when the cell is stimulated with growth factors, thioredoxin peroxidase is inactivated, allowing transient high concentration of H_2O_2 to accumulate locally [23]. The predisposition of thioredoxin peroxidase to transient inactivation has been proposed as a "floodgate" that permits H_2O_2 to accumulate in the highly reducing intracellular environment and to act as a signaling molecule [23].

Targets of ROS

Although O_2 is strongly oxidative with respect to its fully reduced form, water, it is a rare stable di -radical because of the kinetic restriction imposed by its two spin-aligned unpaired electrons. O_2 can only react with transition metals or organic radicals with unpaired electron and is a very week oxidant that cannot efficiently oxidize amino acid or nucleic acid [24]. In contrast, its partially reduced products including superoxide, H_2O_2, and hydroxyl radical ($HO\cdot$) are more reactive.

Nevertheless, the anionic charge of superoxide inhibits its electrophilic activity toward electron-rich molecules. Therefore, superoxide could only oxidize few biomolecules such as enzymes containing the [4Fe-4S] clusters (aconitase or dehydratase as examples) [24]. The locally positively charged iron atom attracts superoxide electostatically and is particularly susceptible to superoxide damage. H_2O_2 is also a weak oxidant due to the stable oxygen-oxygen bond that limits its reactivity [24]. H_2O_2 can oxidize the cysteine (-SH) or methionine residues ($-SCH_3$) of proteins. However, the reaction is very slow unless the cysteine residues are rendered more negatively charged by adjacent positively charged residues to form thiolate anion ($-S^-$) [22]. The thiolate form of cysteine residue is the most nucleophilic amino acid that can react with H_2O_2. Several protein kinases, phosphatases, and transcription factors with important physiological function contain thiolate residues that can be oxidized reversibly by H_2O_2. In contrast, hydroxyl radical reacts readily with most biomolecules including lipids, amino acids, and nucleic acids [24]. Theoretically, the damaging effects of H_2O_2 are mainly due to its conversion to hydroxyl radical by Fenton reaction in the presence of free metal such as copper or iron. Superoxide can also react with another poor oxidant nitric oxide ($NO\cdot$) to generate a very strong oxidant, peroxynitrite ($ONOO^-$), that reacts with most biomolecules [24].

Superoxide and hydroxyl radical are short-lived (estimated intracellular half-life: 10^{-6} and 10^{-9} sec) with very low intracellular concentration (10^{-10} and 10^{-15} M) [19]. On the other hand, H_2O_2 is relatively stable (half-life: 10^{-5} sec) with higher intracellular concentration (10^{-5} M) [19]. H_2O_2 is non-polar and can diffuse freely across membrane with a very long diffusion distance (1.5 mm in the presence of 2 mM glutathione) [19]. The relative long diffusion distance of H_2O_2 and its ability to reversibly oxidize specific protein residues make it a suitable molecule for signal transduction [25].

Molecular mechanism linking nutrient excess to insulin resistance

The molecular mechanism by which nutrient excess is linked to insulin resistance is still under debate. One of candidate theories, the "ectopic fat" hypothesis, has gained wide support [26,27]. The hypothesis comes from the observation that both excessive fat tissue, as seen in common obesity, or the inability of store fat, as seen in congenital or acquired lipodystrophy, are associated with insulin resistance [26,27]. Both conditions are associated with increased FFA flux into circulation with ectopic accumulation of fat in the skeletal muscle and liver. Using magnetic resonance spectroscopy, Schulman *et al.* demonstrated that intracellular accumulation of triacylglycerol (TG) and fatty acids intermediates (e.g., phosphatidic acid, lysophosphatidic acid, diacylglycerol and ceramide) in muscle and liver was associated with insulin resistance in human [28,29]. Fatty acids intermediates have been shown to activate the PKC-θ, a serine/threonine kinase that can phosphorylate serine residues of IRS and thus attenuate insulin signaling [30-32]. In support of

this hypothesis, fat infusion increases in intramyocellular fatty acids intermediates and induces insulin resistance whereas pharmacological inhibition of lipolysis decreases FFA flux into bloodstream and improves insulin sensitivity in human [33]. Furthermore, pharmacological inhibition of ceramide production or genetic knockout of PKC-θ prevented fat-induced insulin resistance in mice and human [32, 34].

