Reactive Oxygen Species Play a Causal Role in Multiple Forms of Insulin Resistance

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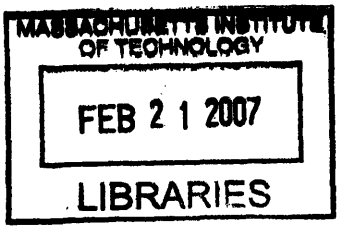

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ABSTRACT

Insulin resistance is a cardinal feature of type 2 diabetes and is characteristic of a wide range of other clinical and experimental settings. Little is known about why insulin resistance occurs in so many contexts. Do the various insults that trigger insulin resistance act through a common mechanism? Or, as has been suggested, do they utilize distinct cellular pathways? Here, we report a genomic analysis of two cellular models of insulin resistance, induced by treatment with tumor necrosis factor-α and dexamethasone. Gene expression analysis suggested that reactive oxygen species (ROS) levels were elevated in both models, and this was confirmed through measures of cellular redox state. ROS have been previously proposed to be involved in insulin resistance, although evidence for a causal role has been scant. To test this hypothesis, six treatments designed to alter ROS levels, including two small molecules and four transgenes, were tested in cell culture; all ameliorated insulin resistance to varying degrees. One treatment was tested in obese, insulin resistant mice and was shown to improve insulin sensitivity and glucose homeostasis. Our results suggest that elevated ROS levels are an important trigger for insulin resistance in multiple settings.

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Chapter 1. Problem Foundations

This thesis is about the molecular mechanisms underlying insulin resistant glucose uptake. As background we describe a conceptual framework for analyzing experimental evidence supporting such a mechanism. We then use it to examine in some detail a handful of current molecular hypotheses (section 1.4). But before we delve into insulin resistance we first step back to ask where it fits into the more general problem of hyperglycemia in the setting of type 2 diabetes (T2D).

In section 1.2 we consider a general question regarding hyperglycemia itself: is it the principal cause of the debilitating consequences of diabetes, namely diabetic complications? While the evidence for a causal role of hyperglycemia is substantial, a close examination reveals the ways in which it is incomplete. Moreover, it sets the stage for an intriguing conclusion about the direct contribution of the underlying pathology of insulin resistance to diabetic complications. We take up this point in Chapter 6.

In section 1.3 we describe a physiological model of glucose metabolism and identify which features change in T2D. This model serves four purposes. First, with respect to the ultimate goal of reversing hyperglycemia, the model demonstrates that insulin mediated glucose uptake is but one of many glucose sources and sinks. Second, a model of glucose homeostasis at the level of organ systems and circulating hormones allows one to recognize architectural features of metabolism. These global features influence hypothesis generation and serve to naturally constrain the degrees of freedom in our experimental choices. Third, consideration of each glucose source and sink in turn establishes a pattern of thinking regarding their regulation. By seeing the regulation of

insulin sensitivity as part of this logical pattern, questions pertaining to mechanism can be posed in a systematic fashion. Fourth, an understanding of the full physiology is critical for interpreting the most stringent tests of mechanism, namely the results of intervention in animals or humans. Since the control of blood glucose is distributed over many organ systems, understanding the forces driving a rise or fall in glucose level requires an appreciation for all of the variables in the physiology model.

We begin this chapter by describing the tragic facts that are the driving force behind our work and that of many others, that is to say the statistics of the growing problem of type 2 diabetes.

1.1 Scope of the problem

Diabetes is a worldwide epidemic[1]. The past two decades have seen the number of cases rise from 30 million to a staggering 230 million worldwide. By 2025 this number is expected to reach 350 million. Greater than 90% of these people have the type 2 form of the disease. India and China now have the largest number of diabetics (35 million and 24 million). In certain Middle Eastern countries such as the United Arab Emirates and Qatar the diabetes prevalence is ~20%. Moreover the number of cases in Africa, the Eastern Mediterranean, the Middle East, and South-east Asia are expected to more than double by 2025.

In 2005 the total prevalence of diabetes in the United States was 21 million people or 7% of the population[2]. It is also the 6th leading cause of death. Having the disease increases one's risk of death twofold over diabetes-free individuals of a similar age.

In addition to the mortality risk, the morbidity burden is overwhelming. Poorly controlled diabetes results in disease of small blood vessels (microvascular) and of large blood vessels (macrovascular), leading to complications that include vision loss, kidney failure, neuropathy and amputation, high blood pressure, pregnancy complications, and cardiovascular disease. In the US diabetes is the leading cause of blindness in adults. It is the leading cause of kidney failure, and it is the leading cause of non-traumatic amputation. Diabetes raises the heart death rate and the risk of stroke by 200 to 400%. And in pregnancy, poorly controlled diabetes is responsible for 5-10% of birth defects and 15-20% of spontaneous abortions[2].

In the US, the cost of diabetes was $132 billion in 2002. It is an unfortunate fact that much of the suffering due to this disease could be alleviated with better diagnosis and modest preventive measures. Roughly one third of individuals with diabetes do not know they have it. Of those at risk, 90% are obese, and modest weight loss (5-7% of body weight) could lower that risk by at least 60%.

As challenging as diabetes is as a health problem, it is also a tremendously difficult research problem. We now know that tight control of blood glucose levels can significantly reduce the risk of diabetic complications[3,4], but we are in need of better therapeutic interventions to routinely achieve euglycemia. To rationally identify such interventions we need to logically connect the perturbation of a molecular target to its successive physiologic consequences and finally to its effect on diabetic complications. But conceiving, studying, and finally validating a disease model in humans is a mammoth task. It is worth appreciating the challenges involved. Retinopathy, nephropathy and other complications take years to develop, necessitating multi-year time frames to study each

intervention. The physiology of diabetes encompasses multiple interacting organ systems, making it a formidable task to untangle their individual roles. A molecular target must be found that when perturbed in isolation is powerful enough to exert an influence on diabetic complications. And lastly, the therapeutic agent has to be safe enough to take for decades. For historical perspective, from 1889, when the function of the endocrine pancreas was discovered, to 1922, when insulin was isolated, to 1999, the year that it was conclusively demonstrated that insulin treatment could prevent T2D complications, 110 years passed.

1.2 Defining type 2 diabetes as a disease of glucose homeostasis

The word diabetes was coined by the Greek physician Aretaeus of Cappadocia. It comes from the greek "diavaino" which literally means "passing through" or "siphon" and reflects the clinical symptoms he first recorded in the 2nd century AD. An excerpt of the original English translation appears below[5]:

"Diabetes is a wonderful affection, not very frequent among men, being a melting down of the flesh and limbs into urine. Its cause is of a cold and humid nature as in dropsy. The course is a common one, namely the kidneys and bladder; for the patients never stop making water, but the flow is incessant, as if from the opening of aqueducts. The nature of the disease then is chronic, and it takes a long period to form, but the patient is short-lived if the constitution of the disease be completely established; for the melting is rapid, the death speedy."

Aretaeus described the symptoms of what today we call type 1 diabetes (T1D). The presenting symptoms include polyuria (frequent urination), polydipsia (constant

thirst), and polyphagia (excessive hunger). Despite nutrient intake the type 1 diabetic's metabolism behaves as if it were starving. The disease most often strikes in childhood and if untreated leads to death in less than a year.

It had also been long observed that the urine of diabetics was sweet on account of its sugar content. This fact was codified by Thomas Willis, a 17th century English physician, when he appended to the name diabetes the word mellitus, from the greek "melli" meaning honey. By the early 19th century biochemical tests for glucose were becoming available, enabling physicians to distinguish the polyuria of diabetes from that of other conditions. This test also set the stage for one of the earliest and most beautiful examples of rational drug discovery.

The cause of T1D was discovered through a triumphant series of logical steps. First a hypothesis was put forward concerning what aspect of the anatomy had changed in diabetics. Autopsies of their bodies sometimes found evidence of a damaged pancreas. Tellingly, individuals with a damaged pancreas always had diabetes. The first causal experiment was performed by Minkowski and Mering: after successfully carrying out total pancreatectomies in dogs they witnessed the development of polyuria. With the use of a biochemical test for glucose Minkowski confirmed that the dogs had developed diabetes. Subsequent work eliminated pancreatic digestive secretions as the glucose lowering substance. The breakthrough causal experiment was performed by Banting and Best in 1921-2 when they successfully isolated a pancreatic extract that was able to keep a pancreatectomized dog alive by preventing its diabetes. Insulin was found in these extracts and by 1923 Eli Lilly was mass producing it. Its impact was sensational: "Those who watched the first starved, sometimes comatose, diabetics receive insulin and return

to life saw one of the genuine miracles of modern medicine. They were present at the closest approach to the resurrection of the body that our secular society can achieve[6].

The biochemical glucose assay was not only a critical tool in the discovery of insulin, but beginning in the early 20th century had become a fairly routine urine test in hospital settings. This revealed quite a number of individuals who were mildly diabetic. Moreover the course of their disease was quite different: in these individuals the thirst and urination often developed gradually and they suffered from chronic conditions such as cataracts, blindness, sever foot disease, and leg infections. This is in contrast to the acute onset of symptoms, the rapid deterioration and quick death observed in the more recognizable group of "juvenile" diabetics. Those with the slower course of the disease had other distinguishing features as well: they were often over 40, wealthy, and overweight.

With the discovery of insulin, Himsworth determined how to classify these two groups of diabetics based on a difference in their pathophysiology. He discovered that injecting the adult onset group with insulin produced a very modest, if any reduction in blood sugar, whereas injection of the juvenile onset group produced hypoglycemia[7-9]. He went on to distinguish between "insulin sensitive" and "insulin in-sensitive" diabetics, hypothesizing that the sensitive group suffered from insulin deficiency while the in-sensitive group resulted from inefficient insulin action. It would take the development of insulin assays and a more rigorous test of insulin sensitivity (the hyperinsulinemic euglycemic clamp) to confirm Himsworth's hypothesis[10]. This culminated in the formal classification of diabetes in 1979[11] into two subtypes, today known as type 1 diabetes and

type 2 diabetes, whose characteristics are remarkably similar to the groups Himsworth characterized some 40 years earlier.

Type 2 diabetes is defined in terms of blood glucose. Specifically, a fasting glucose concentration greater than 126mg/dl, if repeated on two different occasions, leads to a diagnosis of diabetes[2]. There is no reference to the underlying pathophysiology causing the glucose abnormalities. In other words today's diagnostic criteria could just as easily have been used in the early 1800s when the first glucose assays were developed. The criteria are useful because they accurately predict of the development of complications. But are glucose levels a good predictor because hyperglycemia is the cause of complications?

The purpose of considering the historical context of these discoveries is to appreciate how much this history has shaped our view of diabetes[12]. It is worth standing back to ask some key questions. Does hyperglycemia cause the complications of type 2 diabetes? While insulin therapy lowers blood sugar and has been proven to lower complications[4], insulin has numerous metabolic actions in addition to glucose disposal. How are we to know that glucose is the culprit? Consider also that while blood sugar may have been the only recognized feature of T2D in the early 20th century, today many other abnormalities are recognized. Could one of these be the cause of complications? Direct proof will require human testing of an intervention that exclusively targets glucose or prevents the vascular pathology attributable to glucose. Until then the case must be made with indirect evidence from humans and evidence from animal models, some of which we summarize below. First, in both animal models and humans the risk of complications correlates with the degree of chronic hyperglycemia[4]. Second, insulin therapy based

glucose control lowers risk of all complications. Third, an independent agent, metformin, which also lowers blood glucose has been shown to decrease risk of complications[13]. Fourth, the full spectrum of diabetic complications are not known to be part of any disease that does not display hyperglycemia. Fifth, T1D and T2D share few clinical features other than hyperglycemia, yet poor glucose control in either case results in the same characteristic complications[14].

