Redox regulation of the insulin signalling pathway

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ABSTRACT

The peptide hormone insulin is a key regulator of energy metabolism, proliferation and survival. Binding of insulin to its receptor activates the PI3K/AKT signalling pathway, which mediates fundamental cellular responses. Oxidants, in particular H2O2, have been recognised as insulin-mimetics. Treatment of cells with insulin leads to increased intracellular H2O2 levels affecting the activity of downstream signalling components, thereby amplifying insulin-mediated signal transduction. Specific molecular targets of insulin-stimulated H2O2 include phosphatases and kinases, whose activity can be altered via redox modifications of critical cysteine residues. Over the past decades, several of these redox-sensitive cysteines have been identified and their impact on insulin signalling evaluated. The aim of this review is to summarise the current knowledge on the redox regulation of the insulin signalling pathway.

1. Introduction

Insulin, an anabolic peptide hormone secreted by pancreatic β-cells, is a key regulator of important metabolic processes such as glucose and lipid homeostasis, as well as a determinant of longevity [1,2]. The actions of insulin are mediated by the so-called insulin signalling pathway, initiated by the binding of insulin to its receptor, which triggers a sequence of intracellular phosphorylation events [2]. The insulin pathway maintains metabolic homeostasis by redirecting post-prandial glucose into muscle and adipose tissues, and by suppressing glucose production in the liver. Furthermore, insulin regulates the generation of energy storage, such as glycogen and triacylglycerides [3]. In this context, insulin resistance is defined as the decreased ability of a tissue to adequately respond to the actions of insulin, which is a risk factor for developing type-2 diabetes (T2D) [4]. The worldwide prevalence of T2D has grown concerningly over recent decades, and is estimated to increase further from 9.3% in 2019 (463 million people) to 10.9% by 2045 (700 million people) [4–6]. Furthermore, T2D is an important risk factor for the development of co-morbidities including cardiovascular and kidney diseases, resulting in reduced quality of life [7]. An undeniable connection exists in Western societies between behaviour, diet, obesity and T2D. The growing incidence of T2D is linked, at least partly, to decreased energy expenditure due to our increasingly sedentary lifestyles, and to increased consumption of processed foods and drinks containing high levels of sugar [8,9]. The pathological hallmarks of T2D in mammals include: i) the resistance of peripheral tissues to insulin signals, leading to ii) hyperglycaemia and compensatory hyperinsulinaemia, and iii) impaired/abnormal insulin secretion by pancreatic β-cells [4]. Most individuals with T2D are obese, and obesity itself can cause insulin resistance [4].

Several studies have implicated the involvement of oxidative stress in the development of insulin resistance and T2D. Oxidative stress arises from the aberrant production or defective scavenging of reactive oxygen species (ROS), leading to damage of macromolecules [10]. For example,
ROS associated with several abnormalities, such as hyperglycemia, non-enzymatic glycosylation, inflammation and/or dyslipidemia, may cause decreased insulin expression and/or an impaired response to the insulin signal [11-13]. Thus, the increased supply of energy substrates and the inflammatory environment under T2D conditions are thought to result in the excessive generation of mitochondria-derived ROS that suppress the insulin signalling cascade and thereby promote the development of insulin resistance [13,14].

Besides these harmful and cell damaging high levels of ROS, lower doses of ROS, in particular hydrogen peroxide (H$_2$O$_2$), play essential roles in fine-tuning the insulin signalling pathway and are therefore indispensable for its optimal functioning. Several phosphatases, including those counteracting the insulin-stimulated phosphorylation cascade, contain critical cysteine residues at their active site that can be oxidised in response to H$_2$O$_2$, resulting in their inactivation. Similarly, several kinases that mediate insulin signalling are prone to redox regulation.

In this review, we summarise the current state of knowledge on redox signalling events that contribute to regulating the insulin signalling pathway. Furthermore, we show that key redox-active cysteine residues are evolutionarily conserved from invertebrates to humans, which is consistent with their significant physiological role.

2. Redox signalling

2.1. Cellular sources of ROS

ROS is a commonly used term that includes both short-lived and more stable products from the reduction of molecular oxygen (O$_2$). The stepwise transfer of single electrons during O$_2$ reduction results in the formation of the superoxide radical anion (O$_2^-$), as well as non-radical species such as H$_2$O$_2$ [15]. In the presence of iron ions, H$_2$O$_2$ can form the hydroxyl radical (•OH), which is highly reactive (diffusion-limited) and therefore more implicated in oxidative damage rather than redox signalling. Superoxide is generated ‘accidently’ by the mitochondrial respiratory chain as a by-product of aerobic metabolism [16]. Moreover, superoxide can be produced deliberately by the NADPH oxidase (NOX) family of enzymes in response to stimuli, including growth factors such as insulin [17]. H$_2$O$_2$ is the most abundant ROS in eukaryotes with a cellular steady-state concentration of ~1–10 nM [15]. H$_2$O$_2$ is formed from superoxide by the action of superoxide dismutase (SOD) enzymes [15]. H$_2$O$_2$ can also be produced directly, for instance by xanthine oxidase in purine catabolism or by the endoplasmic reticulum oxidoreductase ERO1 during disulphide bond formation by the protein folding system [18].

While excessive formation of ROS is associated with the development of many diseases in humans including T2D, it has become increasingly apparent over recent years that low levels of specific ROS, particularly H$_2$O$_2$, are required for normal cellular function, and are involved in the regulation of many physiological processes such as signal transduction, cell differentiation and proliferation [15]. H$_2$O$_2$ is formed enzymatically by NOXs together with SODs in response to growth factor signals, e.g. insulin. This localised transient H$_2$O$_2$ burst is essential for optimal tyrosine-phosphorylation-dependent signalling events by modifying the activity of kinases. These include the insulin receptor (IR) kinase domain itself (see Section 4.2), as well as phosphoinositide 3-kinase (PI3K) and AKT. Furthermore, the activity of phosphatases, such as PTEN, PTP1B and PP2A, that counteract insulin signalling by dephosphorylating and thereby inhibiting insulin-responsive kinases, is modulated by H$_2$O$_2$ (see Section 4.3). Thus, the localisation and levels of ROS have an impact on the progression of the insulin signalling cascade.