The "ectopic fat" hypothesis is compatible with the observations that skeletal muscle from insulin-resistant subjects displays reduced mitochondrial oxidative phosphorylation capacity and reduced expression of genes involved in mitochondrial biogenesis [28, 35-37]. It is proposed that either inherited or acquired (e.g. ageing) mitochondrial dysfunction is the primary event that leads to decreased fatty acid β-oxidation and fat accumulation in skeletal muscle or liver [38].

However, an alternative hypothesis linking chronic nutrient overload to insulin resistance has been recently proposed. When excessive nutrient enters the metabolic pathways, a surplus of reducing equivalents is generated with an increased rate of electron flux through the mitochondrial ETC. The electron leak from respiratory complex I and III of ETC will increase accordingly, leading to increased production of superoxide and subsequently H_2O_2 from the mitochondria. ROS have been shown to activate the stress-sensitive serine/thronine kinase c-*jun* N-terminal kinase (JNK), which in turn phosphorylates IRS at serine residues and thus attenuate insulin signaling [39]. The precise mechanism by which ROS activates JNK remains uncertain. It has been shown that H_2O_2 can inhibit thioredoxin binding to the apoptosis signal-regulating kinase (ASK1), a member of mitogen-activated protein kinase kinase kinase (MAP3K), by modification of the cysteine residues in the thioredoxin binding domain of ASK1 [40,41]. The unbinding of ASK1 from thioredoxin leads to the full activation of ASK1, which in turn activates JNK. H_2O_2 has also been shown to activate JNK through inhibition of MAP kinase phsophatase by cysteine residue oxidation [42].

In support of these findings, high-fat diet has been shown to increase mitochondrial ROS emission and shift the cellular redox environment to a more oxidized state in skeletal muscle of rodents or humans [43-45]. Attenuating mitochondrial ROS emission by antioxidant treatment or over-expression of catalase or MnSOD prevents high fat diet-induced insulin resistance in mice [43]. These data indicate that increased mitochondiral ROS generation by excessive metabolic flux induces insulin resistance. Furthermore, transgenic mice engineered to increase fatty acid β-oxidation develop severe insulin resistance despite being protected from obesity [46]. However, genetically engineered mice with reduced mitochondrial oxidative phosphorylation capacity were protected from insulin resistance [47]. These observations suggest that reduced mitochondrial oxidative phosphorylation may actually prevent insulin resistance instead of promoting insulin resistance. Recent evidence also demonstrated that mitochondrial dysfunction observed in insulin resistance is not a primary event but is secondary to ROS-induced damage, which can be prevented by antioxidant treatment [44].

In addition to high fat diet-induced insulin resistance, increased mitochondnrial superoxide generation has also been shown to be the underlying mediators of multiple forms of insulin resistance models including inflammation- or glucocorticoid-induced insulin resistance [48,49]. TNF-α or dexamethasone treatment increases mitochondrial superoxide generation and induces insulin resistance whereas antioxidant treatment rescues these effects. Induction of mitochondrial superoxide by chemicals is sufficient to drive cellular insulin resistance, which can also be rescued by antioxidant treatment [48,49].

Another important mechanism linking nutrient excess to insulin resistance is endoplasmic reticulum (ER) stress [50]. The ER is responsible for protein synthesis and proper folding of the proteins. The lumen of ER has a very high concentration of protein (> 100 mg/dl). Physiological stresses that increase the demand of protein synthesis or disrupt protein folding lead to accumulation of unfolded or misfolded protein in the ER, a condition referred as "ER stress" [51]. The eukaryote cells have evolved a set of transcriptional and translational programs, the unfolded protein response (UPR), to cope with ER stress [51]. Several UPR mediators including inositol-requiring enzyme-1 (IRE-1), PKR-like endoplasmic-reticulum kinase (PERK), and activating transcription factor 6 (ATF6), are localized in the ER and are bound by