Even if hyperglycemia per se explains only a component of the risk of diabetic complications they must at least have a common cause. Consequently understanding the pathophysiology of hyperglycemia is an important goal and will be the subject of what follows.

1.3 Glucose accounting: sources and sinks

The goal of this section is to develop a systematic way of thinking about whole body glucose homeostasis. To understand how blood glucose levels are set we ask the following accounting question: what are the sources of glucose and what are its sinks? Knowledge of the molecular parameters that govern source and sink output together with data on the state of these parameters should be sufficient to predict glucose levels. In general terms, control of glucose production and consumption by individual tissues is a function of circulating regulatory factors, circulating metabolic substrates, and intracellular pathways of substrate transport and metabolism. For each source and sink, we describe specific examples of these parameters and outline the functional organization of glucose processing. The level of detail is meant to be deep enough to capture salient features and to reveal a structured approach to the problem. In the case of insulin

stimulated glucose disposal we take up an in depth investigation of the individual molecules involved (section 1.4). Finally, we describe the specific changes to source and sink output that lead to hyperglycemia in type 2 diabetes.

The overall architecture of glucose homeostasis is a useful context within which to think about the details of source and sink function. At least two key design goals are discernible. As glucose is an essential nutrient for all cells, the first goal is to sustain a continuous supply of blood glucose in the face of daily fluctuations in consumption. The second goal is to be able to preferentially divert available glucose to tissues like the brain or the fetus in conditions such as starvation or pregnancy. These goals are achieved via several processes: glucose synthesis and storage, regulated cellular consumption, and glucose shunting. They work together to withstand perturbations in glucose needs and supplies that arise in different physiologic or disease states such as exercise, fasting, eating, pregnancy, T2D, and sepsis, to name a few. Each of these states calls for its own balance of source and sink activity.

In a given physiologic state the balance of glucose production and consumption is orchestrated by the specific complement of factors circulating in the blood. While our ultimate aim is to recognize the factors associated with any possible state, the current state of knowledge is best developed for the states of feeding and fasting in the context of the healthy or T2D individual. The fed and fasted states are two complementary stages of an idealized response to a meal. The fed state refers to the acute physiologic changes set in motion by an ingested glucose load. In particular, glucose is absorbed into the blood, whereupon it is sensed by the beta cells of the pancreas, which in turn release insulin into the circulation. The role of insulin is to shut off all endogenous glucose sources and in

synchrony amplify certain glucose sinks. Tissues take up glucose and use it for fuel or set it aside for storage. The fasting state refers to reciprocal metabolic adjustments made once the glucose load has been absorbed. Insulin levels fall and additional hormones such as glucagon and epinephrine appear, leading to the activation of glucose sources and a reduction in consumption by insulin sensitive glucose sinks. Maintaining constant glucose levels in the fasted state is particularly important for tissues that use it as their primary fuel source: important examples include the brain and red blood cells, both of which take up glucose constitutively and are unregulated by insulin. In summary, we have lumped normal metabolism into two states and noted that circulating factors such as insulin are used to orchestrate the switch.

In what follows we use a uniform pattern in relating the functional organization of each glucose source and sink: identify the circulating substrates, identify the intracellular factors that control substrate transport and processing, and then describe how circulating regulatory factors modulate the intracellular pathways. This information is known in some detail for a small number of physiologic states like feeding and fasting. It is incomplete in so much as it leads to a telescoping series of questions about how substrate supply or the levels of these regulatory factors are in turn controlled. But for our purposes, it will suffice to sketch enough proximal mechanisms to allow the predictable control, either experimentally or therapeutically, of source and sink function.

1.3.1 Glucose sources

Glucose sources fall into three classes: food intake, storage release, and *de novo* synthesis. The latter two sources serve one primary purpose: to maintain blood glucose

levels in the fasted state. The storage form of glucose is known as glycogen. Many tissues have a small cache of it available to satisfy acute disruptions in their glucose supply. If the disruptions are prolonged, large glycogen stores harbored by the liver are released into the circulation. During the first 22 hours of fasting, liver glycogenolysis (glycogen to glucose conversion) contributes between 34 and 50% of glucose released[15]. The remaining glucose need is met by *de novo* synthesis, known as gluconeogenesis. After 24 hours this source accounts for virtually all of the blood glucose supply, 80% of which is contributed by the liver with the rest coming from the kidneys[16]. The importance of these sources is exemplified by the small supply of glycogen in the brain – without blood glucose it would run out in minutes [17]. In the fed state there is no need for glucose from endogenous sources so insulin acts to suppress both glycogenolysis and gluconeogenesis.

What are the cellular determinants of gluconeogenic output? Glucose is synthesized from a set of substrates that includes amino acids, lactate, and glycerol, with the reactions catalyzed by a set of enzymes, important examples being PEPCK, fructose-1,6-bisphosphatase, and glucose-6-phosphatase[18]. Total flux through gluconeogenesis is a function of intracellular substrate levels and the activity of the gluconeogenic pathway. Intracellular substrate supply is in turn determined by the amount of substrate in the blood and transport to the inside of the cell. The activity of the gluconeogenic pathway is controlled by circulating levels of factors such as insulin, glucagon, adrenaline, and cortisol. In the fed state, insulin, through receptors on the hepatocyte plasma membrane, acutely blocks gluconeogenesis. Its direct effect is to inhibit the expression of the key liver gluconeogenic enzyme PEPCK, while its indirect effects include the suppression of glucagon release from the pancreas, lowering blood levels of glycerol by inhibiting fat

cell lipolysis, and lowering blood levels of amino acids by directing their incorporation into muscle protein[19]. Conversely, in the fasted state glucagon acutely activates gluconeogenesis by leading to the phosphorylation and activation of fructose-2,6-bisphosphatase which in turn activates fructose-1,6-bisphosphatase by lowering the levels of its allosteric inhibitor fructose-2,6-bisphosphate. Prolonged fasting is referred to as starvation. In such conditions cortisol is released, causing the transcriptional upregulation of the gluconeogenic enzymes glucose-6-phosphatase and PEPCK.

What determines the amount of blood glucose contributed by liver glycogen breakdown? Glucose production from glycogen is a function of the liver's intracellular glycogen stores and the activity of the enzymes used to break it down. Glycogen is a polymer formed from glucose, the supplies of which total 100g in the adult liver[18]. The enzymes of glycogenolysis include glycogen phosphorylase, phosphoglucomutase, and glucose-6-phosphatase. Glucagon, released in the fasting state, regulates this pathway by activating glycogen phosphorylase kinase, which in turn phosphorylates and activates glycogen phosphorylase. Conversely, in the fed state, insulin stimulates a phosphatase to deactivate glycogen phosphorylase.

The last glucose source is perhaps the most basic: food intake. Ironically, it is the least understood, being influenced by many factors, a review of which is beyond the scope of this exposition. But briefly, the contribution of an ingested load to blood glucose is a function of its size and its absorption in the intestine. Glucose is absorbed in the small intestine through the SGLT1 transporter. For complex carbohydrates to be absorbed they must first be digested to glucose by alpha-amylase and alpha-glucoside hydrolases. These proteins are therapeutic targets to be discussed in more detail shortly. Owing to the

central role of insulin, we mention some interesting facts concerning its ability to regulate food intake. One of the characteristic symptoms of type 1 diabetes is hunger that persists despite polyphagia. This suggests that insulin or one of its downstream effects signals satiety. It turns out, quite surprisingly, that there is a direct effect of insulin on the brain, as demonstrated by a compelling genetic experiment. When brain insulin receptors were knocked out in mice, they responded by increasing their food intake and subsequently their weight[20]. We will conveniently sidestep the challenges of predicting the overall magnitude of this glucose source, opting instead to imagine it being of fixed size (in clinical diagnostic applications, it is 75g of glucose).

1.3.2 Glucose sinks

We now turn our attention to glucose sinks, of which there are two classes: excretion in the urine and cellular consumption. In the normal individual excretion is not an appreciable sink as the kidneys reabsorb all but 0.1% of the glucose they filter. Glucose disposal can be broken down by organ: in the fasting state brain accounts for 60%, skeletal muscle 17%, the heart 11%, and the rest of the tissues 12%[21]. In the fed state the breakdown shifts: of the total glucose consumed as much as 95% is accounted for by skeletal muscle[22]. This shift occurs due to feeding induced insulin release which amplifies glucose disposal into skeletal muscle and adipose tissue ~15 fold[21]. The brain continues to consume glucose at the same rate as during the fasting state – the percentage shifts because the large meal-derived glucose bolus is almost entirely disposed of into muscle.

The rate of glucose consumption is in principle determined by two factors: the regulated entry of glucose into cells (the "push") and the rate of its utilization by distinct biochemical pathways (the "pull"). Glucose enters most cells by passive diffusion down its concentration gradient through specialized channels from the GLUT family; the exception is the intestine and kidneys where it is actively transported against a gradient via transporters in the SGLT family. The regulation of transport rates is best understood in muscle and fat cells. It is further classified as non-stimulated (basal) or stimulated. Basal glucose transport in muscle and fat is mediated by the constitutive presence of Glut1 on the plasma membrane. Stimulated glucose transport in muscle can result from either insulin action, muscle contraction[23], or hypoxia[24], and works by recruiting the activity of a second glucose transporter, Glut4. In fat cells insulin is the only known stimulus. The particular signaling events that mediate insulin action will be discussed in detail in section 1.4. Regarding the mechanism by which hypoxia and contraction augment glucose transport, little is known outside of the fact they use signaling pathways distinct from insulin and may involve the gene AMPK[25]. Thus to predict the rate of glucose transport into a given sink assuming a fixed concentration gradient, one must know the levels of basal uptake transporters like Glut1, the state of transport stimuli such as insulin, and the sensitivity of the Glut4 response to these stimuli.

The utilization side of glucose disposal is subdivided into three principal cellular processes: glycogen synthesis, fuel metabolism (ATP generation), and NADPH synthesis. Insulin boosts the capacity of all of them to metabolize glucose. In the case of glycogen synthesis it mediates activation of the enzyme glycogen synthase by phosphorylating glycogen synthase kinase[18]. The generation of ATP is split between glycolysis and

oxidative phosphorylation. Insulin is thought to activate glycolysis by promoting the synthesis of fructose-2,6-bisphosphate, an allosteric activator of phosphofructokinase 1[26,27], and oxidative phorphorylation by increasing mitochondrial protein synthesis as well as post-translational activation of TCA cycle enzymes[28]. Finally, to increase NADPH synthesis insulin increases the expression of glucose-6-phosphate dehydrogenase[29] and acutely promotes NADPH consumption (by stimulating fatty acid synthesis), which has the effect of drawing in more glucose to replenish it[30]. Through careful isotope studies it has been determined that in the fed state glycogen formation is the chief subcellular sink in muscle cells[31], accounting for 90% of glucose flux[32]. Moreover it appears that glucose transport, as opposed to utilization, is the rate-limiting step in this tissue. That said, it is formally possible to imagine disposal rates limited by utilization, were it sufficiently depressed in some disease state. In summary, predicting the contribution of glucose sinks to blood glucose involves calculating transport rates into a given sink (as described earlier) and knowing the capacities of the different utilization pathways present in that sink, be it their basal capacity or as it is affected by the circulating milieu of the fed or fasted state.

Why might metabolism designate some tissues to be insulin dependent and others to be independent? One could imagine a metabolism in which muscle and fat take up the glucose they need without a specific signal, like all other tissues. Why use an ON switch? One plausible explanation is that it provides the flexibility to turn these sinks OFF. Earlier in the discussion we commented on the need to prioritize sinks according to their dependence on glucose, with the brain topping the list. During the fasting state and especially during starvation it is necessary to actively prevent muscle from taking up

glucose that the brain needs. This is a reasonable trade in light of muscle's ability to use alternative fuels. Though insulin levels fall during fasting, it may not be possible to lower them enough to achieve an adequate balance, as insulin is required for many other functions. To solve this problem there are other circulating factors that actively repress insulin stimulated glucose disposal. Epinepherine and cortisol are prime examples. Such a state of diminished insulin action is known as insulin resistance, and in conditions like starvation it can be viewed as a glucose shunt.