2.2. Cysteine-based redox modifications

Amongst the various ROS, H$_2$O$_2$ exhibits low overall reactivity, but relatively high selectivity towards specific thiol groups (R–SH) of cysteine residues (Fig. 1). Therefore, redox-based post-translational modification (PTM) of cysteines in target proteins can modulate a wide range of biological processes [15,19]. The cysteine proteome serves as an adaptive interface between the genome and the external environment of an organism. The reversible oxidation of cysteines involved in redox signalling processes depends on their location at the protein surface, and hence their accessibility to oxidants and antioxidants. The human genome encodes ~214,000 cysteine residues - a frequency lower than expected (~2.26% versus 3.28%) [20]. Cysteines play important functions as structural disulphides in mediating protein folding, or as catalytic active sites in enzymes such as phosphatases and proteases. This biochemical reactivity explains why cysteine residues are highly evolutionary conserved, but selected against if non-functional [21,22].

Cysteines in their deprotonated thiolate form (R–S$^-$) can undergo oxidative modification by H$_2$O$_2$ to form a sulfenic acid (R–SOH), intra-/inter-molecular disulphides (R–SS–R), or higher oxidation states, such as sulfenic (R–SO$_2$H) and sulfonic acids (R–SO$_3$H). Cysteine oxidation to sulfenic acid and disulphides is completely reversible. The reduction of sulfenic acids is mediated either by the glutaredoxin (Grx) and thioredoxin (Trx) systems that allow rapid enzymatic reduction, or by free low molecular weight thiols such as glutathione (GSH) (Fig. 1) [23]. GSH is a tripeptide synthesised from the amino acids glutamate, glycine, and cysteine within the cytosol, and transported to other cellular compartments. The synthesis of GSH is enzyme-catalysed in two ATP-dependent reactions that are strongly inducible by the transcription factor NRF2 [24] (see Section 5.1).

As a low molecular weight thiol-containing antioxidant, GSH is present...
in all eukaryotic cells [25]. GSH directly scavenges free radicals by donating two electrons when two GSH molecules react together to give an oxidised disulphide form (GSSG). Furthermore, GSH is necessary for the function of glutathione peroxidase (GPX) enzymes, which detoxify H\textsubscript{2}O\textsubscript{2}, lipid peroxides, or other organic peroxides [26]. S-Glutathionylation can occur in two ways: i) as a nucleophilic attack by GSH on an oxidised thiol, or ii) by the reverse reaction. This process can happen either enzymatically catalysed or uncatalysed, and occurs under conditions of both oxidative and nitrosative stress [15]. S-Glutathionylation acts as a reversible mechanism to modulate the function of target proteins (in most cases, associated with their inactivation), as well as to protect cysteines from irreversible hyper-oxidation that would lead to permanent damage [15,24,27].

The redox-reactivity of each cysteine varies considerably depending on its structural context and local environment [38]. Analysing the redox proteome (i.e. redoxome) of several mouse tissues revealed that redox-sensitive cysteine residues exist in a local environment that tunes the side-chain for oxidative modifications [29]. A common amino acid signature was enriched surrounding highly modified cysteine residues: selection against acidic amino acids and selection for basic amino acids. This specific spanning signature can be explained by the effects of the proximal charge on the cysteine side-chain. The thiol/thiolate equilibrium is sensitive to electrostatic changes, with a positively charged residue (e.g. arginine) stabilising the negatively charged thiolate [29]. Conversely, the negative charge of a phosphorylated side-chain is predicted to antagonise cysteine oxidation, and indeed the % cysteine modification was negatively correlated with the propensity of phosphorylation proximal to this site [29]. In contrast, no correlation was observed in adipocytes treated with the drugs BCNU (bis-chloroethyl nitrosourea) and auranofin, suggesting that changes in protein phosphorylation in response to simultaneous thioredoxin reductase (TrxR) and glutathione reductase (GR) inhibition were unlikely a direct result of oxidation of these proteins. Thus, a global protein phosphorylation change in response to oxidative events might be the result of redox-dependent changes in the activity of upstream kinases [30]. In this sense, the oxidation of a cysteine residue that is necessary for catalytic function, e.g. in cysteine-dependent phosphatases, would interfere with the formation of the thiol-phosphate intermediate during the dephosphorylation process, therefore maintaining the target protein in a phosphorylated state. A similar scenario and direct redox regulation of cysteine-dependent kinases is possible, where oxidation for signalling purposes might involve structural re-configuration, resulting in enhanced, altered or decreased kinase activity [31].

Superoxide anions and H\textsubscript{2}O\textsubscript{2} are efficiently removed by cellular antioxidant systems (e.g. SOD, peroxiredoxins (Prx), GPX, catalase), thus contributing to generally low intracellular H\textsubscript{2}O\textsubscript{2} levels. Given the high cellular abundance of antioxidant enzymes such as Prx and their strong reactivity with H\textsubscript{2}O\textsubscript{2} [32], it is currently debated how the oxidation of a target cysteine can occur by H\textsubscript{2}O\textsubscript{2} in this environment to allow redox signalling. So far, three main models have been established: i) localised formation of a H\textsubscript{2}O\textsubscript{2} burst that allows the direct oxidation of a less reactive target protein [33], ii) indirect oxidation via a redox relay mediated by a highly redox-sensitive protein, such as Prx [34], and iii) formation of more reactive molecules such as peroxymonocarbonate (HCO\textsubscript{3}\textsuperscript{-}) to form the highly reactive peroxymonocarbonate anion (HCO\textsubscript{3}\textsuperscript{-}), that can directly oxidise the target (3).