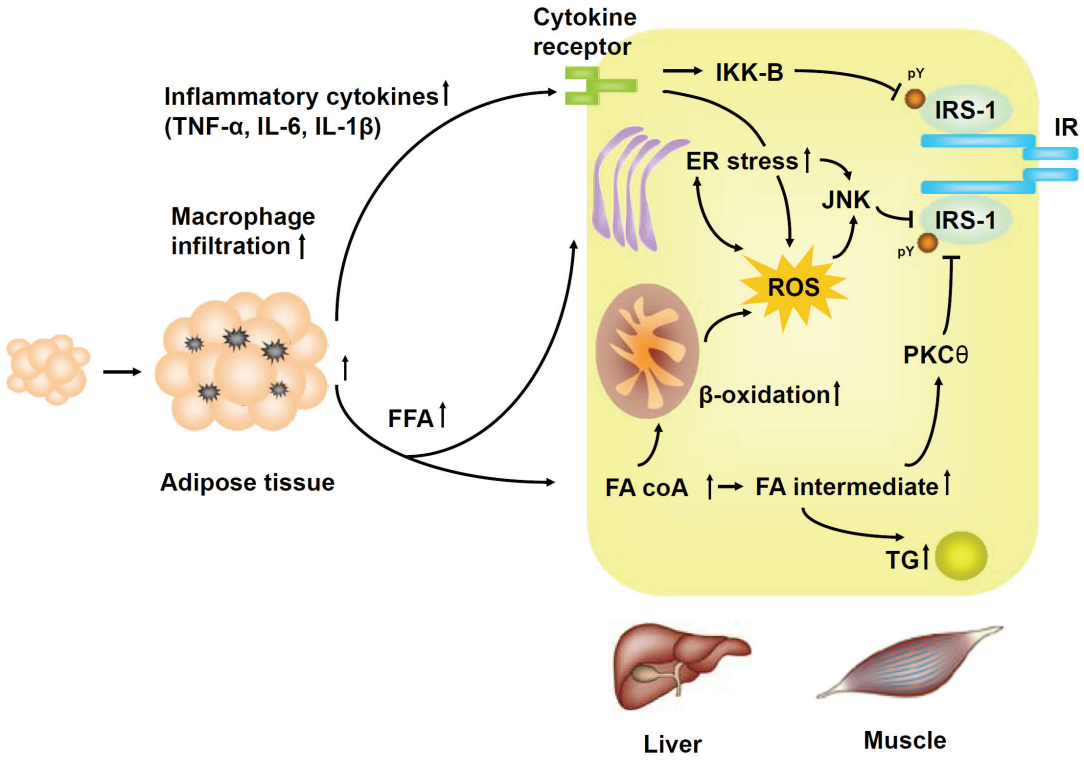

Figure 2. Molecular mechanism linking nutrient excess to insulin resistance. IR, insulin receptor; IRS-1, insulin-related substrate 1; JNK, c-jun N-terminal kinase; PKC θ, protein kinase C θ; IKK, IκB kinase; FFA, free fatty acid; FA coA, fatty acid co-enzyme A; TG, triglycerides; TNF-α, tumor necrosis factor-α;IL-6, interleukin 6; IL-1β, interleukin 1β; ROS, reactive oxygen species.

the abundant ER chaperones BiP/glucose-regulated protein 78 (GRP78). In stressful conditions, ER chaperones are sequestered by binding to mis-folded protein and UPR mediators are released [51]. These mediators initiate a cascade of reactions to reduce protein translation and increase misfolded protein degradation, chaperone synthesis, and cell apoptosis. IRE-1 can further activate JNK, which in turn phosphorylates IRS at serine residue, leading to attenuated insulin signaling. In support of this notion, both genetic and diet-induced obesity cause ER stress with activation of JNK in mice [50]. Treatment of chemical chaperones or overexpression of BiP/GRP78 alleviate ER stress, restore systemic insulin sensitivity, and resolve fatty liver in obese mice [53,54]. Both genetic knockout of JNK and treatment of cell-permeable JNK -inhibitory peptide improve insulin resistance and ameliorates hyperglycemia in diabetic mice [39,55].