We have now itemized the individual sources and sinks, described what happens to them during states such as feeding and fasting, outlined how this change comes about as a result of circulating factors and their influence on intracellular circuitry, and tried to infer why they behave the way they do. Needless to say, the qualitative information we have provided is inadequate for quantitative predictions of glucose dynamics. Moreover we have left out the regulatory influences of many other circulating factors. On the other hand we have included enough detail to establish a pattern of logical synthesis that is scalable - it should be able to accommodate the inevitable escalation of variables that must be considered. The next step is to describe how source and sink behavior changes in type 2 diabetes.

1.3.3 How do source and sink behaviors change in type 2 diabetes?

The chronic hyperglycemia of T2D is at its roots the result of increased source output and decreased sink disposal. This leads to higher glucose excursions in the fed state as well as higher fasting glucose levels. The major contributors to the rise in glucose are increased liver gluconeogenesis, decreased glucose disposal in muscle, and elevated

food intake. The contribution to hyperglycemia during fasting is accounted for by a rate of liver gluconeogenesis that is ~90% greater than normal[33]. After a meal, the hyperglycemic response is due in large part to the reduced action of insulin, both as a consequence of insulin resistance and its inadequate secretion. Insulin fails to fully restrain gluconeogenesis, in what amounts to a 50% defect in its repressive action[34]. Its ability to stimulate muscle glucose transport is depressed by 50%[32], and on average 70% less insulin is secreted per unit of glucose sensed by the beta cells[35]. Though diabetics are often overnourished and obese, paradoxically in the fed state their metabolism resembles starvation! The remaining possible contributors to hyperglycemia include glycogenolysis (source) and non-insulin mediated glucose disposal (sinks), which in turn can be separated into basal and contraction induced glucose disposal. None of these appear to be different in T2D[34,36-38].

The model of physiology together with knowledge of its disturbances in T2D allows us to make sense of how current therapies work as well as to recognize the special challenges to developing new ones. It is a remarkable fact that many current agents used in T2D were discovered serendipitously. These include metformin, sulfonylureas, and thiazolidinediones (TZDs). Encouragingly, they modulate the sources and sinks from our model in predictable ways. Metformin decreases gluconeogenesis[39], sulfonylureas stimulate insulin release[40], and TZDs enhance insulin stimulated glucose transport as well as basal glucose transport[41,42].

While these agents attempt to correct an underlying defect in T2D, there are other agents which augment the normal function of certain glucose sinks or depress the normal function of glucose sources. Acarbose works by blocking intestinal breakdown of

disaccharides, a step needed for glucose absorption. SGLT2 is the glucose transporter that the kidneys use to reabsorb filtered glucose. Interestingly there exist natural product inhibitors of SGLT2, a long studied example of which is phlorizin[43]. Moreover pharmaceutical companies are developing additional members of this class for therapeutic purposes[44]. The use of such inhibitors creates a new glucose sink: urinary excretion. Finally TZDs increase insulin sensitivity, but they also increase basal glucose transport, augmenting an existing glucose sink which is unaffected by T2D.

What is surprising to appreciate is that one of the oldest drugs has the potential to cure hyperglycemia. That drug is insulin. While diabetics may not be to control their glucose levels by making enough endogenous insulin, they can always do so with a large enough injection of exogenous insulin. Unfortunately there is a very serious drawback. If blood sugar falls too low as a result of an inappropriately high insulin dose, the brain could starve, leading to diabetic coma and death. This danger will exist with any agent that ignores or attempts to bypass the endogenous, self-regulating glucose control system.

What kind of therapeutics might be able to circumvent the risk of serious hypoglycemia? At least three possibilities can be imagined: an insulin delivery device that mimics the function of the beta-cell by carefully titrating insulin release, an agent which increases the capacity of the beta-cell to release insulin without itself inducing release, and finally an agent that improves the peripheral sensitivity to insulin. Beta-cell transplants are an example of a cell therapy that follows the first approach, although they are still in the research phase. Yet another serendipitous discovery has lead to the development of the first agent employing the second approach: GLP1 is a hormone that sensitizes beta-cells to the stimulatory effect of glucose. Agents that mimic GLP1, or

increase endogenous GLP1 levels by blocking its degradation lead to greater insulin release and tighter glucose control, with minimal risks of hypoglycemia. TZDs are the only validated agents in the third class. However, they have the unfortunate side effect of causing fluid retention by activating PPARgamma in the kidney[45]. Many diabetics have heart disease for whom fluid retention is a dangerous risk, thus limiting the use of this class of drugs.

Parametrizing glucose metabolism in terms of tissues and circulating factors results in a useful tool for studying and eventually treating diabetes. It naturally partitions the physiology into sub-problems, allowing them to be studied independently and on a cellular scale. Such a breakdown affords the possibility of taking any environmental or genetic perturbation and predicting its net effect on blood glucose from an understanding of how individual parameters are affected.

Another important use of a parameterized physiology is for diagnostic applications. Here the problem is that despite the existence of techniques to measure circulating factors and individual tissue contributions to blood glucose, there are important clinical settings where their use is impractical. For example, in large epidemiologic studies or large clinical trials it would be desirable to know how much glucose the liver is producing or how much glucose is being consumed by muscle in response to insulin. However, in this setting the only measurements that are practical to make might be of blood glucose and insulin. To assess the underlying metabolic lesions requires reasoning in the opposite direction: infer the behavior of the individual tissues and regulatory factors solely from a glucose and insulin level. This situation also arises when interpreting experimental data from interventions in human or mouse models of

diabetes. In all of these settings an understanding of the major glucose sources and sinks is necessary to be able to list the combinations of responses that could have given rise to the observed data.

We have made an effort to describe the whole problem of glucose metabolism, but for the rest of this thesis will focus on one specific aspect of it: insulin stimulated glucose uptake. While we cannot say with certainty how much of diabetes would be eliminated by improving insulin resistance, there is evidence to suggest it would be a contribution of some consequence. Moreover, it turns out that there are multiple diseases in which insulin resistance arises. Consequently its successful treatment may be of significant value in conditions outside of type 2 diabetes. We devote section 1.4 of this chapter to a reductionist account of this diabetes sub-problem. In subsequent chapters we present the results of our studies.

1.4 Resistance to Insulin Stimulated Glucose Uptake

The purpose of this section is to assess the evidence supporting certain pathways in the etiology of insulin resistant glucose uptake. To accomplish this we first review the state of knowledge concerning the normal sequence of molecular events by which insulin stimulates glucose transport. The next step is to enumerate the different types of mechanisms that could in principle subvert this process. It would be useful to have a framework in which to weigh experimental data pertaining to a given mechanism. So a second goal of this section is to clearly articulate a set of criteria that would constitute a transparent basis for the interpretation of such data. The first mechanism of insulin resistance we evaluate involves the genes IRS1 and 2. Observations from this analysis are

used to elaborate in more detail on our choice of criteria. Finally, these criteria are applied to the evidence supporting a set of prominent mechanisms thought to cause resistance to insulin stimulated glucose uptake.

1.4.1 Insulin Signal Transduction

Insulin stimulates glucose uptake by initiating a sequence of rapid molecular signaling events. This signaling pathway culminates in the translocation of Glut4 from intracellular locations to the cell surface, a process that occurs in minutes[46]. A striking visual demonstration of this appears in Fig.2 in which adipocytes harboring a Glut4-EGFP fusion have been treated with insulin[47]. Adipose and muscle are the only tissues that support insulin mediated glucose uptake (IMGU), and few if any known differences in insulin signaling exist between them. We therefore describe the signaling events without reference to cell type. Is the insulin receptor required for IMGU, or could there be other receptors mediating this effect? In fact it is required, as insulin receptor knockout cells, be they myocytes or adipocytes, do not support IMGU[48,49]. In the case of Glut4 knockout cells, there is still some residual IMGU in both adipocytes and myocytes, although it is drastically reduced in both cases[50,51]

The following is a short description of what are widely held to be the proximal signal transduction events triggered by insulin binding to its receptor[52](see Fig.3): **1.** insulin receptor (IR) stimulation leads to activation of its kinase domain and the subsequent tyrosine phosphorylation of several scaffold proteins including IRS1-4, Cbl, and APS (among others). **2.** these phosphorylated scaffolds then dock with proteins harboring SH2 domains and propagate the insulin signal through mechanisms including

allosteric activation and subcellular localization; tyrosine phosphorylated IRS1 and 2 in particular mediate the recruitment of the p85 subunit of PI3Kinase which then leads to allosteric activation of its catalytic subunit, p110. **3.** increased PI3Kinase activity leads to the phosphorylation a family of membrane lipids known as myoinositols, in particular phosphatidylinositol 4,5-bisophosphate (PtdIns(4,5)P2), generating PtdIns(3,4,5)P3. **4.** membrane PtdIns(3,4,5)P3 recruits two kinases, Akt2 and PDK1 by binding to their pleckstrin homology domains whereupon PDK1 phosphorylates Akt2 at Thr308. **5.** PDK2, now known to be the mTOR/Rictor complex[53], phosphorylates Akt2 at Ser473 leading to its activation.

Accumulating evidence supports a role for several additional proteins in the insulin to Glut4 axis. Akt2 is thought to communicate with Glut4 in part through phosphorylation of a protein known as AS160. AS160 possesses a Rab GTP-ase activating domain, which makes it an interesting candidate for regulating Glut4 translocation given the well known role of Rab proteins in vesicle trafficking. There is also important work addressing events proximal to Glut4 translocation, in particular the role of TUG[54], as well as the identification of a second signaling pathway originating at the insulin receptor involving the proteins CAP and c-Cbl[55]. It is entirely unclear how complete our knowledge of the insulin signaling pathway really is. Does Akt2 lie three steps away from Glut4, or thirty? Mass spectrometry based proteomics has recently made possible an eye opening experiment. At least 89 different adipocyte proteins were identified as being tyrosine phosphorylated in response to insulin stimulation[56]. Any of them could in principle be part of the insulin to Glut4 circuit.

1.4.2 Insulin resistance

Defects in insulin mediated glucose uptake can be thought of as arising from one or more lesions in the insulin signaling cascade. How do insulin resistant individuals acquire these signaling defects? One possibility is to think of them as being inherited: e.g. loss of function alleles of the signaling genes could constitute the genetic basis of the disease. To date, however, no common polymorphisms associated with insulin resistance have been found in any member of the canonical insulin pathway. A second possibility is that one or more signaling proteins, perhaps even the entire cascade, are under the control of an explicit regulatory pathway. Perturbations of this regulation, either by genetic or environmental means, could then lead to insulin resistance. Consider the gene harboring the only known polymorphism which increases risk of diabetes and has clear links to insulin resistance, PPARgamma[57]. PPARgamma itself is not a signaling protein in the insulin to Glut4 cascade[58], but is in fact a transcription factor. When activated by the thiazolidinedione class of drugs it somehow increases insulin sensitivity. For an example of an environmental perturbation consider glucocorticoid administration: when given to healthy individuals it causes insulin resistance. The glucocorticoid receptor (GR) is not part of the insulin to Glut4 cascade either – it is also a transcription factor. How do PPARgamma or GR influence insulin signaling? As both of these factors exert their effects on chronic time scales one could imagine the process involves a lengthy series of steps following the first wave of transcription. Eventually this could manifest itself as changes in protein levels of signaling genes, the expression of novel regulatory kinases, or even as post-translational modifications of signaling proteins that occur with slow

kinetics. Arriving at the causal basis of insulin resistance will require the elucidation of these regulatory cascades as well as the insulin signaling cascade.