![Fig. 2. Mechanisms of redox signalling. Activation of growth factors leads to the induction of superoxide anion (O\textsuperscript{2-}) formation by NOX enzymes, which is subsequently converted to H\textsubscript{2}O\textsubscript{2} catalysed by extracellular SOD3. H\textsubscript{2}O\textsubscript{2} enters cells by facilitated passive diffusion via aquaporins, which results in a localised intracellular H\textsubscript{2}O\textsubscript{2} burst. Alternatively, superoxide is generated in mitochondria as a by-product of respiratory metabolism and converted to H\textsubscript{2}O\textsubscript{2} by SOD2 in the matrix. The reactivity of H\textsubscript{2}O\textsubscript{2} with antioxidant enzymes such as peroxiredoxins (Prx) is several orders of magnitude higher than towards the thiol of a ‘normal’ target protein. Therefore, several models have been proposed to explain how redox modifications of target proteins are possible. The target protein is located in close proximity to the site of H\textsubscript{2}O\textsubscript{2} formation, enabling close-range direct oxidation (1). In the redox relay model, a highly reactive sensor, e.g. Prx, acts as a redox messenger protein, transmitting the oxidation to a subsequent target cysteine, which allows longer-range signalling (2). The reactivity of H\textsubscript{2}O\textsubscript{2} can be enhanced by interaction with cellular bicarbonate (HCO\textsubscript{3}\textsuperscript{-}) to form the highly reactive peroxymonocarbonate anion (HCO\textsubscript{3}\textsuperscript{-}), that can directly oxidise the target (3).](image-url)

3. Signalling by insulin

Insulin plays an essential role in controlling nutrient and metabolic homeostasis. Insulin promotes glucose storage in the liver and glucose uptake into fat and muscle cells via the glucose transporter GLUT4. In response to insulin, GLUT4 is translocated from the cytoplasm to the plasma membrane. Mice lacking GLUT4 are insulin resistant [37]. The precise mechanism whereby insulin leads to the translocation of GLUT4 is still not fully understood, but the PI3K/AKT pathway is known to play an essential role.

The insulin receptor (IR) is a α/β heterotetrameric glycoprotein belonging to the receptor tyrosine kinase superfamily, with 20 subfamilies described in humans, based on sequence homology, structure and ligand affinity [38,39]. Besides the IR, other members of the receptor tyrosine kinase superfamily include endothelial growth factor receptor (EGFR) with its ligand EGF, and platelet-derived growth factor receptor (PDGFR) with its ligand PDGF [39].

Binding of insulin to the IR extracellular domain induces a conformational change of the IR that enables binding of ATP at its cytoplasmic β domain, resulting in rapid IR auto-phosphorylation. This intracellular activation of specific tyrosine residues in the IR kinase domain is essential for mediating all the metabolic effects of insulin, starting with the recruitment of downstream signalling molecules such as insulin receptor substrates (IRSs) [2]. Tyrosine phosphorylation of IRS enables the binding of various proteins containing an SH2 domain, importantly PI3K, which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) to phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3}) at the plasma membrane. Increased intracellular levels of PIP\textsubscript{3} attract 3-phosphoinositide-dependent protein kinase 1 (PKD1), a serine/threonine kinase, and protein kinase B (PKB) also known as AKT. Phosphorylated PKD1 in turn phosphorylates and thereby activates AKT, which is the key player in insulin-mediated regulation of metabolism and gene expression [40]. The AKT family consists of three isoforms (AKT1,2,3) in mammals, but AKT2 specifically regulates insulin-dependent glucose homeostasis [41]. Transgenic AKT2-knock-out (KO) mice develop insulin resistance and a T2D-like phenotype [42]. Moreover, significant defects in glucose uptake were observed in cultured adipocytes obtained from mice lacking AKT2 [43]. The missense loss-of-function mutation AKT2-Arg274His
leads to hyperinsulinemia and insulin resistance in humans [44], highlighting the importance of AKT2 in mediating insulin signalling. Conversely, the activating mutation AKT2-Glu17Lys within the PH domain of AKT2, required for docking to PIP2, leads to hyperinsulinemia and insulin resistance in humans [44], showing the importance of the PH domain and PIP2 in activating AKT2.

AKT activation induces glycos storage via inhibition of glycosyn synthase kinase 3β (GSK3β), and promotes the uptake of glucose via translocation of GLUT4 vesicles to the cell membrane [2]. Although the PI3K/AKT pathway regulates long-term effects such as cellular differentiation and proliferation, its primary goal is the acute regulation of glucose metabolism in insulin-target tissues.

Several mechanisms modulate insulin signalling, including the regulation of ligand production and availability, the endosomal internalisation and downregulation of the IR, the degradation of IRSs, and the dephosphorylation of insulin targets by specific phosphatases. In this context, the protein tyrosine phosphatase 1B (PTP1B) is described to dephosphorylate the IR kinase domain, PISK and AKT, thus acting as an important negative regulator of insulin signalling [46]. PTP1B-deficient mice are characterised by increased insulin sensitivity and are resistant to high fat diet (HFD)-induced obesity [47,48]. Furthermore, PTP1B polymorphisms in humans are associated with morbid obesity [49].

Therefore, optimal regulation of glucose metabolism by insulin requires tight regulation, and a balance of phosphorylation and dephosphorylation events. In the following sections, the redox-mediated fine-tuning of key components from the insulin cascade will be described.

4. Insulin-mediated \( \text{H}_2\text{O}_2 \) generation regulates insulin signalling

Chronically high ROS associated with hyperglycaemia are recognised to have pathophysiological roles in the progression of T2D, e.g. impairment of \( \beta \)-cell function, or the development of further comorbidities such as vascular complications. Studies in healthy humans and rodents further revealed that the adipose tissue experiences oxidative stress as a result of excessive caloric intake leading to the development of insulin resistance [50,51]. Using a model of physiologically derived oxidative stress by inhibiting TrxR and GR simultaneously in adipocytes, >2000 genes were found to be differentially expressed compared to untreated cells [10]. Interestingly, this response shared many similarities with changes observed in insulin resistance models. Providing these cells with an antioxidant induced only minor transcriptional changes, but rescued the insulin resistance. This indicates that the transcriptional changes observed in response to oxidative stress are not the cause of insulin resistance. Thus, oxidative stress must have effects in addition to transcriptional changes to cause insulin resistance [10].