ER stress and oxidative stress are closely interlinked processes [20]. The folding of protein into correct conformation requires the formation of intra-molecular and inter-molecular disulfide bonds, which is an energy-consuming oxidative process. The ER is a unique intracellular environment with oxidizing status. The oxidized glutathione to reduced glutathione ratio (GSSG/GSH) is 1:1 to 1:3 in the ER, in contrast to the ratio of > 60:1 in the cytosol [56]. During the formation of disulfide bond, two cysteine residues are oxidized and two electrons are released. The released electrons are then transferred to the protein disulfide isomerase (PDI), passed to the ER oxidoreductin (ERO1), and finally react with O_2. This process mimics the ETC in mitochondrial respiration. Like mitochondrial respiration, the ER electron transfer during oxidative protein folding is a source of ROS production [20]. It is estimated that up to 25 % of ROS generated in a cell results form oxidative protein folding in the ER [57]. Accumulated ROS will also deplete the reduced glutathione required for protein folding, further exacerbating

ER stress. In support of these findings, antioxidant treatment was shown to alleviate ER stress and improve protein secretion both *in vitro* and *in vivo*, indicating a close interaction between ER stress and oxidative stress [58] (**Figure 2**).

Inflammation, ROS and insulin resistance

Chronic low-grade inflammation is one of the hallmarks of obesity and type 2 diabetes [59]. Biomarkers of inflammation, such as tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP), are elevated in obese subjects [59]. Unequivocal epidemiological and experimental evidence in animal and human indicate that inflammation is crucial for the development of obesity-induced insulin resistance [59-62]. The major source of inflammation is believed to be the activated macrophages in adipose tissue. It is estimated that about 10 % of the cells in adipose tissue of lean animals or human are composed of macrophages [63,64]. This percentage increased markedly to 40 % in obese counterparts. The infiltrating macrophages in the intramuscular fat of obese mice are approximately 3 times more than those in lean mice. Inflamed adipose tissue is capable of secreting a variety of pro-inflammatory mediators including TNF-α that induce peripheral insulin resistance.

A central question remained unanswered is how the inflammation is triggered in the adipose tissue. One proposed mechanism is based on the observation that macrophages form ring-like structures surrounding dead adipocytes. As adipose tissue expands during the development of obesity, certain regions become hypoperfused, leading to adipocyte microhypoxia and cell death. Adipocyte hypoxia and death triggers a series of pro-inflammatory program, which in turn recruit new macrophages [65]. Another proposed mechanism is the activation of inflammatory pathway by oxidative stress and ER stress. High-fat diet induces ER stress in adipocytes and activates JNK and IKK that triggers inflammatory response in adipose tissue [66]. High-fat diet has also been shown to increase ROS production in adipose tissue via NADPH oxidase activation in adipocytes [43]. Treatment with NADPH oxidase inhibitor reversed ROS production in adipose tissue and improved systemic insulin resistance [43].

The emerging role of ROS as an insulin-signaling molecule

Although excessive oxidative stress is generally linked to the development of insulin resistance, it became apparent in recent years that low-level of H_2O_2 is actually required for normal insulin signaling [25]. The potential involvement of ROS in normal cellular signaling has been observed for more than 30 years [25]. H_2O_2 is rapidly and transiently generated in response to various stimuli, including insulin, growth factors, and cytokines. H_2O_2 itself can exert insulin-mimicking effects and elimination H_2O_2 attenuates normal insulin signaling. Subsequent studies revealed that insulin receptor is coupled to several types of NADP(H) oxidase at the plasma membrane through small GTP-binding protein in non-phagocytic cells including adipocytes, myocytes, hepatocytes, and vascular cells [21]. Upon insulin stimulation, NADP(H) oxidase generate superoxide, which is then converted to H_2O_2.

Abundant evidence suggests H_2O_2 is required for optimal activation of many signaling pathways, especially those mediated through protein phosphorylation [25]. H_2O_2 is ubiquitous, relatively long-lived, and are readily permeable to membranes. Although H_2O_2 is generally considered as a weak oxidizing agent that cannot directly oxidize DNA or lipid, it is capable to oxidize certain protein residues. Certain protein residues such as the thiolate (deprotonated) form of cysteine ($-S^-$) are particularly susceptible to the electrophilic attack by H_2O_2. However, the cysteine residues of most cytosolic proteins are protonated (-SH) due to the low pH environment of cytosol and are resistant to oxidation by H_2O_2 [20-22]. Only cysteine residues that are more negatively charged by nearby positively charged amino acid can exist as thiolate anions in cytosol. Proteins with susceptible cysteine residues at catalytically active site may become inactive upon oxidation by H_2O_2.