1.4.3 Framework for evaluating evidence: application to the IRS hypothesis

We believe there are at least five considerations that are useful for gauging the experimental evidence that a particular factor is a cause of insulin resistant glucose uptake. (1) Is the state of the factor different in insulin resistance as compared to insulin sensitivity and is this change measurable in cells that support IMGU such as muscle or fat? (2) Does the factor have a causal effect i.e. manipulating it either leads to the induction of insulin resistant glucose uptake or it leads to restoration of IMGU in a setting of insulin resistance? (3) Does the manipulation employed directly implicate a cellular mechanism of action, such as when performed in isolated cells or when it involves tissue specific genetics, or could indirect effects also account for the data, as might happen with whole body genetic perturbations? (4) Are the effects of the manipulation measured on insulin mediated glucose uptake or are surrogate markers used, such as insulin signaling events? (5) Are there any confounding effects of the manipulation, and are there additional observations from related manipulations that are difficult to reconcile?

The role of IRS1 and 2 in insulin resistance has been heavily studied, resulting in a rich body of data addressing each of the five points elaborated above. First of all, the state of IRS proteins changes in type 2 diabetes. At least two different modifications have been observed: decreased protein levels of IRS1/2 and increased serine phosphorylation

of IRS1. Moreover insulin resistant conditions other than diabetes show similar state changes[59],[60]. These genes are thus candidates for causal involvement.

Two classes of questions can be asked about the causal nature of these state changes. The first asks whether they are sufficient to cause insulin resistance on their own. If so, one should be careful about concluding too much. For example, it might be tempting to propose the following: if mice with a deletion of IRS1 are insulin resistant, and IRS1 expression decreases in diabetic muscle, then loss of IRS1 expression in diabetic muscle must be a cause of diabetes induced insulin resistance. To draw this conclusion, however, is to implicitly suggest that increasing the levels of IRS1 would restore insulin sensitivity. But it could be that in the diabetic cell multiple signaling deficits exist such that reversing any single deficit may have no restorative effect on IMGU. Observations supporting sufficiency really only address the effect of IRS function on the state of the healthy cell. The second type of question addresses necessity: are deficits in IRS signaling necessary to cause insulin resistance within the cellular context of diabetes? This question is often rephrased as: would restoring IRS signaling in diabetes ameliorate insulin resistance? Since the disease context is made explicit in this question there are no implicit assumptions. One final comment: many features of the cellular state do not change in diabetes – that doesn't imply they can have no causal influence. However, they are not considered here as there is no obvious way to pick out such causal factors from the background of non-casual factors.

What are the data supporting a causal role for changes in protein levels of IRS1 and 2? *In vivo*, both IRS1 null mice and IRS2 null mice display whole-body insulin resistance[61,62]. This is strong evidence that a depression in IRS1/2 levels is sufficient to

have a causal impact. On the other hand, to our knowledge there is no evidence that restoration of IRS1/2 protein levels improves insulin sensitivity in obesity or diabetes. Some data exist to the contrary: in 3T3-L1 adipocyte cell culture, glucocorticoid treatment both decreases IRS1 expression and causes insulin resistance. Yet when IRS1 is overexpressed in these cells, they show no improvement in insulin stimulated glucose uptake[63]. The implications of the mouse knockout data for human insulin resistance or obesity mouse models are also weakened by the observation that in these conditions protein levels of IRS1 and IRS2 from muscle are reduced by only ~50%[64-66] or not at all[67-72], depending on the study. IRS1 and IRS2 heterozygote mice display a 50% drop in their respective protein levels, yet are phenotypically normal. So the state changes created in the null mice may be too severe to fairly model the diabetic condition. Another possibility is that when modest deficits in both IRS1 and IRS2 are combined a more severe phenotype results – however, data from mice that are IRS1/2 double heterozygotes provide little support for this hypothesis[73] (at least not on a genetic background of insulin receptor haploinsufficiency). The IRS knockout phenotypes no doubt illustrate important functions of IRS1/2, but they may not be an accurate model of insulin resistant conditions such as obesity or diabetes.

Although knockout of the IRS1/2 genes causes whole body insulin resistant glucose uptake, from this observation it is not possible to deduce their effect on cellular insulin sensitivity. An attractive hypothesis is that the knockout phenotype is due to a cell-autonomous effect on the insulin signaling pathway. However given that the IRS genes were knocked out in every cell of the animal, it is conceivable that some pathological response in a tissue other than muscle indirectly leads to muscle insulin

resistance. Data gathered in various cell culture systems shed light on this issue. Isolated IRS2-/- muscle cells mediate IMGU just as sensitively as wildtype cells[74]. Furthermore RNAi mediated inhibition of IRS2 had no effect on IMGU in L6 muscle cells[75]. In contrast, when IRS1 protein levels were lowered by >80% with RNAi in cultures of primary muscle cells they developed a severe reduction in IMGU[76]. Moreover, primary IRS1 null adipocytes display a 50% defect in IMGU[77]. However, this latter result has to be qualified due to a confounding effect: differentiation of IRS1 null cells from preadipocytes (which do not support IMGU) to adipocytes is inhibited by 60%[78]. The case of IRS2 illustrates the potential danger of translating a whole body phenotype to a cellular phenotype. On the other hand IRS1 is capable of generating cellular insulin resistance if sufficiently inhibited, but in some cases this may be on account of developmental effects.

An alternative mechanism by which IRS1 might cause insulin resistance is through changes in its serine phosphorylation state. Multiple serine phosphorylated residues are observed in IRS1 from muscle and adipose tissue in both human diabetics and mouse models of obesity[67,79]. We focus on the role of serine 307 as it is the best studied. An attractive but arduous strategy for testing its causal role would be to create transgenic mice with a knock-in mutation at that site and evaluate their susceptibility to diet induced insulin resistance. Instead cell culture experiments of a similar spirit were performed. 32D cells, a myeloid cell line that lacks endogenous IRS1 or insulin receptor, were simultaneously transfected with IRS1 mutants and the insulin receptor. Insulin sensitivity in these cells was then quantified as the extent of insulin stimulated IRS1 tyrosine phosphorylation. Insulin resistance was modeled by TNF-alpha (TNF) treatment,

which both impeded insulin stimulated IRS1 tyrosine phosphorylation (IRS-pY) and induced phosphorylation of serine 307. Mutating this residue to alanine prevented TNF's effect on insulin stimulated IRS-pY, demonstrating its causal role[80,81]. This system is a clever way to isolate and study IRS1 biochemistry. However, 32D cells differ significantly from muscle or fat cells: they grow in suspension and do not support many of the characteristic metabolic actions of insulin, in particular IMGU. It is therefore difficult to extrapolate from these studies what effect these mutations would have in muscle or fat, and as yet there are no reported results that resolve the issue. An important question is highlighted by these data: to what extent can the measurement of proximal signaling events in the canonical pathway be used as a surrogate for insulin mediated glucose uptake? On the other hand the signaling could be regarded as important in its own right.

An assessment of the IRS1 hypothesis would not be complete without an evaluation of confounding or conflicting data. An alternative explanation for the role for serine phosphorylated IRS1 is that it is an *in vivo* marker of insulin resistance. In addition to the many proteins that become tyrosine phosphorylated in response to insulin[56], several proteins are serine phosphorylated in response to insulin. In particular, insulin receptor signaling leads to IRS1 serine phosphoryation (IRS1-pS)[82]. Since insulin resistant individuals are very frequently hyperinsulinemic, serine phosphorylated IRS1 could be a reporter of hyperinsulinemia and thus a marker of insulin resistance[67]. To maintain that it is a cause and not just a marker implies that it is involved in a negative feedback circuit. Insulin mediated insulin resistance is known to be possible, so IRS1-pS could be a component of this mechanism. This question will be resolved when the effect of mutant

IRS1 proteins can be assessed in the appropriate cell type and in the appropriate model of insulin resistance. Finally, there are some conflicting pharmacologic observations: the drug rapamycin has been found to block the ability of insulin to stimulate IRS1 serine phosphorylation[83]. If IRS1-pS is part of a mechanism of feedback inhibition then rapamycin would be predicted to promote insulin sensitivity, but rapamycin administration to both mice and cells in fact causes insulin resistance[84,85].

Having discussed many aspects of the IRS1/2 hypothesis in the five-point framework outlined previously, we now expand further on the criteria of whole body vs. cellular insulin sensitivity, insulin signaling vs. glucose uptake, and confounding factors.

The value of distinguishing between whole body and cellular insulin resistance is further illuminated by considering some examples of non cell-autonomous processes on muscle. It is a remarkable fact that there are more examples of interventions in non-muscle tissues that exert an influence on muscle IMGU than there are muscle specific interventions with this effect. The liver best exemplifies this phenomenon: knockout of the insulin receptor[86], induction of ER stress[87], knockout of the IGF1 receptor[88], or expression of constitutively active IKK-beta[89], are just a few examples of liver specific interventions that affect muscle insulin sensitivity. Examples of adipose tissue specific interventions that also influence muscle include leptin knockout[90], adiponectin overexpression[91], and Glut4 knockout[50]. Interventions in myeloid cells are a recent addition to the list of non cell-autonomous mechanisms regulating muscle insulin sensitivity, an example being IKK-beta knockout[92].

In vivo data from muscle specific genetic interventions go a long way toward enhancing our understanding of the intracellular regulation of insulin sensitivity.

However even this kind of data present challenges to interpretation: compensatory changes in whole body physiology can still occur, leading to indirect effects on the tissue of interest by means of circulating factors[93]. Experiments with isolated cells allow controlled perturbations of the physiology because the extracellular environment can be kept constant. The challenge in this case is to accurately model that environment. Thus causal evidence from both types of systems has characteristic utilities in the understanding of cellular insulin sensitivity.

It is worth noting the multitude of examples demonstrating the dissociation between the insulin sensitivity of glucose uptake and that of proximal signaling events. Such examples lead to a more nuanced view of how to interpret the causal role of individual signaling events. This dissociation occurs in two ways: a treatment induced improvement in glucose uptake displaying no improvement in signaling, or a treatment induced decrease in glucose uptake despite normal signaling. Two examples of the first type include exercise or thiazolidinedione treatment of diabetic humans or mice, which uniformly improve insulin stimulated glucose uptake but display either no improvement in insulin stimulated IRS1-pY, Akt-pS/T, PI3K activation, or AS160-pS[94-96] or improvement in only a subset of this pathway[97,98]. Multiple examples of the second type also exist: insulin resistance due to obesity, diabetes, growth hormone excess, or lipid infusion (among others) uniformly lowers glucose uptake, but results in either no change in insulin signaling[99,100] or changes in just a subset of signaling events[101,102]. There are several different ways to interpret these observations. It could be that current knowledge of the insulin signaling cascade contains many gaps, which could account for the cases where the canonical pathway shows no changes despite a reduction or improvement in

glucose uptake. Another possibility is that the limits of current techniques together with small study sizes result in insufficient power to detect small changes in signaling events. One could imagine that a modest deficiency of multiple signaling events leads to sizeable deficits in glucose uptake. The truth is likely to lie somewhere between these two alternatives, but the latter expanation introduces a heavy experimental burden. It suggests that boosting one step of the signaling pathway is unlikely to improve glucose uptake, so that a demonstration of its causal involvement would require manipulating multiple steps, a technical challenge with current techniques. Moreover, accurately modeling insulin resistance may require inducing modest changes in multiple signaling molecules, also technically demanding.