In this context, it may seem ‘paradoxical’ that the over-expression of antioxidant enzymes leads to the development of insulin resistance in mice [52], and that growth factors such as insulin stimulate the generation of localised ROS, which play a role in facilitating downstream insulin signalling [53–55]. The insulin-mediated production of \( \text{H}_2\text{O}_2 \) was first described in 1979 in rat adipocytes and is coupled with the translocation of GLUT4, AKT2 activation even under serum-starved conditions in HeLa cells, and a tonic nuclear exclusion of the AKT target FOXO1 from the nucleus [44,45], thereby recovery of IR activity. The precise mechanism still needs to be elucidated, but potentially involves the formation of a disulphide between Cys1245 and Cys1308 [68]. Interestingly, \( \text{H}_2\text{O}_2 \) also increases IR.
PTEN dephosphorylates PIP$_3$ Ser473, leading to full activation. This process is counteracted by phosphatases: attract AKT and PDK1, bringing both enzymes in contact, and allowing PDK1 to phosphorylation, which generates ADP. This ADP can act as a negative feedback on AKT activity. The binding of insulin to the insulin receptor (IR) leads to IR auto-phosphorylation, which generates ADP. This ADP can act as a negative feedback inhibitor of IR kinase activity. Binding of insulin to the IR also results in the activation of NOX enzymes and the formation of localised H$_2$O$_2$, which oxidises two redox-sensitive cysteine residues (Cys1245 and Cys1308) in the IR kinase domain, that interferes with the inhibitory effect of ADP, thereby prolonging IR signalling activity. Active IR stimulates PI3K, a kinase that phosphorylates PIP$_2$ to PIP$_3$. Enhanced PIP$_3$ levels in the plasma membrane attract AKT and PDK1, bringing both enzymes in contact, and allowing PDK1 to phosphorylate AKT2 at Thr308. In parallel, mTORC2 phosphorylates AKT2 at Ser473, leading to full activation. This process is counteracted by phosphatases: PTEN dephosphorylates PIP$_3$, whereas PTP1B dephosphorylates the IR and AKT2, thus terminating insulin signalling. Both phosphatases are susceptible to redox regulation and are inactive when oxidised. IR-mediated H$_2$O$_2$ leads to the formation of a disulphide between Cys77 and Cys124 in PTEN, whereas PTP1B undergoes sulphenamide formation and S-glutathionylation at Cys215. (c) AKT2 activity is also fine-tuned by redox mechanisms. Insulin-stimulated H$_2$O$_2$ generation leads to the formation of a disulphide located in the PH domain of AKT2 (Cys60-Cys77), which enhances its recruitment to the plasma membrane, and subsequent activation by phosphorylation. However, high levels of ROS lead to the formation of a second inhibitory disulphide (Cys297-Cys311) located in the kinase domain of AKT2, resulting in diminished activity.

### 4.3. Phosphatases are inhibited by insulin-induced H$_2$O$_2$

Purified IRs maintain their phosphorylation even after removal of insulin. Therefore, cellular phosphatases are recognised as playing a significant role in terminating and regulating IR activity and its downstream components [81, 82]. Protein tyrosine phosphatases (PTPs) function as negative regulators of insulin signalling by dephosphorylating and thereby inactivating insulin targets, including the IR and AKT [46].

PTPs share a common conserved motif at their catalytic site: (I/V) HXAXGXR (S/T/G), including a critical cysteine residue which is necessary for their enzymatic function [46]. This cysteine performs a nucleophilic attack on the downstream phospho-tyrosine target, exhibits a low pK$_a$ and is therefore also prone to oxidation. In general, the catalytic cysteine of PTPs forms a cyclic sulphenamide with the nitrogen of a neighbouring serine residue or becomes glutathionylated upon exposure to oxidants [83, 72] (Fig. 3b). An exception is PTEN where the catalytically essential cysteine forms an inhibitory disulphide with a close-by cysteine [69]. Re-activation of oxidised PTP enzymes is achieved by reduction of the catalytic cysteine by Trx [84].

**Protein-tyrosine phosphatase 1B (PTP1B)** is a negative regulator of insulin signalling through dephosphorylation of the IR and AKT. Permanently high activity of the H$_2$O$_2$-detoxifying enzyme glutathione peroxidase 1 (GPX1) has been positively correlated with the early development of insulin resistance and T2D in animal models [52, 93]. Conversely, GPX1-KO mice exhibit enhanced ROS levels, increased PTP1B oxidation, PI3K/AKT activation, and enhanced insulin sensitivity [55, 94]. PTP1B-KO mice display lower circulating levels of fasting insulin, accompanied by increased IR phosphorylation in liver and muscle tissues. Moreover, these mice are more sensitive to stimulated endogenous or injected insulin as measured by glucose and insulin tolerance tests [95-97].

Members of the PTP superfamily act in a substrate-specific manner and employ a common biochemical mechanism for phosphate hydrolysis involving transient cysteinyl-phosphate intermediates. In vivo, PTPs undergo essentially two regulatory processes - they are inhibited through reversible oxidation of the active-site cysteine residue, and are activated through tyrosine phosphorylation of specific tyrosine residues located near the catalytic site [98]. Whereas the catalytic centre of PTP1B is located in the cytosol, the whole enzyme protein is anchored...
Table 1

| Protein Category | Residue (location) | Modification | Oxidant | Interaction | Effect | Ref. |
|------------------|--------------------|--------------|---------|-------------|--------|------|
| Insulin receptor | Cys1245 (kinase domain) | Intramol. disulphide | GSH | Activation, promotes AKT2 recruitment to the PM | Inactivation, promotes the release of inhibitory ADP from the kinase domain | [68] |
| PTEN Phosphatase | Cys77 (kinase domain) | -glutathionylation | S | Inactivation | [69, 70, 71] |
| FOXO1 TF | Cys612 (PH domain) | Intramol. disulphide | Basal conditions | FOXO1 acetylation, cell cycle arrest and induction of pro-apoptotic genes | Full transactivation activity | [76] |
| FOXO3 TF | C31 (Forkhead domain) | Intermol. disulphide | H | Inactivation | [78] |
| FOXO4 TF | Cys477 (linker domain) | Intermol. disulphide | P300/CBP | FOXO4 acetylation, cell cycle arrest and induction of pro-apoptotic genes | Nuclear translocation | [79] |

*intramol., intramolecular; intermol. intermolecular; PM, plasma membrane; TF, transcription factor.*
family that negatively regulates insulin signalling by dephosphorylating AKT at Ser473. Inhibition of PP2A by small-molecule compounds leads to a dose-dependent increase in AKT phosphorylation and its downstream targets FOXO1 and GSK3α [118]. PP2A contains a critical cysteine residue within its catalytic active site, that is potentially susceptible to oxidation. To-date, studies on the redox regulation of PP2A are limited, although some reports provide evidence for reversible inhibition upon oxidation [119,120].

