Insulin resistance is a cellular antioxidant defense mechanism

Kyle L. Hoehn1,2,3, Adam B. Salmonb1, Cordulia Hohen-Behrens9, Nigel Turnera, Andrew J. Hoy2, Ghassan J. Maghzaia, Roland Stockerc, Holly Van Remmenb, Edward W. Kraegena, Greg J. Cooneya, Arlan R. Richardsonb, and David E. James1

1Diabetes and Obesity Program, Garvan Institute of Medical Research, 384 Victoria Street, Darlinghurst, NSW 2010, Australia; 2Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900; and 3Centre for Vascular Research, School of Medical Sciences (Pathology) and Bosch Institute, University of Sydney, Sydney, 94 Parramatta Road, Camperdown, NSW 2036, Australia

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We know a great deal about the cellular response to starvation via AMPK, but less is known about the reaction to nutrient excess. Insulin resistance may be an appropriate response to nutrient excess, but the cellular sensors that link these parameters remain poorly defined. In the present study we provide evidence that mitochondrial superoxide production is a common feature of many different models of insulin resistance in adipocytes, myotubes, and mice. In particular, insulin resistance was rapidly reversible upon exposure to agents that act as mitochondrial uncouplers, ETC inhibitors, or mitochondrial superoxide dismutase mimetics. Similar effects were observed with overexpression of mitochondrial MnSOD. Furthermore, acute induction of mitochondrial superoxide production using the complex III antagonist antimycin A caused rapid attenuation of insulin action independently of changes in the canonical PI3K/Akt pathway. These results were validated in vivo in that MnSOD transgenic mice were partially protected against HFD induced insulin resistance and MnSOD+/− mice were glucose intolerant on a standard chow diet. These data place mitochondrial superoxide at the nexus between intracellular metabolism and the control of insulin action potentially defining this as a metabolic sensor of energy excess.

Insulin resistance (IR) leads to chronic hyperglycemia and/or hyperinsulinemia and these effects contribute to the development of hypertension, type 2 diabetes (T2D), kidney disease, and cardiovascular disease. A major difficulty in the management of metabolic disease concerns the modality of treatment. Some clinical studies, such as the ACCORD trial have strived to normalize blood glucose as a top priority by using a range of therapeutic strategies including insulin injections. Surprisingly, this therapeutic approach was unsuccessful (1). One possibility is that overriding IR may have exacerbated intracellular stress by increasing nutrient delivery to an already stressed cell. Consequently, identifying the mechanistic origin of IR remains a major objective as it may aid therapeutic design.

The origin of IR has been difficult to elucidate in part due to the diverse set of risk factors linked to this condition including overnutrition, physical inactivity, pregnancy, Hepatitis C, polycystic ovarian syndrome, HIV protease inhibitor therapy, and antiinflammatory corticosteroids. Do such factors converge at a common intermediate in the insulin action pathway or does IR represent a collection of distinct cellular disorders? For example, endoplasmic reticulum (ER) stress, proinflammatory responses, oxidative stress, intracellular ceramide accumulation, or the activation of JNK, IKK, or PKC are all currently implicated in the development of IR in overnourished or obese rodents (2, 3). In such models, correcting any one of these intracellular stresses is sufficient to improve IR leading to the possibility that these factors are somehow interconnected. One view is that insulin receptor substrate 1 (IRS1) represents a common convergence point for many defects contributing to IR (4). However, this view has been challenged in that the ability of IRS1-independent receptor tyrosine kinases to activate metabolism is also impaired in IR (5). This is consistent with previous studies that have failed to observe defects in upstream elements of the insulin signaling pathway under insulin resistant conditions (6).

In the present study, we took a comprehensive approach to identify factor(s) that might unify multiple models of IR. Initially, we compared four diverse models of IR including chronic treatment with insulin, corticosteroids, proinflammatory cytokines, or lipid in both muscle and adipose cell lines. Using minimal exposure to these insults, we have previously observed IR with no consistent change in upstream elements of the insulin signal transduction pathway (5); however, we have now identified a direct correlation between mitochondrial oxidative stress in all models. This is intriguing because reduced mitochondrial function is associated with IR in the elderly and first degree relatives of type 2 diabetes (2). Insults such as inflammation (e.g., tumor necrosis factor-α, TNF) and antiinflammatory corticosteroids (e.g., dexamethasone) cause IR are also associated with reduced mitochondrial function (7, 8), however intriguingly IR insults, such as hyperinsulinemia are shown to enhance mitochondrial oxidative phosphorylation (9) suggesting that mitochondrial oxidative stress may be a more reliable predictor of IR than mitochondrial function. In addition to showing that mitochondrial superoxide (O2•−) is increased in all four models of IR, we also show that either pharmacologic or genetic strategies that override mitochondrial O2•− reverse or prevent the onset of IR both in vitro and in vivo. Moreover, selective induction of O2•− using the mitochondrial complex III antagonist antimycin A (AntA) rapidly induced IR and we observed an inverse relationship between the expression of mitochondrial superoxide dismutase (MnSOD) and IR in skeletal muscle of intact mice. We propose that mitochondrial O2•− is a unifying element of IR principally acting as a nutrient sensor in key metabolic tissues to regulate nutrient intake in accord with energy oversupply.