Special attention is warranted by one particular confounding effect that can occur in studies of obesity induced insulin resistance, namely that the intervention under evaluation decreases the proclivity to gain weight. Two recent examples of this are found in JNK1 knockout[79] and S6K[103]knockout mice. Conceptually, the nature of confounding is straightforward: if the insulin resistance inducing stimulus is absent, it is not surprising that insulin resistance is absent as well. It is worth noting that there are now a large number of whole body genetic interventions that diminish weight gain on a high fat diet. In such cases it is tempting to ignore the theoretical imprecision of conclusions about insulin resistance and to instead tout the therapeutic potential of these interventions. However, for the purposes of this thesis we are interested in a reasoned understanding of the factors that influence insulin sensitivity and insist on controlling for the effect of weight gain.

1.4.4 Case studies of insulin resistance mechanisms:

We now evaluate several well studied pathways thought to be involved in insulin resistance, including JNK activation, NFkB activation, free fatty acid excess, OXPHOS depression, and adiponectin depression. In each case we take into consideration the five components of our logical framework: a change in pathway activity, causality (necessity vs. sufficiency), whole body vs. cellular mode of action, glucose uptake vs. signaling, and confounding data.

With the recognition that type 2 diabetes bears similarities to states of inflammation[104], proteins activated by inflammatory cytokines have been evaluated as causes of insulin resistance; JNK, a kinase, is one such protein. JNK activity was found to be elevated in liver, fat, and muscle from insulin resistant obese mice. These mouse models included both diet induced obesity (DIO) and leptin deficiency (ob/ob)[79]. Three lines evidence suggest that JNK plays a causal role in whole body insulin resistance. First, JNK1 null mice are significantly protected from the whole body insulin resistance effects of DIO. Second, injection of a cell permeable peptide inhibitor of JNK improved insulin sensitivity in both DIO and db/db mouse models[105]. Third, obese mice infected with an adenoviral vector expressing a dominant negative mutant of JNK (DNJNK) became more insulin sensitive[106]. What tissues does JNK act on to diminish whole body insulin sensitivity? Elevated JNK activity could depress IMGU by virtue of its action within muscle or fat cells, or it could influence these tissues indirectly, through its actions on the liver for example. The knockout and inhibitor experiments can not address this point as JNK activity was inhibited in virtually all tissues. Adenoviral infection is largely localized to liver cells, suggesting that improvement in whole body insulin sensitivity

observed in the third experiment could be the result of an indirect influence on muscle and fat. There is one report that directly addresses the cellular influence of JNK in a cell type that support IMGU. Free fatty acid treatment of cultured fat cells led to both JNK activation and insulin resistant glucose uptake. Insulin sensitivity could be partially restored by RNAi mediated depression of JNK expression[107]. Importantly, in many of the experiments discussed above the evidence that JNK activation mediates insulin resistance was supported by glucose uptake data as well as insulin signaling data. What are the confounding observations? Interestingly, JNK phosphorylation and activation is yet another normal response to insulin stimulation[108]. So increased JNK activity *in vivo* could be a side effect of the hyperinsulinemia present in the obesity models. The JNK knockout data are significantly confounded by the fact that the null mice gained less weight in response to diet or leptin deficiency. In these mice the absence of JNK may affect food intake or energy expenditure so that the entire improvement in insulin sensitivity is due to the absence of weight gain. In the other two mouse experiments, however, weight loss was not observed.

NF-kappaB is another factor activated during inflammation whose role in insulin resistance has been heavily investigated. It is a heterodimeric transcription factor composed from a family of five subunits that are variably expressed in different cell types[109]; in muscle the predominant dimer is between p50 and p65[110]. Its activity is controlled by an interacting family of proteins known as IkappaB(s), which inhibit its ability to bind DNA. The best studied mechanism of NF-kappaB (NF-kB) activation involves the degradation of IkappaB (IkB) as a result of its phosphorylation by the kinase IKK-beta. Is NFkB activity altered in states of insulin resistance? Elevated NFkB activity

in human diabetic muscle has been inferred from the observation that levels of IkB-beta are depressed[109]. Similarly, elevated NFkB in muscle from a rat model of obesity has been inferred from decreased levels of IkB-alpha[111]. In adipocytes, insulin resistant stimuli such as TNF are known to activate NFkB[112]. Is NFkB a cause of insulin resistance? Fortunately, animal data addressing both sufficiency and necessity are available. Genetic intervention to increase NFkB activity is often achieved by expressing activating mutants of IKK-beta. Inhibition of NFkB is achieved by expressing non-degradable mutants of its inhibitory partner IkB-alpha. Mice with muscle specific expression of a constitutively active IKK-beta do not become insulin resistant, despite increased NFkB activity[113]. Moreover transgenic mice expressing a constitutively active form of IkB-alpha, in a muscle specific fashion, are not protected from obesity induced insulin resistance[113]. This is despite the defective activation of NfKB. Interestingly, mice with liver specific expression of constitutive IKK-beta do become insulin resistant[89], and mice with myeloid cell specific knockout of IKK-beta retain insulin sensitivity on a high fat diet[92]. Thus if one assumes that IKK-beta interventions work solely through NFkB, then although NFkB plays no direct role in the cellular insulin resistance of muscle, it can lead to muscle insulin resistance indirectly.

One attractive hypothesis explaining how obesity leads to insulin resistance proposes that it is due to the lipid build-up inside muscle cells. It is well documented that obesity leads to lipid deposits in tissues other than fat, in particular liver and muscle[114,115]. Moreover it is even found in the muscles of lean insulin resistant individuals[116]. Does intramyocellular lipid cause insulin resistance? Intravenous infusion of free fatty acids (FFA) is sufficient to increase muscle levels of FFA metabolites as well as cause whole-

body insulin resistance in mice and humans[117]. Analagous results have been obtained in both cultured muscle and fat cells, which can be made insulin resistant by treatment with high concentrations of FFAs such as palmitate[107,118]. An important question is whether high FFA levels contribute to the insulin resistance in the setting of obesity, and if so whether the effect is mediated by a direct action on muscle. The answer to both questions appears to be yes: when fatty acid delivery to muscle is specifically inhibited by conditional knockout of the FATP gene, the mice are protected from DIO induced insulin resistance[117]. Encouragingly, insulin signaling inhibition due to FFA exposure is backed up by a similar inhibition of insulin stimulated glucose uptake. There is one provocative instance where the intramyocellular lipid hypothesis makes an incorrect prediction, offering an opportunity to extend our understanding of its mechanism of action. Endurance trained athletes, who are highly insulin sensitive, have been found to have significant lipid deposits in their muscles[114]. A refinement of the hypothesis has been suggested by a recent study that evaluated additional properties of these lipid deposits. Intramyocellular lipid from obese humans differed from that found in athletes by virtue of being heavily oxidized[119]. Whether or not this distinguishing property has causal significance will be addressed later in the thesis.

The role of mitochondrial oxidative phosphorylation (oxphos) in diabetic insulin resistance is an older hypothesis that has received fresh attention on account of powerful new data from multiple groups. Expression of oxphos genes are coordinately downregulated in muscle from diabetic humans[120,121] as well as adipose and muscle tissue from obese rodents[122,123]. This change in expression is mirrored by a functional defect in the ATP synthetic capacity of muscle from insulin resistant humans[124]. Additionally,

glucocorticoid administration, which causes insulin resistance, has also been shown to cause depressed oxphos capacity[125,126]. In contrast to the abundance of data supporting an association between oxphos changes and insulin resistance, less data is available to support a causal connection. The best evidence comes from observations that certain rare mutations that inhibit oxphos function, including the leucyl tRNA mutation in Mitochondrial Diabetes and Deafness as well as frataxin mutations in Friedrich's Ataxia[127], are sufficient to cause insulin resistance. To date there is no experimental data supporting the ability of improved oxphos function to restore insulin sensitivity. The challenge to interpreting the data from rare mitochondrial mutations is that some mutations that significantly affect mitochondrial function do not in fact cause insulin resistance[128]. Lastly, the oxphos hypothesis is confounded once more by the normal function of insulin. Insulin acutely stimulates ATP formation by mitochondria[129], raising the possibility that depressed oxphos is a marker of insulin resistance rather than a cause.

The last hypothesis we examine concerns the function of adiponectin. In this case we do not consider a mechanism for cellular insulin resistance because it is not a cellular factor per se, as it is secreted by fat cells and circulates in the blood. While the effects of adiponectin on cellular function are of great importance, our discussion will be limited to the logic of its role in whole-body insulin resistance. Adiponectin levels are depressed in insulin resistant humans and animals[130]. This depression is causal: adiponectin knockout mice are mildly insulin resistant[131] (sufficiency), and transgenic adiponectin expression or adiponectin injection promotes insulin sensitivity in mouse models of obesity (necessity)[91,132]. While the big picture is fairly straightforward, there are some finer details that require resolution. One perplexing issue concerns the tremendous amount of

adiponectin circulating in the blood. Its levels, even in a state of insulin resistance, are far higher than one might expect are needed to maximally stimulate its receptors[133].

The aims of this section have been twofold: first, to outline a conceptual framework for thinking about and evaluating data pertaining to mechanisms of insulin resistance and second, to use this framework to evaluate some prominent examples of factors thought to cause insulin resistant glucose uptake. As the number of factors with a causal role grows, a more general question emerges: how are mechanisms such as JNK, NFkB, FFA, OXPHOS, adiponectin all interconnected? Furthermore, which of these factors can be exploited for therapeutic purposes?

In the chapter 2 we begin the description of our original experiments, and by chapter 6 we will evaluate the evidence collected within the framework outlined here. But first some background on the biology of reactive oxygen species is needed, as it will be the pathway we focus on as a mechanism of insulin resistance.

1.5 ROS Background

Here we present some background knowledge regarding the biology of reactive oxygen species (ROS). The strategy that lead us to consider its role in the etiology of insulin resistance together with the evidence we collected to support it will occupy the remainder of the thesis. Keeping in mind that there is a tremendous literature associated with the biology of ROS[134], our goal here is a focused review that discusses their manipulation. Within the context of this topic we discuss various aspects of ROS biology, including their molecular properties, the enzymes that scavenge them, and cellular sources of production.

The term "reactive oxygen species" refers to a specific subset of oxygen derivatives. In biological settings three molecules receive the most attention: superoxide, hydrogen peroxide, and hydroxyl radical. An appreciation for some of their basic electronic properties is useful for understanding the chemical reactions in which they participate. O_2 is an oxidizing agent possessing two, antiparallel, unpaired electrons in its outer shell. This particular arrangement favors chemical reactions in which it accepts electrons one at a time. For example, mitochondrial oxidative phosphoryation involves four sequential one-electron reductions of O_2, to generate $2H_2O$. A simple electronic relationship connects O_2, superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical: superoxide is derived from O_2 by a one electron reduction, hydrogen peroxide is derived from superoxide by a second one-electron reduction (plus 2H+), hydroxyl radical is derived from hydrogen peroxide by a third one-electron reduction (plus H+), and a fourth one-electron reduction generates water (see Fig.4). The reactivity of different ROS species depends on their thermodynamic and kinetic properties. Thermodynamically, hydroxyl radical is the most oxidizing of the ROS, followed by superoxide, and hydrogen peroxide. Hydroxyl radical will rapidly oxidize virtually any biomolecule, accounting for its reputation as a damaging agent. On the other hand the kinetics of uncatalyzed superoxide and H_2O_2 reactions are typically slow. Transition metals such as iron and copper are potent catalysts of one-electron transfers involving superoxide and H_2O_2. The Fenton reaction, involving a transition metal and H_2O_2 as substrates, leads to the production of hydroxyl radical. Moreover this reaction is catalyzed by superoxide (see Fig.3). As a result, metals and metal containing cofactors occupy a central role in the biochemistry of ROS.