4.4. AKT2 is activated or inhibited by oxidative modifications depending on the site of action

AKT is a serine/threonine kinase acting downstream of growth factors, and is the key kinase regulating insulin signalling. Active AKT phosphorylates and thereby activates or inactivates its targets with the ultimate goal to enhance anabolic processes. AKT activation is dependent on lipids that are produced by PI3K, and its phosphorylation at two critical sites (Thr309 and Ser474) is mediated by PDK1 and mTORC2, respectively [121] (Fig. 3c).

The redox state of multiple cysteine residues in AKT is important for regulating its activity. AKT2, the AKT isoform that is highly enriched in insulin-target tissues [122], contains two cysteine residues (Cys297 and Cys311) within its activation loop, that form an intramolecular disulphide upon oxidation to inhibit AKT2 activity. Interestingly, these cysteine residues are conserved in the other two AKT isoforms, AKT1 and AKT3, but only modulate the activity of AKT2 [74]. The inhibitory disulphide can be resolved by the Grx system [75]. In addition, Cys124 and Cys311 in AKT2 can undergo S-glutathionylation. The redox regulation of Cys124 has been described in PDGF-stimulated NIH3T3 cells as an AKT2 inhibition site [73] (Fig. 3c, Table 1).

Cys77 of AKT2 is oxidised in response to BCNU/auranofin treatment, associated with hyper-phosphorylation at Thr309 [30]. Several substrates of AKT were also found to be phosphorylated, indicating that AKT2 Cys77 is active when oxidised. However, other AKT targets exhibited decreased phosphorylation, while some didn’t change in response to oxidative stress, showing that not all AKT substrates are influenced by oxidative stress in the same manner. Immuno-precipitation and proteomic approaches revealed that Cys60 and Cys77 of AKT (both located in the PH domain) form a disulphide that acts as a redox switch regulating AKT binding to PIP3 and activation [30]. The PH domain is essential for AKT recruitment to the plasma membrane and subsequent phosphorylation at Thr309 (required for ordering the activation loop and substrate binding) and at Ser474 (to control the positioning of key catalytic residues) in AKT2 [123] (Fig. 3c, Table 1). Insulin stimulates PI3K resulting in the accumulation of PIP3 at the plasma membrane and enhanced binding of inactive cytosolic AKT via its PH domain [66]. Inside the cell, active AKT is predominantly membrane-bound and upon dissociation from the plasma membrane, the PH domain promotes AKT dephosphorylation [124]. Thus, AKT is controlled as a PIP3-sensitive switch, which couples membrane binding to kinase activation [124]. In line with this model, a recent study reported that insulin increases the membrane translocation of AKT2 but not AKT1 in skeletal muscle cells [121].

Phosphorylation of AKT2 at Thr309 was found to be enhanced in response to oxidative stress, a phenomenon that was impaired in an AKT2-Cys60/77Ser mutant stimulated with insulin, implying that either Cys60, Cys77 or both are necessary for insulin-stimulated AKT activation even in the absence of oxidative stress [30], since the Cys60-Cys77 disulphide is formed not only under conditions of oxidative stress but also upon insulin stimulation. This suggests that these residues are sensitive to physiological H\(_2\)O\(_2\) levels [30]. Enhanced formation of the Cys60-Cys77 disulphide in response to insulin increases the affinity of AKT to PIP3, thus promoting its translocation to the membrane and phosphorylation [30]. As the PH domain interacts with and inhibits the kinase domain [124], the redox state of the redox-sensitive cysteines Cys60 and Cys77 within the PH domain may regulate this interaction [30].

Cys124 in the linker region of AKT2 has been identified as another regulatory cysteine, which is isoform-specific to AKT2 and not conserved in AKT1/3. Cys124 forms a sulfenyl group in response to PDGF treatment, leading to the inhibition of kinase activity. Thus, H\(_2\)O\(_2\) can enhance or decrease the activity of AKT2, depending on its levels. Together, the combinations of oxidative modifications to Cys60, Cys77, Cys297 and Cys311 may serve to control the amplitude of AKT activity during signalling [73]. Overall, the insulin-induced formation of H\(_2\)O\(_2\) results in the activation of AKT, the master regulator of insulin signalling.

5. The antioxidant effects of insulin

As described above, insulin exerts pro-oxidant functions via activation of NOX enzymes and subsequent generation of H\(_2\)O\(_2\), which is required for optimal activation and functioning of the insulin signalling (PI3K/AKT) pathway. In addition to the important role of insulin in maintaining glucose homeostasis, insulin also possesses antioxidant effects to protect cells against oxidative stress and oxidative stress-related diseases. Many studies have investigated the antioxidant and cytoprotective functions of insulin. Insulin treatment recovers the cellular GSH/GSSG pool by upregulating the expression and activity of several enzymes with antioxidant functions, including glutamate-cysteine ligase (GCL), GR, glucose-6-phosphate dehydrogenase (G6PD), GSTs, TrxR, SOD and catalase (reviewed in Ref. [125]). Notably, these enzymes are downstream targets of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2).
5.1. Insulin signalling enhances the activity of NRF2

NRF2 plays a major role in the maintenance of cellular redox homeostasis by controlling the response to oxidative stress. Under unstressed conditions, NRF2 is bound to its regulatory partner Keap1 (kelch-like ECH-associated protein 1) and targeted for proteasomal degradation, resulting in the constitutive low abundance of active NRF2. Keap1 contains multiple conserved and highly redox-sensitive cysteine residues, situated close to polar and basic amino acids, which can be modified by H₂O₂ or other electrophiles resulting in the altered conformation of Keap1. Upon oxidative stress or in the presence of other electrophilic compounds, oxidation of the cysteine residues in Keap1 impairs its binding to NRF2, limiting its ability to present NRF2 for proteasomal degradation. Active (released) NRF2 translocates into the nucleus, where it induces the transcription of genes containing an antioxidant response element (ARE) in their promoter regions [126]. However, NRF2 has been implicated not only in redox homeostasis, but also in DNA repair, mitochondrial function, proteostasis, and proliferation [127]. Besides this redox regulatory mechanism, NRF2 is also strongly regulated via other PTMs (e.g. phosphorylation) that prevent or promote its nuclear accumulation (Fig. 4).