In insulin signaling or other receptor tyrosine kinase (RTK) pathway, the binding of ligand *per se* is not sufficient to trigger phosphorylation cascade. Instead, concurrent oxidative inhibition of PTP-1B, a negative regulator of insulin signaling, is required for phosphorylation propagation [67,68]. All PTPs harbors an essential cysteine residue in the active site motif His-Cys-X-X-Gly-X-X-Arg-Ser/Thr [69]. Given that purified PTP-1B is constitutively active, the reversible oxidation of cysteine residues of PTP-1B into inactive sulfonic acid form (Cys-SOH) offers an

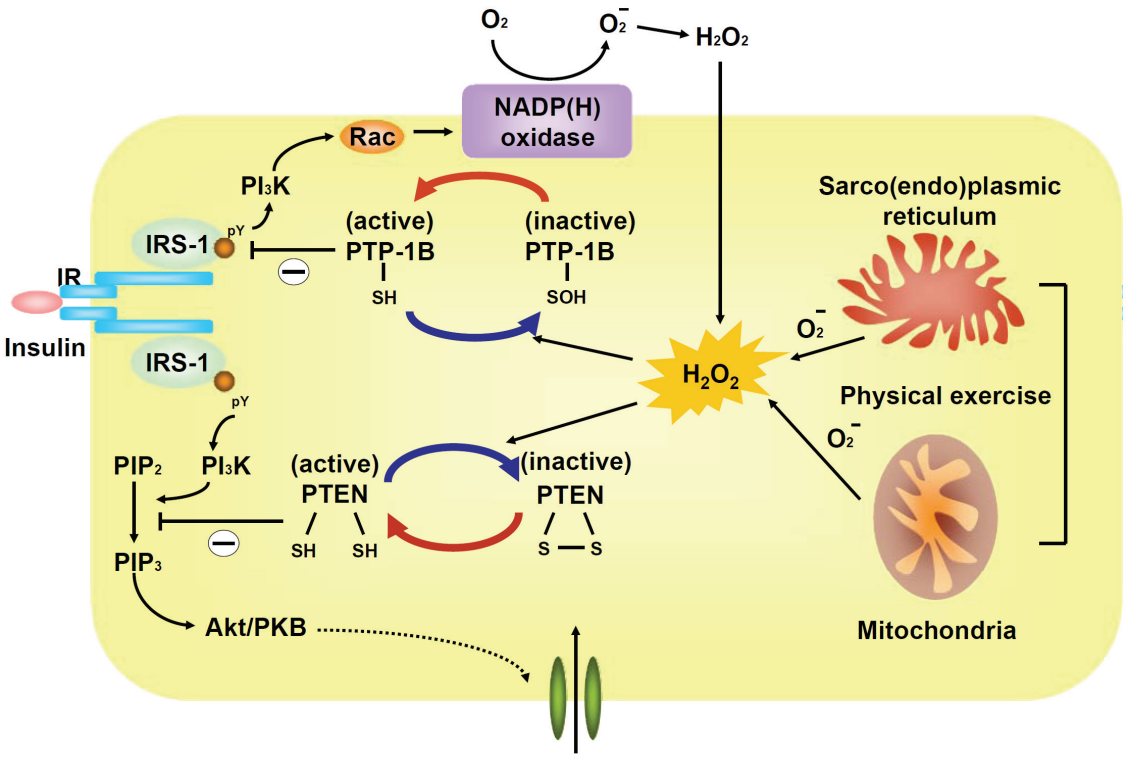

Figure 3. Physiological role of hydrogen peroxide in insulin signaling. IR, insulin receptor; IRS-1, insulin-related substrate 1; PI3K, phosphotidylinisitol-3'-OH kinase; NADP(H), nicotinamide adenine dinucleotide phosphate; PTP-1B, protein tyrosine phosphatase 1B; PTEN, phosphatase and tensin homologue; PKB, protein kinase B.

important regulatory mechanism of insulin signaling. Similar regulatory mechanism was also observed for PTEN, a lipid phosphatase that dephosphorylates PIP_3 to terminate PI3K signaling. The catalytic active Cys-124 in PTEN is oxidized by H_2O_2 to form disulfide bond with Cys-71 [69].