Results

Mitochondrial Superoxide Stress Precedes IR. We have previously described a reproducible system for studying IR in myotubes and adipocytes in culture relying on the translocation of the facilitative glucose transporter GLUT4 to the plasma membrane (5). This


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1K.L.H. and A.B.S. contributed equal data
2To whom correspondence may be addressed. E-mail: d.james@garvan.org.au or klh8st@virginia.edu.
3Present address: Department of Pharmacology, University of Virginia, Charlottesville, VA 22908.

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Mitochondrial Superoxide Is Sufficient to Drive IR.

The above findings have been described for growth factor-induced H2O2 production, which mitigates reactive oxygen species (ROS) production (Fig. 2D).

Next we tested the effects of a series of O2•− scavengers on IR. The mitochondria-targeted superoxide dismutase (SOD) mimic MitoTEMPO (2-(2,2,6,6-tetramethyl-piperidin-1-oxyl-4-ylamino)-2-oxoethyl)-TPP) reversed IR in a dose dependent manner whereas the TPP moiety or TEMPOL had no effect (Fig. 2E). Two other mitochondria-penetrating SOD mimetics, manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP, 300 μM) and manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP, 100 μM) (16, 17), also reversed PALM-induced IR (Fig. 2E). Since MnTBAP can substitute for the endogenous mitochondrial MnSOD in vivo (18) we tested this compound against a range of IR models including CI, TNF, and DEX in both 3T3-L1 adipocytes and L6 myotubes and found that MnTBAP reversed all models of IR (Fig. 2 F and G).

These data suggest that mitochondrial O2•− is a common feature across multiple models of IR. To confirm the findings obtained with these drugs and antioxidants we used a more specific genetic strategy by overexpressing MnSOD. Overexpression of MnSOD by 2.5-fold had no significant effect on insulin-stimulated GLUT4 translocation in control L6 cells but overcame the ability of CI, TNF, DEX, and PALM to impair insulin-stimulated GLUT4 translocation (Fig. 2F). In insulin resistant cells but not in control cells, overexpression of MnSOD led to an overshoot in insulin-stimulated GLUT4 translocation (Fig. 2H). One possibility is that these IR models still induce mitochondrial O2•−, however its rapid conversion to hydrogen peroxide (H2O2) by the overexpressed MnSOD may somehow potentiate insulin action. Such a model has been described for growth factor-induced H2O2 production, which inhibits tyrosine phosphatases to enhance signal transduction (19). However, overexpression of MnSOD overcame IR without significantly modulating insulin signaling (Fig. 2I), suggesting that if H2O2 is responsible for the overshoot it must be mediating its effects via an alternate mechanism.

Mitochondrial Superoxide Is Sufficient to Drive IR. The above findings show that many models of IR are associated with increased mitochondrial O2•− production (2–14), which if reversed may restore insulin sensitivity. We next investigated whether the induction of mitochondrial O2•− within the ETC under defined experimental conditions is sufficient to drive IR. Complex III (cytochrome bc1) of the ETC is a well established source of mitochondrial O2•− so we selectively induced O2•− formation with the Complex III Q site inhibitor antimycin A (AntA) (20, 21). Low doses of AntA (50 nM) caused a rapid (30 min) decrease in insulin-stimulated GLUT4 transloca-

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tion in L6 myotubes (Fig. 3A) concomitant with increased O$_2^••$ production from mitochondria (Fig. 3B). To exclude the possibility that partial inhibition of mitochondrial electron transfer rather than O$_2^••$ production explained the effects of AntA, we performed three further experiments. First, stigmastatin, which blocks AntA-induced O$_2^••$ production by inhibiting the transfer of electrons to the AntA-binding site of complex III (21), was shown to prevent AntA-induced IR (Fig. 3C). Second, the mitochondrial SOD mimetics MnTBAP and MitoTEMPO were protective against AntA-induced IR (Fig. 3D). Again, MnSOD overexpression led to supercompensation of insulin-stimulated GLUT4 translocation only in cells treated with an insult (Fig. 3E), similar to the effect seen with other IR models (Fig. 2F). Notably, the inhibitory effect of AntA on insulin-stimulated GLUT4 was not due to inhibition of signal transduction through Akt as evidenced by normal Akt phosphorylation at both maximal (Fig. 3F) and sub-maximal insulin concentrations (Fig. S1) in the presence of AntA.