Glycogen synthase kinase β (GSK3β) is an important regulator of glycogen metabolism by inhibiting via phosphorylation the activity of glycogen synthase. In addition, GSK3β regulates the cell cycle, as well as apoptosis and insulin signalling [128]. GSK3β is active in unstressed non-stimulated cells, and is regulated via inhibitory phosphorylation of two N-terminal serine residues (Ser9 and Ser21) in response to growth factors. Therefore, active AKT inhibits the activity of GSK3β.

GSK3β phosphorylates many proteins that are targets of the SCFβ-TrCP ubiquitin ligase complex, which promotes proteasomal degradation [129]. NRF2 contains two β-TrCP recognitions sites, one of which contains a functional GSK3β phosphorylation site [130]. Constitutively active GSK3β-ΔSer9 increases the ubiquitination of NRF2 and further reduces its protein levels [130]. Enhancing PTEN activity via selective drugs and genetic manipulation results in GSK3β-mediated phosphorylation of NRF2 at Ser335 and Ser338 residues, and β-TrCP-mediated NRF2 degradation [131]. A second mechanism by which GSK3β inhibits NRF2 is via the activation of tyrosine kinases. GSK3β phosphorylates and activates Fyn, which translocates to the nucleus where it phosphorylates NRF2, resulting in NRF2 nuclear export and degradation [132, 133]. Further evidence for the cross-talk between PI3K/AKT and NRF2 was obtained by microarray data derived from endometrioid carcinomas, showing that PTEN-deficient tumours express high levels of NRF2 and its targets [131].

Due to its strong induction of antioxidant enzymes, the role of NRF2 in disorders such as insulin resistance has been extensively studied over recent years [134–136]. The activation of NRF2 can have either beneficial or harmful effects dependent on the physiological and pathological context [127]. In diseases characterised by chronic inflammation and associated with high levels of ROS, the activation of NRF2 is expected to have protective effects. However, prolonged activation of NRF2 might lead to reductive stress, the disruption of normal redox signalling events, and therefore to the progression of disease, as described for several types of cancer [24].

Prolonged activation of NRF2 may also result in metabolic changes as NADPH-generating enzymes are targets of NRF2. Therefore, NRF2 acts as a direct link between redox homeostasis and energy metabolism [137]. NRF2 tightly controls GSH biosynthesis by regulating the expression of two GCL subunits, which catalyses the rate-limiting step of GSH biosynthesis, namely the reaction of glutamate with cysteine [138]. Cysteine is generated from the reduction of cystine, which is imported into cells via an x⁻ transporter. By activating Slc7a11, the gene coding for the subunit xCT of XCX, NRF2 also directly controls cystine import into cells [138]. In addition, many diverse GSH-dependent enzymes contain an ARE in their promoter sequence, and are regulated at the transcriptional level by NRF2 – for instance, GPXs that use GSH to detoxify H₂O₂, GSTs that transfer GSH to proteins or xenobiotics, or GR that is responsible for the regeneration of reduced GSH from its oxidised form. This provides an advantage to study the functionality of these components under different conditions without potential compensatory effects from paralogues.
GSSG [139].

NRF2 promotes the regeneration of NADPH through the induction of NADP+—dependent enzymes, including malic enzyme (ME1), isocitrate dehydrogenase (IDH1), glucose-6-phosphate 1-dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (PGD), all part of the pentose phosphate pathway (PPP) [139,140]. NRF2 is essential for recycling GSSG to GSH mediated by GR, as well as an important cofactor in anabolic reactions such as lipid synthesis. NRF2-KO mice show lower NADPH/NADP+ ratios, and NADPH levels decrease upon knock-down of NRF2 in tumour cells [141] Thus, decreased NRF2 activity not only results in lower antioxidant capacity, but also affects fundamental metabolic pathways such as lipogenesis, nucleotide biosynthesis, gluconeogenesis and β-oxidation.

As NRF2 exhibits important cytoprotective, antioxidant and anti-inflammatory effects, many studies have examined the role of NRF2 in the development of diseases including insulin resistance and T2D. The initial steps in the development of T2D are thought to be mediated by enhanced ROS formation due to hyperglycaemia that exacerbates redox dysfunction [142]. NRF2-deficient mice are characterised by lower basal insulin levels and longer periods of hyperglycaemia. Conversely, mice with pharmacologically activated NRF2 show lower blood glucose, improved insulin secretion, and higher insulin sensitivity [143,144], indicating anti-diabetic effects of NRF2. NRF2-KO mice also exhibit more profound diabetic complications such as retinopathy, nephropathy and cardiomyopathy compared to wild-type (WT) controls [133, 143–149]. NRF2-deficiency can induce hepatic insulin resistance via activation of the NfkB signalling pathway in mice fed a HFD [150]. In contrast, several studies using transgenic mice found that NRF2-deficiency reduces insulin resistance and improves glucose homeostasis in HFD obesity models [151,152]. However, in a tissue-targeted NRF2-KO mouse model, a HFD enhanced insulin resistance in adipocytes, whereas hepatocytes showed improved insulin sensitivity [151].

The exact mechanisms whereby NRF2 impacts the development of insulin resistance are not fully understood, but lack of NRF2 would potentially lead to: i) enhanced levels of ROS due to diminished anti-oxidant capacity, and ii) impaired re-activation of inactivated PTIPs due to the lack of Trx [84]. Consistent with this model, AKT phosphorylation at Ser473 was greater in the liver and skeletal muscle of NRF2-KO mice compared to their WT counterparts after intraperitoneal insulin injection [153].