The physiological role of ROS in insulin signaling is supported by a recent study showing that mice lacking glutathione peroxidase 1 (Gpx1) are resistant to high-fat diet-induced insulin resistance. Gpx1 is a ubiquitous enzyme responsible for elimination of endogenous H_2O_2, which typically is in micromolar or submicromolar range. Mice lacking Gpx1 did not have wide-spread elevated H_2O_2 and were euglycemic with a regular diet, suggesting the existence of compensatory antioxidant pathways [70]. However, they were protected from the development of insulin resistance under a high-fat diet. Upon insulin stimulation, insulin signaling is more pronounced with more oxidized PTEN in $Gpx^{-/-}$ myotubes as compared with wild-type [70]. In contrast, mice over-expressing Gpx1 were insulin resistant with reduced insulin signaling [71].

The seemingly conflicting role of ROS in insulin signaling may be explained by the degree and context by which ROS is generated. It has been proposed that transient and low-grade oxidative stress be beneficial, whereas sustained oxidative stress may promote insulin resistance [70]. A similar scenario has also been observed for physical exercise-induced enhancement of insulin sensitivity. Exercise-induced improvement in insulin sensitivity was more pronounced in mice lacking Gpx1, which was blocked by antioxidant treatment [70]. In human, physical activity enhances insulin sensitivity and induces expression of anti-oxidant enzymes in skeletal muscle. However, these beneficial effects were also blocked by antioxidants [72]. In nematode, glucose deprivation promotes ROS formation, induces catalase activity, increases resistance to

oxidative stress, and extends life span [73]. Antioxidant treatment prevents the extension of life span. These data suggest that transient low-level oxidative stress may enhance stress resistance or enhance insulin sensitivity, a hypothetical concept commonly referred to as "hormessis" (**Figure 3**).

Oxidative stress and beta-cell dysfunction

Glucotoxicity and lipotoxicity are generally considered as the major contributors of beta-cell failure in the developing stage of type 2 diabetes [7]. Abundant evidences demonstrate that chronic exposure to high circulating glucose or FFA increases ROS production and decreases insulin content and glucose-stimulated insulin secretion of beta-cells both *in vivo* and *in vitro* [74]. Antioxidant treatment or over-expression of glutathione peroxidase or catalase can reverse these effects [75,76]. Beta-cells express low levels of anti-oxidative enzyme including catalase and glutathione peroxidase, which make them particularly susceptible to oxidative stress [77]. Although the exact mechanism remains uncertain, ROS has been shown to reduce insulin gene expression and insulin secretion of beta-cells, probably through post-translational repression of musculoaponeurotic fibrosarcoma protein A (MafA) and pancreatic duodenal homeobox-1 (PDX-1), two key transcriptional factors that bind to insulin gene promoter [78,79]. Superoxide has also been shown to activate uncoupling protein 2 (UCP2), a mitochondrial protein that increases proton leak, reduces ATP production and metabolic coupling, and negatively regulates glucose-stimulated insulin secretion [80-82]. UCP2 knockout mice are prevented from hyperglycemia-induced loss of glucose responsiveness [82].