Direct manipulation of ROS occurs by means of a set of enzymes found in all aerobic organisms, from bacteria to humans. These enzymes catalyze the one-electron reduction reaction of different ROS species (a process referred to as scavenging). Superoxide is converted to hydrogen peroxide by a conserved family of superoxide dismutases. Hydrogen peroxide is converted to water by catalase and glutathione peroxidase (Gpx). In the case of Gpx, H_2O_2 is scavenged with the aid of glutathione (GSH), a cofactor which becomes oxidized to GSSG by an electron from H_2O_2. GSSG must subsequently be regenerated to GSH using the reducing power of NADPH. The chemical reactions are shown in Fig. x. Hydroxyl radical, the most damaging of the oxygen radicals, can be generated in the Fenton reaction where it's formation from H_2O_2 is catalyzed by metals. Consequently tight control of free metals such as iron and copper as well as iron containing molecules such as heme rings from hemoglobin or myoglobin, is an important component of the control of hydroxyl radical formation and oxidative damage. As might be expected, these enzymes are often localized to sites of high ROS production. Catalase is found in peroxisomes where large quantities of hydrogen peroxide are enzymatically generated. SOD2 is found in the mitochondrial matrix and SOD1 in the intermembrane space(and cytoplasm) for protection against respiration generated superoxide.

Manipulation of ROS can also be carried out through perturbations of their cellular sources. By definition, ROS are created in reactions where oxygen is a substrate. In some enzymatic reactions, the catalytic cycle itself may generate superoxide or hydrogen peroxide as a side product and even the main product. In many cases, however, it is an unintended side product produced at a low frequency. Another possibility is that

certain cellular molecules are simply susceptible to attack by oxygen with concomitant generation of superoxide. In either case, one must remember that manipulating a ROS source produces effects due to ROS as well as due to the perturbation of the other products generated by the source.

The first source we consider is mitochondrial oxidative phosphorylation (OXPHOS), considered the source of the majority of cellular ROS. OXPHOS involves the transfer of electrons from NADH and FADH2 to oxygen by means of a chain of intermediate electron carriers. While the fidelity of electron transfer from carrier to carrier is quite high, there is a small probability that a given electron will be transferred to oxygen (generating superoxide), rather than to the next member of the chain. It is estimated that 0.2% of these electrons are lost to superoxide formation[135]. Moreover, this rate is increased by perturbations that cause a blockade of electron transfer. It is thought that by preventing electron transfer at a particular step, all prior electron carriers persist in the reduced state, increasing the likelihood of electron theft by oxygen. Examples include inhibitors of respiration such as Antimycin A (complex III) and a limiting supply of ADP, which leads to hyperpolarization of the mitochondrial membrane and slowing of the electron transfer reactions (state 4 respiration). Finally, an excess of reducing equivalents relative to the capacity of the electron transport chain (ETC) to process them leads to mitochondrial ROS through the same mechanism, namely by favoring the reduced state of electron carriers. Perturbations that completely block entry of electrons to the ETC will prevent ROS formation as well as all its other functions, including membrane polarization and ATP synthesis. Similarly, ROS production is lowered by perturbations that favor the oxidized state of electron carriers, as occurs during uncoupled

respiration. Again, however, uncoupling will both lower membrane potential as well as ATP synthesis.

At least three additional significant sources of ROS are recognized: heme proteins, certain enzymes, and the endoplasmic reticulum (ER). We consider some specific well known examples, particularly because they arise later in the thesis. Heme proteins such as hemoglobin and myoglobin are potential sources of superoxide due to the potential for bound oxygen to steal an electron from the heme iron[134]. Heme itself can be a source of hydroxyl radical as its iron is susceptible to Fenton chemistry. Heme binding proteins s and heme degrading proteins are considered to perform an antioxidant function. Multiple enzymes are capable of generating ROS: prominent examples include NADPH oxidase, cytochrome P450s, and xanthine oxidase. NADPH oxidase is an enzyme utilized by phagocytes to kill bacteria. It specifically transfers electrons from NADPH to oxygen, releasing superoxide within phagocytic vescicles. Xanthine oxidase, which converts xanthine to urate, generates a superoxide molecule during every catalytic cycle. The cytochrome P450s are heme proteins that together with oxygen and an electron source mediate the oxidation of substrates. The combination of the heme iron, oxygen, and an additional source of electrons leads to side reactions in which superoxide is generated. Finally, the ER is a site of ROS production by virtue of its high concentration of cytochrome P450 enzymes. As with mitochondria, the normal function of these enzymes must be taken into considerations when intervening to block ROS formation.

ROS emerged as a pathway of interest to us through wholly unbiased means. The chapters that follow describe how we arrived at it and what was done to manipulate it.

The concepts described here should help illuminate the reasoning behind some of our experimental choices.

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Figure 1. Glucose Accounting.

Diagram of sources and sinks of blood glucose by cellular process and organ system. List of circulating factors that acutely and chronically regulate the activity of these sources and sinks.

Fig. 1

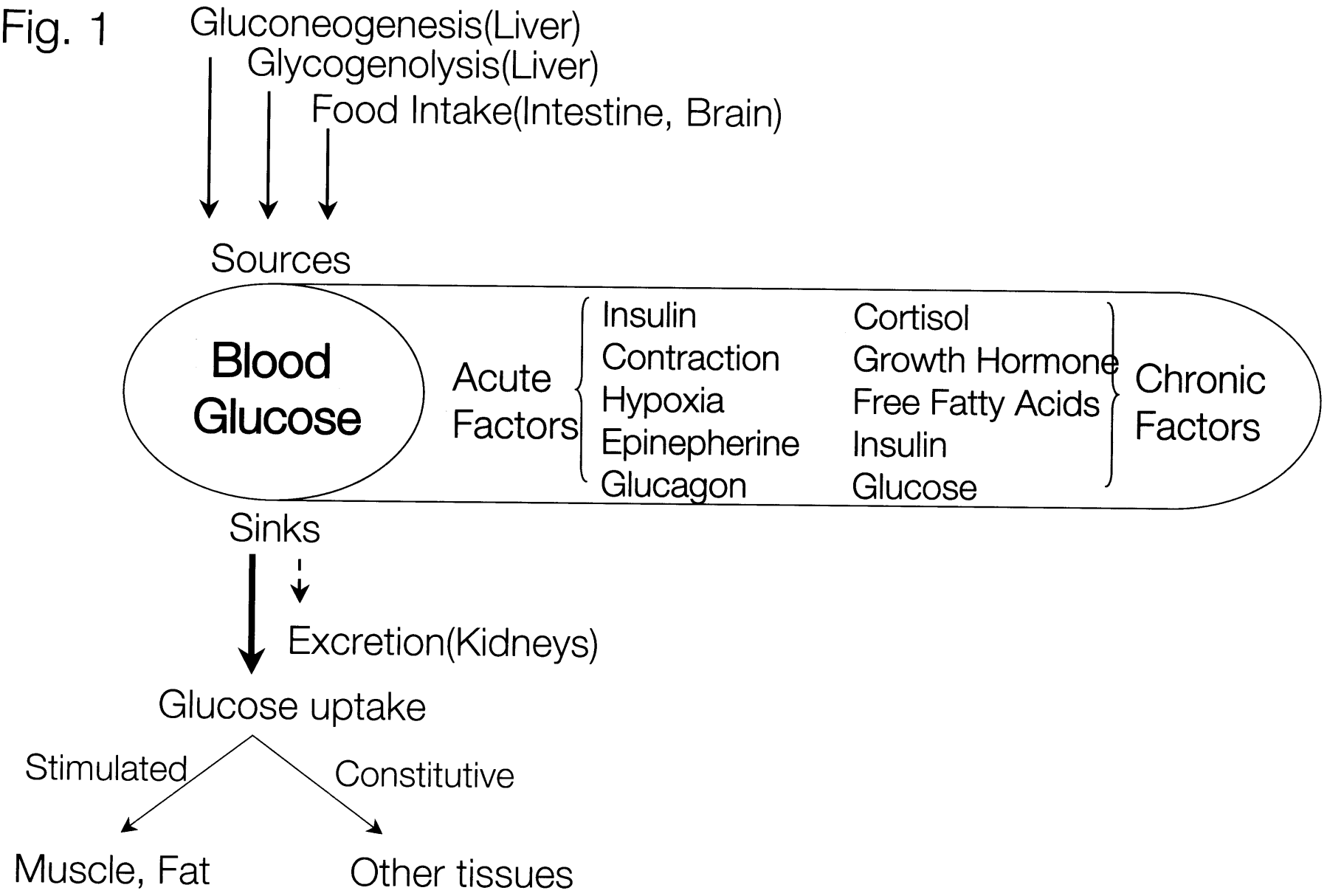
Gluconeogenesis(Liver)
Glycogenolysis(Liver)
Food Intake(Intestine, Brain)
Sources
Blood Glucose
Acute Factors
Insulin
Contraction
Hypoxia
Epinepherine
Glucagon
Cortisol
Growth Hormone
Free Fatty Acids
Insulin
Glucose
Chronic Factors
Sinks
Excretion(Kidneys)
Glucose uptake
Stimulated
Constitutive
Muscle, Fat
Other tissues

Figure 2. Insulin stimulated Glut4-EGFP translocation.

Confocal microscopy of 3t3-l1 adipocytes transfected with a Glut4-EGFP gene fusion. The image depicts subcellular localization of Glut4-EGFP fusion proteins in the presence and absence(basal) of insulin. Taken from Saltiel and Kahn, Nature 2001.

Fig.2

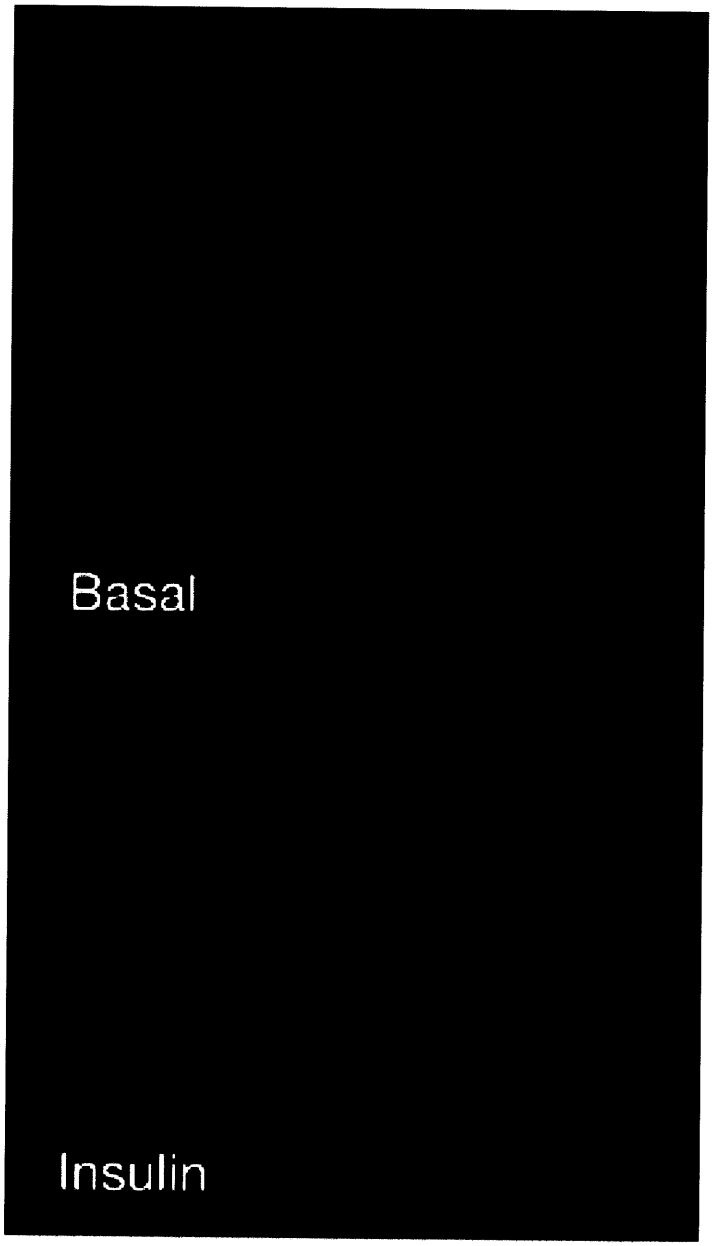

Figure 3. Insulin Signal Transduction.