5.2. Insulin signalling blocks the activity of FOXOs

The forkhead box class O (FOXO) family of transcription factors is named after the Drosophila melanogaster gene fkh (fork head), because mutations in this gene cause developmental defects in adult flies phenotypically appearing as a spiked head [154]. Mammals have four FOXO isoforms: FOXO1, FOXO3 and FOXO4, which share high sequence similarity, and FOXO6, which is less similar to the others, members, has more restricted expression and distinct regulatory mechanisms. C. elegans and Drosophila each have only a single FOXO orthologue, called DAF-16 and dFOXO respectively (Fig. 5).

FOXOs have highly conserved functional domains: the forkhead DNA-binding domain, and domains that control nuclear import/export and transactivation. The best understood mechanism by which FOXO proteins regulate gene expression is via the evolutionarily conserved forkhead domain, a winged-helix DNA-binding domain, that recognises the core DNA motif 5′-TGTTTAC-3′ [155]. To ensure the correct cell type-specific effect is initiated by these widely expressed transcription factors, FOXO proteins interact with a range of binding partners such as STAT3, Smads, P300, C/EBPβ/δ and PPARs, allowing for a much broader transcriptional response [156]. The plasticity of FOXO activity is regulated by several PTMs that determine subcellular localisation, including phosphorylation, acetylation, methylation, glycosylation, protein–protein interactions, nuclear shuttling, and redox regulation.

![Insulin Receptor](image)

**Fig. 6.** The evolutionary conversation of redox-sensitive insulin signalling components. Protein sequence alignment of insulin signalling components reveals that a high proportion of cysteines described to be redox-sensitive are conserved between mammals and invertebrates. The regions surrounding the redox-sensitive cysteines are shown, and the respective cysteines are highlighted in black (numbering corresponds to the position in the upper human sequence). The sequences were obtained from UniProt (www.uniprot.org), aligned by T-Coffee (www.ebi.ac.uk/Tools/msa/tcoffee/) [164] and analysed with JalView [165]. The UniProt IDs for human (Hs), mouse (Mm), Drosophila melanogaster (Dm) and C. elegans (Ce) are as follows: IR: P06213-2 (Hs), P15208 (Mm), P09208 (Dm), Q9689Y (Ce); PTEN: P60484 (Hs), Q00586 (Mm), Q9V0B6 (Dm), GSE01 (Ce); AKT2: P18031 (Hs), P35821 (Mm), Q8HRH4 (Dm), C3JXDB (Ce); AKT2: P31751 (Hs), Q60823, Q8INB9 (Dm), Q9XT7G (Ce); FOXO1,3,4,6 (Hs): Q12778, Q45224, Q98177, Q8M126; FOXO1,3,4,6 (Mm): Q9R1E9, Q9W1H4, Q9W1H3, Q7OKV4; FOXO1,3,4,6 (Ce): Q95V55 (Dm), O16850 (Ce).

FOXO1 is highly expressed in metabolically active cells, such as hepatocytes and adipocytes, where it regulates the transcription of genes involved in adipocyte differentiation and trans-differentiation, oxidative stress response, lipid metabolism, and the induction of gluconeogenic genes during conditions of low energy intake [157]. Under starvation, FOXO1 responds to increasing ROS levels in adipocytes, determining the transcription of mitochondrial anti-stress response genes [158]. FOXO acts as a nutrient sensor in response to insulin signalling via nuclear-cytoplasmic shuttling, and is therefore a crucial regulator of metabolism. In this context, insulin has an inhibitory function on FOXO proteins, as active AKT phosphorylates FOXO at three sites leading to its inactivation via decreased DNA-binding to its consensus response elements, enhanced association with 14-3-3 proteins, and nuclear exclusion [157] (Fig. 4).

FOXO transcription factors regulate a wide range of genes that can be clustered in the following categories: cell fate decisions (cell cycle arrest, apoptosis), metabolism (gluconeogenesis, food intake, redox balance), protein homeostasis (mitophagy, proteasomal degradation, autophagy), signalling (increased PI3K signalling, increased EGF signalling, decreased mTORC1 signalling, increased mTORC2 signalling, decreased MYC signalling), and cell type-specific functions (pluripotent maintenance, immune system) [157]. FOXO targets include intra- and extracellular antioxidants that interfere with all levels of oxygen reduction that would otherwise lead to the formation of ROS and cause oxidative damage [159]. Hyper-activation of FOXOs is associated with hyperglycemia, hypertriglycerideremia, and insulin resistance [160,161]. Transgenic mice expressing constitutively active FOXO1 in either hepatic cells or...
pancreatic β-cells show increased hepatic gluconeogenesis and decreased β-cell function, respectively [162]. The activity of FOXO is upregulated upon nutrient restriction in adipocytes leading to improved antioxidant responses and lipid catabolism, as well as white-to-brown adipocyte remodelling [163].

FOXO transcription factors contain several cysteine residues that are potentially prone to redox regulation in response to changes in cellular redox status. Depending on the respective redox modification, the subcellular localisation, DNA binding capacity, and/or transcriptional activity of FOXO might be altered. Recent studies have confirmed that FOXO proteins undergo redox regulation (reviewed in Ref. [159]). Initial evidence was provided by a study of FOXO4 in HEK293T cells, where all the cysteine residues in FOXO4 were mutated to redox-inert serines. Redox-inactive FOXO4-ΔCys did not bind to the acetylase p300/ CBP, unlike the WT control [79]. Next, using knock-in mutants where all cysteines but one were systematically replaced by serines, Cys477 was identified as being responsible for the interaction with p300/CBP upon exogenous H₂O₂ treatment [79]. This cysteine is conserved amongst the different FOXO isoforms in humans and mice, as well as in invertebrates such as Drosophila (Fig. 6). Interestingly, Cys612 in FOXO1 (corresponding to Cys477 in FOXO4) was subsequently shown to be necessary and sufficient for full FOXO1 transactivation under unstressed conditions, and required for co-activation by CBP and PGC1α [77,76]. ROS also directly regulate the nuclear import of FOXO4 by mediating heterodimerisation with TNPO1 through an intermolecular disulphide between TNPO1 with Cys239 of FOXO4 [80]. The formation of this disulphide enhances the accumulation of FOXO4 in the nucleus, and intriguingly, this mechanism is conserved in C. elegans [80]. Prx1 was found to undergo disulphide-dependent heterodimerisation with FOXO3a, dependent on Cys31 and Cys150 in FOXO3a [78]. Absence of Cys31 and Cys150 diminishes FOXO3a translocation into the nucleus [78]. To-date, several cysteine residues in FOXO isoforms have been identified to be redox-sensitive (Table 1), however most of these studies were conducted in human cell culture systems combined with exogenous H₂O₂ treatment, and whether insulin-induced H₂O₂ affects redox regulation of FOXO is currently unknown.