However, recent studies reveal that ER stress may be the fundamental mechanism of beta-cell failure under chronic metabolic stress. Pancreatic beta-cells are heavily loaded endocrine cells. It is estimated that one million proinsulin molecules are synthesized per minute per cell upon glucose stimulation [83]. The synthesis of one proinsulin molecule requires formation of three intra-molecular disulfide bonds, which imposes a high oxidative protein folding demand on beta-cells. Using a transgenic mice model expressing XBP-I and green fluorescent protein fusion protein for *in vivo* monitoring of ER stress, pancreas has been shown to be the only tissue expressing intense fluorescence in normal condition [84]. In support of this notion, mice deficient for PERK, an UPR transducer responsible for attenuating global protein translation, display reduced beta-cell mass and reduced insulin secretion, and develop neonatal diabetes [85,86]. The phenotype is reminiscent of the Wolcott-Rallison syndrome in human, an autosomal recessive disorder resulting from PERK mutation characterized by severe infantile diabetes [87]. PERK phosphorylates the eukaryotic initiation factor 2 (eIF2α) on Ser 51 and the phosphorylated eIF2α mediates PERK singling to attenuate global protein synthesis. Mice homozygous for mutant eIF2α at Ser 51 have a severe diabetic phenotype with increased oxidative stress, unregulated proinsulin translation, mitochondrial damage, and beta-cell apoptosis [88,89]. Another UPR mediator, the transcription factor C/EBP homologous protein (CHOP) has been shown to trigger beta-cell apoptosis under ER stress. Mice lacking CHOP were protected from ER stress-induced beta-cell apoptosis [90]. Deletion of CHOP preserves beta-cell mass, improves beta-cell function, and reduces oxidative stress in several mouse model of diabetes [91]. Furthermore, over-expression of the ER chaperone, BiP/GRP78, reverses hyperglycemia-induced inhibition of insulin synthesis and secretion [92]. These data indicate UPR and ER chaperones alleviate ER stress in beta-cells and are crucial for the maintenance of beta-cell function and survival under high metabolic burden.

Stress-sensing transcription factors regulate glucose metabolism

In addition to post-translational modification of insulin signaling pathway, oxidative stress also influence glucose metabolism through transcriptional regulation. The FoxO family of Forkhead transcription factor can be activated by oxidative stress through the formation of cysteine-thiol disulfide-dependent complex with the p300/CBP acetyltransferase and subsequent acetylation of FoxO [93]. FoxO can also be activated by SIRT1 deacetylase or beta-catenin upon oxidative stress [94,95]. Mammalian FoxOs control various biological functions including cell cycle arrest, antioxidant response, DNA repair, and apoptosis [96]. FoxOs also regulates gluconeogenesis, adipocyte differentiation and beta-cell proliferation [97-99]. Gain-of-function mutation of FoxO1 in mice causes

Table 1. Major randomized placebo-control trials investigating the effects of antioxidant supplement on prevention of diabetes or glucose homeostasis

Study	Study Population	Duration (Years)	Antioxidants (daily dose)	Endpoint	Results
Women Health Study	38,716 healthy US women	10	Vitamin E (α-tocopherol 600 IU)	Incident diabetes	No effect
Women's Antioxidant Cardiovascular Study	6,574 non-diabetic US women at high risk of cardiovascular disease	9.2	Vitamin E (α-tocopherol 300 IU), vitamin C (500 mg), and beta-carotene (25 mg)	Incident diabetes	No effect
Physician Health Study	22,071 healthy US male physician	12	Beta-carotene (25 mg)	Incident diabetes	No effect
Aalpha-Tocopherol, Beta-Carotene Cancer Prevention Study	27,379 non-diabetic male Finnish smokers	12.5	Vitamin E(α-tocopherol 50 mg) and Beta-carotene (20 mg)	Incident diabetes	No effect
Supplementation with Antioxidant Vitamins and Minerals study	3,146 non-diabetic French	7.5	Vitamin C (120mg),Vitamin E (30mg) Beta-carotene (6mg), Selenium (100μg), and Zn (20mg)	Fasting glucose	No effect

diabetic phenotype arising from increased hepatic glucose output and reduced beta-cell mass. Conversely, deletion of FoxO1 in diabetic mice reverses the diabetic phenotypes [99].

Another example is p53, a pivotal tumor suppressor involved in apoptosis, cell senescence, and DNA repair [100]. p53 is primarily induced by genotoxic stress but also by oxidative stress. A recent study shows that either genotoxic stress or oxidative stress in adipose tissue triggers the activation of p53, increases pro-inflammatory gene expression, and promotes senescence-like changes of adipocytes. Inhibition of p53 ameliorates the senescence-like change, decreases inflammatory cytokines and improves insulin sensitivity [101]. Conversely, over-expression of p53 induces inflammatory response and insulin resistance in adipose tissue [101]. These examples indicate oxidative stress may influence glucose metabolism through complex transcriptional regulation.