The molecules that transduce the insulin signal, specifically the IRS arm of the pathway. The connection between AS160 and Glut4 trafficking is currently under study. Taken from Watson and Pessin, TiBS 2006.

Fig. 3

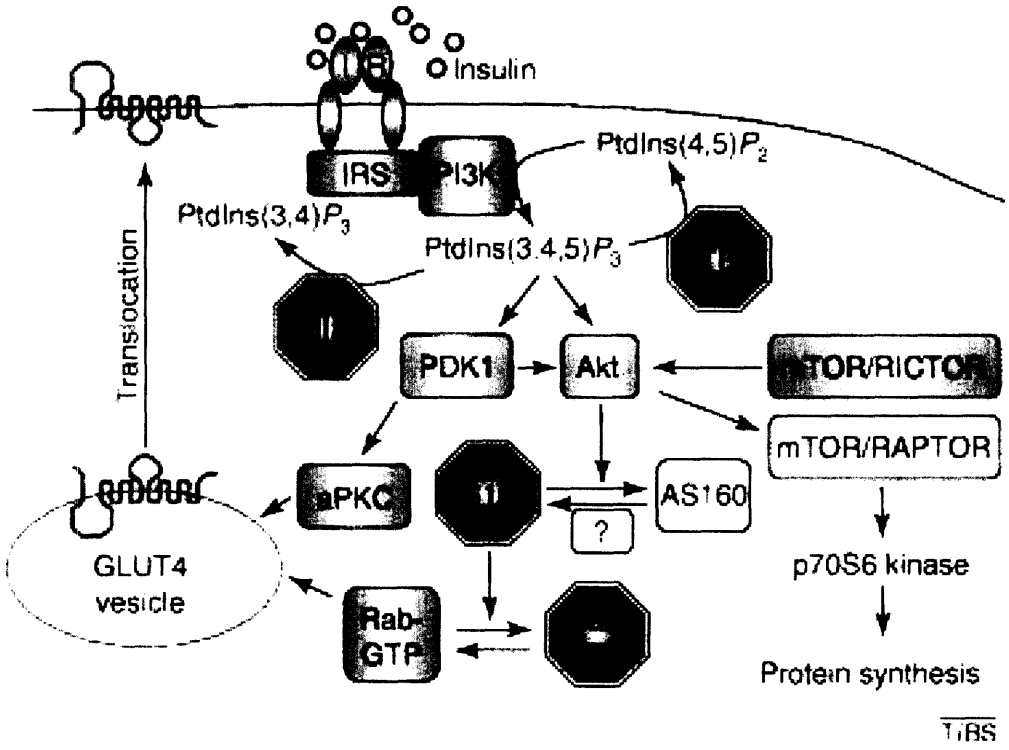
Insulin
IRS
PI3K
PtdIns(4,5)P_2
PtdIns(3,4)P_3
PtdIns(3,4,5)P_3
PDK1
Akt
mTOR/RICTOR
mTOR/RAPTOR
aPKC
AS160
?
Translocation
GLUT4
vesicle
Rab-
GTP
p70S6 kinase
Protein synthesis
TiBS

Figure 4. ROS synthesis and scavenging.

The synthesis reactions illustrate the simple relation between the three principle reactive oxygen species. The Fenton reaction depicts an important role for transition metals in hydroxyl radical formation. ROS scavenging enzymes catalyze 1-electron reduction reactions.

Fig. 4

ROS Generation

$$O^{\bullet}{}_2^- + e^- + 2H^+ \rightleftharpoons H_2O_2$$

$$H_2O_2 + e^- + H^+ \rightleftharpoons H_2O + OH^{\bullet}$$

$$OH^{\bullet} + e^- + H^+ \rightleftharpoons H_2O$$

Fenton Reaction: $H_2O_2 + Fe^{2+} \longrightarrow Fe(III) + OH^- + OH^{\bullet}$

ROS Scavenging Enzymes

Superoxide Dismutase: $O^{\bullet}{}_2^- + O^{\bullet}{}_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

Catalase: $2\,H_2O_2 \longrightarrow O_2 + 2\,H_2O$

Glutathione Peroxidase: $H_2O_2 + 2GSH \longrightarrow 2\,H_2O + GSSG$

Chapter 2. Cellular Models of Insulin Resistance

How might genes or other factors that cause insulin resistance be identified? In Chapter 1 we discussed the criteria used to evaluate the causal role of a given factor. Here we map out a strategy for generating a list of candidate genes enriched for those with causal relevance. The strategy is based on an observation about functional similarities between different clinical states of insulin resistance. This observation also motivates a series of experimental choices regarding the cellular model system used to identify and validate candidate factors. Finally we discuss our efforts to calibrate this cellular model in order to maximize the probability that results derived from it have *in vivo* relevance.

2.1 Strategy for identifying causal factors

The first criterion a proposed causal factor must satisfy is that its state be altered during insulin resistance. An important goal then should be to catalog all such cellular state changes. But if the list of these changes is long an important new question arises: how should the list be weighted to enrich for causes over correlates? It turns out that there are many clinical conditions displaying insulin resistance: they include pregnancy, sepsis, cushing's syndrome, cancer cachexia, obesity, starvation, burn injury, acromegaly, the metabolic syndrome, the response to certain drugs, and ofcourse type 2 diabetes. If it were true that a common cellular response mediates insulin resistance in these different clinical states then a natural weighting scheme suggests itself: identify the changes associated with each state of insulin resistance and concentrate our validation efforts on those that are common to all.

Using a comparative approach to prioritize factors that are commonly regulated in insulin resistant states makes a strong assumption, namely that there is in fact a causal pathway common to all of these states. We offer a hypothesis for why such a strong assumption might be plausible: insulin resistance could be a physiologic response whose function is to shunt glucose disposal. Among the clinical states mentioned above, a handful can be identified in which insulin resistance is actually a desirable attribute: pregnancy, starvation, severe infection, and traumatic injuries such as burns. By turning down glucose disposal in muscle it can be diverted to other sinks: in pregnancy this would be the fetus[1], in starvation the brain, in infection it might be the immune cells, and in trauma it could be the site of injury[2]. It is thus conceivable that each such condition employs its own unique stimulus to activate a common cellular pathway leading to «physiologic insulin resistance». The settings in which the response is pathological could reflect inappropriate activation of this same pathway. One could even imagine a purposeful hijacking of this pathway: in the case of tumor induced insulin resistance the target sink might be none other than the tumor itself. Finally, we must acknowledge the possibility that our assumption about a common pathway could be wrong. In this case using a comparative analysis would not be helpful in identifying a causal factor. Instead it might simply allow us to make the weaker assertion that insulin resistant states are not commonly regulated.

To translate the comparative strategy in to an actual experiment one could ask how the extracellular environment of insulin sensitive tissues is altered in settings of insulin resistance. A handful of circulating factors have been identified that change in these settings. Examples include free fatty acids, growth hormone, glucose, insulin,

dexamethasone (Dex), and TNF-alpha (TNF), all of which are elevated in one or more of these conditions. Moreover, when any one of these factors are experimentally elevated in healthy mice they cause insulin resistance[3-8]. Finally, cultured muscle and fat cells exposed to these same agents also become insulin resistant[9-17]. It is this last observation that serves as the starting point for our experimental approach.

We chose to study insulin resistance arising from exposure of 3T3-L1 adipocytes with either TNF or Dex. The state changes we considered were mRNA levels. We used gene expression profiling to catalog the expression changes occurring in response to each agent. A molecular pathway was inferred from the intersection of these expression changes and became the focus of our validation efforts.

At least two variations on this approach were possible. The first would have been to examine muscle or fat harvested from humans in different states of insulin resistance. The second possibility would have been to harvest tissues from different animal models. Our decision to pursue the cell culture approach was a judgement call based on two suspicions: first, we anticipated that even under controlled conditions there would be significant biological noise due to interindividual variability. We believed that the cell culture environment could be controlled with much greater precision. Second, we suspected that validation of candidates identified in an in vivo system could be very challenging. They could be validated directly in vivo by engineering transgenic mice, but the difficulty of this task together with the risk of finding a correlate were too great to make such an approach feasible. Alternatively candidates generated in vivo could be tested in a heterologous system such as cell culture. Again however, we foresaw challenges in translating the results across systems and worried they would present a

barrier to rapid testing. We thus settled on identifying the candidates in a cell culture system because both the identification and validatation could be performed in one system with relative ease. Ofcourse the danger was that the cellular model would be a poor one and whatever was found would have little relevance to the in vivo setting. So we set out to choose our model conditions carefully.

2.2 Model of cellular insulin resistance

There were a number of advantages to choosing Dex and TNF as our resistance inducing agents. Both are well-validated experimental models of insulin resistance, and both have physiological relevance in vivo. Mice show impaired insulin sensitivity in response to TNF or Dex treatment and are protected from obesity-related insulin resistance by related physiological blockades (genetic ablation of TNF or TNF receptor[18]; treatment with glucocorticoid antagonists[19]). Glucocorticoid treatment is also a frequent cause of insulin resistance in humans. Furthermore, elevated levels of TNF, glucocorticoids, or both have been shown to be associated with insulin resistant states such as obesity[20,21], cancer cachexia[22], sepsis[23], burn trauma[24,25], pregnancy[26,27], metabolic syndrome[28]and starvation[29]. Despite these similarities between TNF and Dex, their cellular response pathways could not be more distinct: TNF signals through a cell-surface cytokine receptor, while Dex signals through a nuclear hormone receptor. Moreover, TNF has pro-inflammatory properties, while Dex is a prototypical anti-inflammatory agent. We reasoned that a powerful approach to understand the cellular basis of insulin resistance would be to compare the effects of these two very different, physiologically relevant treatments: pathways fundamental to insulin resistance might be

expected to show responses in both settings.

In addition to the biological reasons for our choice of TNF and Dex there were strategic reasons. TNF and Dex induce insulin resistance over chronic time scales i.e. days of exposure. This suggested that transcription would be involved, so that recording gene expression changes was likely to be informative. The biology of TNF and Dex has been studied for more than two decades. There is an enormous wealth of literature to use to interpret the changes we would observe. The final reason is more subtle: TNF and Dex mediate their effects through very specific ligand receptor interactions, so the induction of insulin resistance could be viewed as a physiologic and thus evolutionarily designed response (keeping with our earlier discussion). This is to be contrasted with the inappropriate induction of insulin resistance by some novel environmental insult such as a «MacDonald's diet». We anticipated it would be easier to dissect a physiologically relevant response than to analyze how a «monkey wrench» inflicts its damage.

TNF and Dex were also interesting choices because limited knowledge exists about how they cause insulin resistance. Few cellular variables have been identified that change upon TNF or Dex treatment and which, when reversed, restore the ability of cells to take up glucose in response to insulin. In the case of TNF several causal variables have been put forward including serine phosphorylation of IRS1, p38 activation, JNK activation, and NfKB activation. Of these only reversal of p38 activation has been shown to restore insulin stimulated glucose uptake[de Alvaro, 2004]. The other variables have been shown to mediate the effect of TNF on insulin signaling[aguirre,2000] and gene expression[31]. In the case of Dex the best studied variables suggested to mediate its effects are acute insulin signaling proteins. In particular IRS1 has been shown to be

downregulated by Dex, and decreased signaling through PI3K[32] and Akt[33] have also been observed. However, increasing IRS1 expression, PI3K activation, or Akt activation does not reduce Dex's effect[32] on IMGU.