6. The insulin signalling pathway including its redox-sensitive cysteines is conserved

The insulin signalling pathway is associated with metabolism and growth, and is highly evolutionarily conserved from invertebrates to mammals (Fig. 5). Indeed, many seminal discoveries in the field of insulin signalling, especially how down-regulation of this pathway is linked to longevity, were initially made in C. elegans and Drosophila, and subsequently translated to mammals [166–168].

The insulin signalling cascade is comprised of several phosphorylation steps that lead to the activation of AKT, which mediates the ultimate downstream events. The initial step is the binding of insulin to its receptor. Whereas worms and flies contain ~40 and 8 different insulin-like ligands, respectively, mammals have only 3: insulin, IGF-1 and IGF-2. In contrast, worms and flies each have only a single orthologue of the IR, while mammals have multiple types of IR (IR-A, IR-B, IGF-1R). Similarly, worms and flies have a more restricted number of IRS and AKT isoforms compared to mammals [169] (Fig. 5).

So far, most research dissecting the role of cysteine-mediated redox regulation within specific proteins involved in metabolism has been conducted in cell culture systems. Analysis of thousands of random PDB and ModBase structures with less than 70% sequence identity between any two proteins showed that cysteines are the least exposed amino acid residue [170]. In addition, it has been estimated that ~80% of all cysteines possess some functional importance due to their extreme pattern of conservation [21], being either highly conserved or highly degenerated [170]. This observation was interpreted as a result of selective pressure to preserve cysteines in functionally relevant positions, and remove cysteines from other positions where they could be detrimental due to their high reactivity. Exposed and isolated cysteine residues might be direct targets for a wide range of oxidants, as they were found to be more reactive than buried and isolated ones (pKₐ 7.4 vs pKₐ 9.5). In conclusion, the high reactivity of cysteines appears to have shaped their extreme conservation pattern compared to other amino acids, and cysteine usage at protein surfaces is limited and avoided unless functionally important [170].

To determine whether insulin signalling orthologues of redox-sensitive targets described in the mammalian system are conserved in invertebrates, we performed protein sequence alignments (Fig. 6). Several cysteine residues shown to be redox-sensitive and important for regulating the activity of key insulin signalling components in mammals are conserved in Drosophila and C. elegans, indicating that similar redox-regulatory mechanisms are likely present.

Given the strong evolutionary conservation of both the insulin signalling pathway and redox-sensitive cysteines within pathway components, model organisms such as C. elegans and Drosophila are excellent systems to study the role of physiological ROS levels and their impact on signalling cascades in vivo. Indeed, flies share many metabolic features with mammals, and develop similar T2D-like phenotypes including hyperglycemia, insulin resistance and obesity when reared on a high sugar diet [171]. In C. elegans, down-regulation of insulin signalling lowers intracellular glucose levels, induces oxidative non-glucose metabolism, which is associated with ROS generation and lifespan extension [172]. In addition, redox proteomic analysis of adult flies revealed that nutritional status dramatically impacts the redox state of cysteines, with fasting inducing a pronounced oxidising shift compared to fed controls [173]. Further studies will be useful to dissect how fasting impacts cellular redox status, and the role played by low molecular weight thiol compounds, such as GSH, in this process.

Compared to mammals, C. elegans and Drosophila have generally fewer isoforms of the respective key players in their insulin signalling pathways (Fig. 5). This lower genetic redundancy facilitates the generation of KO, redox-null, and specific cysteine mutants without potential compensatory effects from other isoforms. In addition to the high conservation of genes identified to cause diseases in humans [174,175], counterparts of organs and tissues fulfilling metabolic functions in humans are also found in Drosophila [176]. Many powerful genetic tools are also available, allowing tissue-specific and spatio-temporal experiments, as well as various strategies to manipulate the antioxidant capacity and redox state of the fly (reviewed in Ref. [177]). Finally, technological advances enabling the identification and manipulation of redox-sensitive cysteines can equally be applied to the C. elegans and Drosophila model systems.

7. Conclusions and future directions

The redox modification of cysteine residues is an efficient modulator of protein function under both physiological and pathophysiological conditions. Evidence obtained from in vitro experiments highlights that H₂O₂ is essential for normal cellular phosphorylation signalling events by influencing key regulatory nodes such as AKT. Studies in humans support the physiological importance of redox signalling in vivo – for instance, antioxidant treatment prevents the ROS-mediated insulin-sensitising benefits of physical exercise [178].

As described, ROS affect insulin action in two ways: i) insulin-induced H₂O₂ formation is essential for mediating the insulin message to downstream targets, and ii) ROS are also involved in the development of insulin resistance and T2D. Therefore, combating insulin resistance via the general manipulation of the redox system is challenging. Several interventions manipulating redox status to improve insulin action have not been as effective as anticipated (reviewed in Ref. [179]). Further work will help define the regulatory components, mechanisms and redox networks, by identifying specific cellular targets susceptible to oxidative modification by insulin-stimulated H₂O₂ in vivo. Overall, improving our understanding of how and when physiological H₂O₂ is...
involved in the regulation of insulin signalling under healthy conditions, and how H\textsubscript{2}O\textsubscript{2} contributes to the progression of metabolic diseases will improve prospects for successful therapeutic interventions.

Declaration of competing interest

The authors declare that there are no competing interests associated with the manuscript.

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