Implication for therapy

Despite the promising results of antioxidant treatment on glucose homeostasis in animals and the strong inverse associations between dietary antioxidant intake and risk of developing type 2 diabetes in observational studies, several large randomized controlled trials (RCT) failed to support a beneficial effect of anti-oxidant supplements (vitamin E, beta-carotene, and vitamin C) on the prevention of type 2 diabetes (**Table 1**). In the Women's Health Study involving 38,167 healthy US women, supplement of vitamin E provided no benefit on prevention of type 2 diabetes over 10-year follow-up [102]. In the Women's Antioxidant Cardiovascular Study involving 6,574 non-diabetic women with high cardiovascular risk, supplement of vitamin C, vitamin E, or beta-carotene also did not offer benefit on prevention of type 2 diabetes after over 9.2 years [103]. In the Physician Health Study recruiting 22,071 healthy US physicians, supplements of beta-carotene for an average of 12 years had no effect on the prevention of type 2 diabetes [104]. In the Aalpha-Tocopherol, Beta-Carotene Cancer Prevention Study, vitamin A plus beta-carotene for 12.5 years did not prevent type 2 diabetes in 29,379 male smokers [105].

Multiple small clinical trials investigating the effect of antioxidant vitamins, alpha-lipoic acids, and taurine on glycemic control in diabetic patients have yielded inconsistent results. Although some trials reported promising results on glycemic control [106-109], most trials reported neutral effects [110-115]. In the Supple-

mentation with Antioxidant Vitamin and Minerals study involving 3,146 non-diabetic subjects, daily supplement with vitamin C, vitamin E, beta -carotene, selenium, and zinc for 7.5 years had no effect on fasting plasma glucose [116]. These neutral effects of antioxidants on glucose homeostasis are consistent with their neutral or even harmful effects on cardiovascular disease or cancer prevention [117-125]. Vitamin E supplement increased risk of heart failure [119] and beta-carotene appeared to increase cancer incidence and mortality in smokers [123]. In a meta-analysis pooling 68 randomized trials, vitamin A, beta-carotene, and vitamin E supplement are associated with increased mortality whereas vitamin C and selenium had no effects on mortality [125].

The overwhelming failure of antioxidants in clinical trial in contrast to their promising effects in animal or observation studies remained an open question. Understanding how specific ROS and antioxidants act in normal physiology may be critical when attempting to elucidate this paradox. Antioxidants are divided into two broad classes referred as preventive antioxidants and chain-breaking antioxidants. Preventive antioxidants such as catalase or peroxidase reduce the initiation of radical chain reaction, whereas chain-breaking antioxidants such as vitamin E interfere with radical chain propagation by trapping radicals. However, scavenging of radicals generates a secondary radical. The antioxidant activity of a chain-breaking antioxidant depends on the production of a resonance-stabilized and less reactive radical.

An antioxidant is considered protective only if the product is less reactive than the initial damaged molecule. The enzymatic conversion of two -election oxidants by enzymatic antioxidants such as catalase or peroxidase fulfills this criterion. Vitamin E is converted tocopheroxyl radical with low reactivity that needs to be removed by a secondary antioxidant such as ascorbic acid (vitamin C). If the removal of tocopheroxyl radical by a secondary antioxidant is delayed, it may promote lipid peroxidation. Vitamin C is an excellent antioxidant because ascorbyl radical is usually converted disproportionately to ascorbic acid and dehydroascorbate [19]. Beta-carotene exhibits good radical trapping activity in low partial oxygen pressure. However, at higher oxygen pressure, beta-carotene shows an autolytic prooxidant effect, which may explain its carcinogenic potential in the lung [126]. Therefore, conventional chain-breaking antioxidants may become pro-oxidant under certain circumstances. The development of small-molecule catalase or peroxidase mimetics may provide new hope into the field of antioxidant medicine.

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