**Mitochondrial Superoxide Production Regulates Insulin Action in Vivo.** The studies described above provide strong evidence for the involvement of mitochondrial O$_2^••$ production in multiple models of IR in muscle and fat cells in vitro. To confirm these findings in a physiological setting we used three approaches. First, we showed that acute administration of MnTBAP to high fat fed mice caused a significant improvement in glucose tolerance (Fig. 4A and B) principally due to increased peripheral insulin sensitivity in muscle and fat (Fig. 4C and D). Next, we examined the effects of MnSOD overexpression using transgenic (TG) mice expressing this enzyme under the control of the endogenous mouse promoter. The MnSOD-TG mice displayed a 2- to 2.5-fold increase in MnSOD expression in muscle and fat (Fig. S2I and J). Notably, the empty vector control myotubes displayed marked IR under the control of the endogenous mouse promoter. The MnSOD-TG mice displayed a 2- to 2.5-fold increase in MnSOD expression in muscle and fat (Fig. S2I and J). Notably, the empty vector control myotubes displayed marked IR under the control of the endogenous mouse promoter.
Discussion

While a general link between IR and oxidative stress has been proposed (23–25), evidence supporting a specific role for mitochondrial O$_2^{\bullet-}$ in IR in muscle and fat cells is scarce (26). Mitochondrial O$_2^{\bullet-}$ is primarily formed at Complex I (NADH dehydrogenase) or Complex III (cytochrome bc$_1$) of the ETC (11, 14, 21). Since these electron transfer steps are nonenzymatic, any process that increases the reduced state of these electron carriers will increase the probability for electron extraction by high reduction potential molecules, such as molecular oxygen (to create O$_2^{\bullet-}$) (11). Thus, increased mitochondrial oxidative phosphorylation due to increased influx of nutrients in the absence of increased ATP consumption may be expected to increase mitochondrial O$_2^{\bullet-}$ production due to depletion of ADP availability and increased occupancy of electron carriers. Similarly, a reduction in mitochondrial number without a concomitant reduction in nutrient uptake will increase net substrate flux through the remaining mitochondria resulting in increased O$_2^{\bullet-}$ production per energy unit. Additionally, aged mitochondria become damaged and concomitantly produce more mitochondrial ROS per unit mitochondria compared to young mitochondria (27). Thus, mitochondrial O$_2^{\bullet-}$ production may represent the link between mitochondrial function and IR.

Mitochondrial O$_2^{\bullet-}$ has previously been linked to hyperglycemia-induced metabolic dysfunction in endothelial cell systems (28) and in inflammation in adipocytes (29). A major advance of the present study is the observation that mitochondrial O$_2^{\bullet-}$ is upstream of IR in skeletal muscle and adipose tissue. Using a mitochondria-targeted dye that is highly specific for O$_2^{\bullet-}$ we show increased O$_2^{\bullet-}$ production in four separate models of IR, and it has recently been reported that in vitro preparations of muscle mitochondria from mice fed a high diet are more susceptible to ROS production (25). However, in these studies mitochondrial H$_2$O$_2$ rather than O$_2^{\bullet-}$ was proposed as the link to IR. In our study we have overexpressed MnSOD in cell lines and in animals and show that this alone has significant insulin sensitizing properties under various cellular and physiologic stresses. This is intriguing since MnSOD selectively decreases O$_2^{\bullet-}$ levels at the expense of increased H$_2$O$_2$ production and so this supports a role for O$_2^{\bullet-}$ rather than H$_2$O$_2$ in IR. It is also of interest that overexpression of MnSOD in L6 cells had no effect on insulin action in control cells whereas in insulin-treated cells we observed a compensatory effect on insulin action. This indicates that O$_2^{\bullet-}$ production is increased under these conditions and its rapid dismutation to H$_2$O$_2$ may be mediating a positive effect on insulin action (26). It should be noted that longer term exposure of cells to high levels of H$_2$O$_2$ leads to IR (26). However, this occurs via covalent modification of Akt, an effect that we have not observed in more physiological models of IR (5).

There are several features of O$_2^{\bullet-}$ that make it an ideal sensor of cellular nutrient homeostasis, which are not shared by H$_2$O$_2$. Unlike H$_2$O$_2$, O$_2^{\bullet-}$ is membrane impermeable and so its production will be relatively localized and concentrated allowing for reliable detection and restricting its cadre of substrates. Additionally, the chemical spin state of O$_2^{\bullet-}$ makes it relatively target specific such as its known substrates include a brief list of other radicals such as nitric oxide, Fe/S containing proteins, and phosphatases such as PTP1B and PP2B (30). Finally, O$_2^{\bullet-}$ is proximal to H$_2$O$_2$ thus it likely represents a very early sign of cellular stress. O$_2^{\bullet-}$ may act as a conduit to induce a range of cellular preservation pathways directed toward minimizing further O$_2^{\bullet-}$ production. Other known roles for O$_2^{\bullet-}$ include increasing mitochondrial uncoupling and increased expression of antioxidant genes. Based upon the present study, O$_2^{\bullet-}$ also leads to IR, which may represent a third key component of this antioxidant defense mechanism. This hypothesis is exciting for a number of reasons. For example, it suggests that IR may be a protective mechanism, in which case we
should perhaps reconsider using therapeutic strategies to overcome unless they also eliminate the primary defect. Moreover, it suggests that cells have evolved sophisticated mechanisms to not only guard against nutrient lack, such as the AMPK pathway, but also nutrient excess (Fig. 4L). For most organisms the latter is presumably quite rare or at least an intermittent phenomenon emphasizing why the current situation of constant nutrient oversupply is not easily tolerated.

Two significant questions arise from this study. First, how does $O_2^\cdot-$ produced in the mitochondria regulate the translocation of GLUT4 to the plasma membrane? Intriguingly, cells that lack mitochondria are not insulin responsive (31), suggesting that the insulin signal must traverse this organelle as a checkpoint before signaling to GLUT4. Second, is mitochondrial antioxidant therapy likely to be of benefit in the treatment of metabolic disease? One potential problem is that while this strategy may alleviate oxidative damage it may impair hormetic feedback pathways or lead to alternate problems of cellular over nutrition including advanced glycation end products, which are also linked to IR. Thus, care must be taken when considering this type of intervention.

In summary, the fact that mitochondrial $O_2^\cdot-$ is upstream of IR is of major significance suggesting that IR may be part of the antioxidant defense mechanism to protect cells from further oxidative damage. Thus, IR may be viewed as an appropriate response to increased nutrient accumulation as originally suggested by Unger (32), representing part of the cells attempt to return to an energy neutral situation. This concept potentially changes our thinking concerning therapeutic modes of treating metabolic disease.

![Diagram](image-url)
in GLUT4 expression. GLUT4 was normalized to total cellular GLUT4 thus taking into account changes in the concentrations of GLUT4 in the plasma membrane (PM) as described in ref. 5. Models of IR were performed as described above. Chronic insulin (C) treated cells were cultured in serum free DMEM media with 0.2% BSA (stepdown media) and given doses of 10 nM insulin at 12 PM, 4 PM, 8 PM, and 16 PM the following day. In experiments where drugs were added to the IR models the compounds remained on the cells with the insulins throughout the entire duration of the final 6 h, except in the case of G1 where the drugs were washed out during the PBS washes but replaced for the final 90 min. MnSOD cDNA was obtained from JA Melendez (Albany Medical College, NY) and cloned into the retroviral vector pBABE-puro. MnSOD and empty vector control retroviruses were produced in Platinum-E cells and used to infect L6 myoblasts overexpressing HA-GLUT4 driven from a pWZL-derived retrovirus. Cells were stably selected with G418 (400 μg/mL) and puromycin (2 μg/mL) for >3 passages before differentiation and experimentation. TNF and DEX treatment increased cellular GLUT4 levels by 22 and 94%, as described in ref. 5, however in all experiments cell surface GLUT4 was normalized to total cellular GLUT4 thus taking into account changes in GLUT4 expression.

Measurement of Mitochondrial ROS. MitosOX Red was administered essentially as described by the manufacturer (Molecular Probes); however, the two cell lines required different concentrations and incubation times. L6 myotubes were incubated with 5 μM MitosOX Red for the final hour of IR treatment while 3T3-L1 adipocytes were incubated with 1 μM MitosOX Red for the final 30 min of treatment. Cells were cultured in low-absorbance, black-walled 96-well plates. After MitosOX treatment cells were quickly washed with PBS and fluorescence was detected on a Fluo-Star plate reader with excitation at 490 and 495 nm and emission settings at 590 nm. ROS production by isolated mitochondria was measured by monitoring Amplex Red oxidation to Resorufin in the presence of H2O2 (ex/em at 540/590 nm).