The second major decision was to use 3T3-L1 adipocytes as the cell type. Alternatives included primary adipocytes, primary muscle, and muscle cell lines. We opted against primary cells because of the challenges of routine isolation, their limited lifespan in culture, and the technical difficulty of modifying them genetically. The muscle cell lines have the disadvantage that they are incompletely differentiated and thus lack many important characteristics of muscle tissue: they can not contract, they express very little Glut4, their glucose uptake response to insulin is minimal, and it remains minimal even when the cells are transfected with excess Glut4[34,35]. In contrast, 3T3-L1 adipocytes possess many of the characteristic metabolic properties of adipose tissue, including lipid storage, lipolysis, and sensitivity to metabolic hormones such as insulin and epinepherine. In particular, the insulin mediated glucose uptake response is both sensitive (EC50 of 100pM) and pronounced (>15-fold over basal uptake). Moreover, they become insulin resistant in response to many of the same agents that can cause insulin resistance *in vivo*. Finally, 3T3-L1s can be modified genetically with routine and straightorward techniques that allow the creation of stable cell lines[36]. Their close relation to *in vivo* adipocytes and the enormous technical experience that exists in the scientific community made the 3T3-L1 model an attractive choice. At the same time we recognized the danger of studying adipocytes when in fact our aim is to understand cellular insulin resistance in muscle, which is the chief insulin sensitive glucose sink *in vivo*.

2.3 Model calibration

Cultured 3T3-L1 adipocytes exposed to TNF or Dex become insulin resistant within several days, as assayed by the ability of insulin to stimulate glucose uptake[10,11]. To maximize the physiological relevance of this model, we calibrated the treatment regimen so that (i) insulin-dependent glucose uptake was decreased by ~50%, a degree similar to that seen in the clinical setting; (ii) the defect in insulin action was reversible by washing out the agent; and (iii) the defect could be rescued by pioglitazone (Fig.1a,b), a member of the thiazolidinedione (TZD) class of insulin-sensitizing drugs.

In addition to preserving the model's physiologic relevance the use of positive controls had a practical goal: gauging to what extent an experimental intervention could be expected to reverse insulin resistance. If washing out Dex or TNF led to a very modest recovery, than we would infer they had done irreversible damage and it would be hard to imagine any putative insulin sensitizer performing better. Similarly if pioglitazone, a clinically effective treatment, was ineffective or only weakly sensitizing in our system then there would be no precedent for potent insulin sensitization and our a priori probability of finding such an intervention would be low. After carefully exploring the conditions of the treatment protocol we were able to find a dose and duration of exposure that satisfied our calibration criteria. This was encouraging because it is known that in humans insulin resistance is often reversible, be that by cessation of glucocorticoid treatment, weight loss, or after childbirth, to name a few examples. Moreover, it is also known that in humans[37] and rats[8] TZD treatment is an effective treatment for Dex induced insulin resistance.

While searching for culture conditions to calibrate the insulin resistance model we

encountered some unexpected difficulties. Two tissue culture variables that are difficult to control were found to exert a profound effect on our positive controls. The first variable is an obligate component of media, the serum. It is a complex, protein rich liquid that is incompletely defined and varies from manufacturer to manufacturer and even between different lots from a single manufacturer. Serum affected two of our metrics: sensitivity to TNF and dex as well as rescue by pioglitazone (Fig.2). The second variable with a surprising effect on our metrics is the particular clone of 3T3-L1 preadipocyte. As with serum, clones vary based on commercial lot number. Their properties also change the longer they are passaged in culture. We observed that clones from certain lots do not support pioglitazone mediated rescue of insulin resistance(Fig.3). The serum variation problem will only be solved once completely defined media with our desired properties becomes available. The clone variation problem will only be solved with the advent of a genetically stable preadipocyte line. In the meantime the solution will require a devout commitment to the use of positive controls. Serum lots and preadipocyte clones must be simultaneously screened to identify a combination that satisfies the three calibration criteria.

We've broken down the problem of diabetic complications in to a series of subproblems, transforming it in to a specific question about insulin resistance. Reasoning from an observation about the relationship between different insulin resistant conditions this question was formulated as follows: do TNF and Dex mediate insulin resistance in 3T3-L1 adipocytes by a common pathway. In Chapter 3 we describe the results of our strategy for identifying components of this pathway.

Methods

Reagents. Pioglitazone (Alexis Biochemicals). Dexamethasone (Biomol). TNF (Cell Sciences). Mannitol, Insulin, Isobutylmethylxanthine, Digitonin (Sigma). DMEM (Invitrogen). Fetal Bovine Serum, Bovine Calf Serum (HyClone). 3T3-L1 preadipocytes were obtained from ATCC.

Cell Culture. Early passage 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium supplemented with Glutamax, 10% bovine calf serum, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were maintained at 37°C, 10% CO_2 and mycoplasma-free. Differentiation medium was added to pre-adipocytes two days after reaching confluence (Day 0). Differentiation medium consisted of DMEM supplemented with 10% Fetal Bovine Serum (FBS), to which was added a cocktail containing 0.11 mg/ml isobutylmethylxanthine, 1ug/ml insulin, 250nM dexamethasone and 1.5uM pioglitazone. On Day 2, the medium was replaced with DMEM/10%FBS containing 1ug/ml insulin and 1.5uM pioglitazone. From Day 4 onward, cells were maintained in DMEM plus 10%FBS with a media change every other day until experimental treatments were initiated. Batches of pre-adipocytes and FBS were screened for their ability to give rise to adipocytes satisfying the positive control behavior depicted in Fig.1a,b.

Induction of insulin resistance. Dex (20 nM) or TNF (4 ng/ml) treatment was initiated with mature adipocytes anywhere from day 8 to day 14 of differentiation. Media was changed daily for TNF for a total incubation time of 4 days. Dex media was changed every other day for a total of 8 days. For experiments in which transgene expression was induced, doxycycline was added to cells on day 4 of differentiation and TNF or Dex treatment was always initiated on day 8. For washout experiments cells were made insulin resistant as above at which point media absent TNF or Dex was added and refreshed every two days for a total of 8 days.

Glucose Uptake Assay: Cells in 12 well dishes were washed twice with KRP (127mM

NaCl, 4.7mM KCl, 0.9mM $MgSO_4$, 10mM $NaPO_4$, 0.9mM $CaCl_2$) and incubated with pre-warmed KRP (670ul) containing 0.2% fatty acid free BSA and the appropriate concentration of insulin (10nM insulin as a maximum stimulus). The dish was then allowed to float in a 37°C water bath for 30min. After this period, tritiated 2-deoxyglucose (2DG) and unlabeled 2DG (total vol. 75ul) were dispensed into each well for a final concentration of 1uCi/ml and 0.1mM respectively. Cells were incubated for an additional 5 min. at 37°C and the reaction was stopped by immersing the entire dish in a reservoir of ice cold PBS. While submerged, the plate was gently swirled for 30 sec. The plate was then dried, excess PBS was aspirated, and 740ul of digitonin release buffer (100mg/ml Mannitol, 1mg/ml digitonin) was applied to each well. After 10 min. at room temperature, 665ul from each well was counted in a scintillation counter. Fresh media was always added to cells 24 h prior to assay.

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Figure 1. Characterization of the insulin resistant state.

(a) Rates of glucose transport in TNF-treated cells. Basal glucose transport (grey) and insulin-stimulated glucose transport (white) are shown. Cells were untreated, treated with TNF alone, TNF plus pioglitazone, or TNF washout (TNF followed by medium). Basal rate refers to the rate of glucose transport in the absence of insulin. Insulin-stimulated rate was calculated as the rate of transport in the presence of insulin minus the basal rate. All values are normalized to the insulin-stimulated rate from untreated cells. Results are mean +/- s.e.m. Asterisk indicates a significant difference (p-value < 0.05, t-test) compared to TNF alone.
(b) Rates of glucose transport in Dex-treated cells. Data are analogous to (a).

Fig. 1

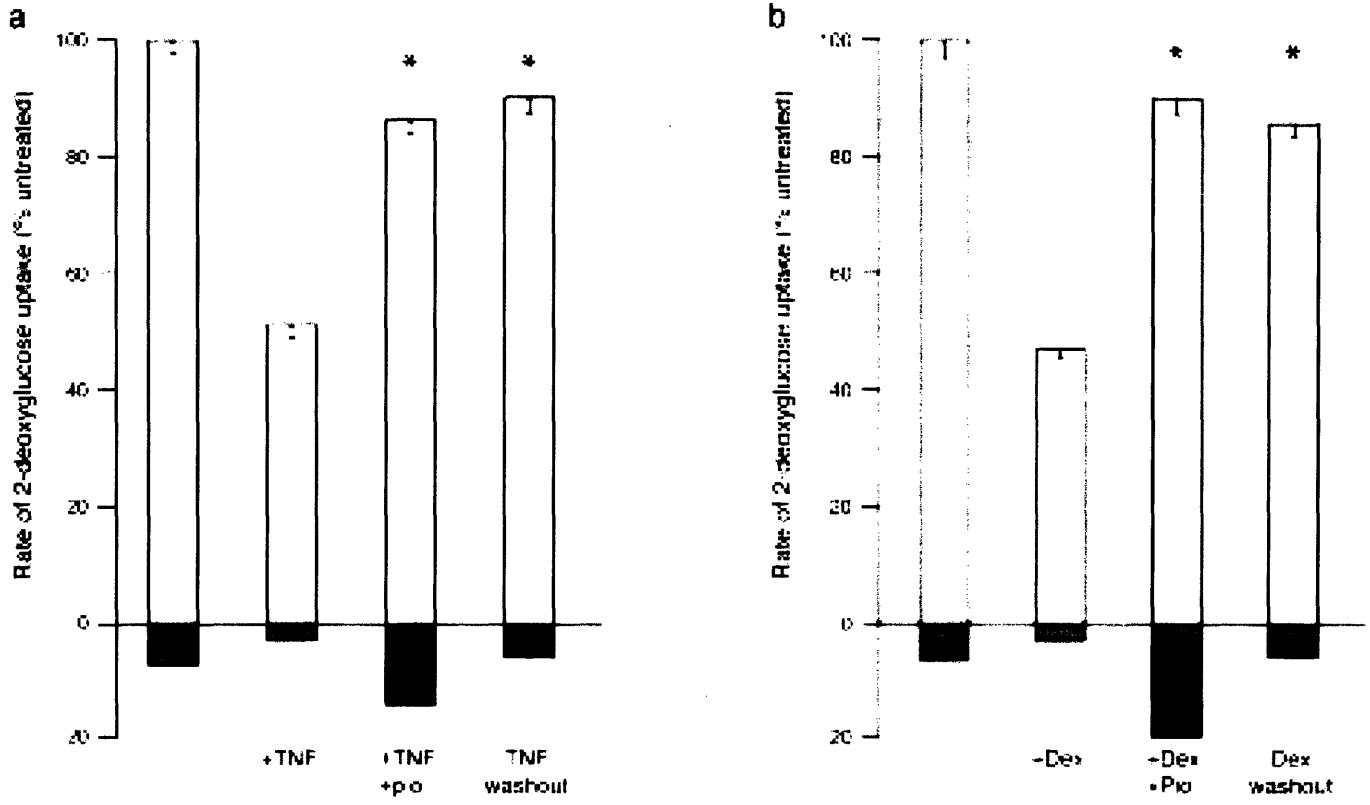

a
b
100
80
60
40
20
0
-20
*
*
+TNF
+TNF
+pio
TNF
washout
-Dex
-Dex
+Pio
Dex
washout

Figure 2. Effect of serum source on TNF induced insulin resistance.

Rates of insulin-stimulated glucose transport in TNF-treated cells. Cells were untreated, or treated with TNF in the presence of different sera. Insulin-stimulated rate was calculated as the rate of transport in the presence of insulin minus the basal rate. All values are normalized to the insulin-stimulated rate from untreated cells incubated with the corresponding serum.

Fig. 2

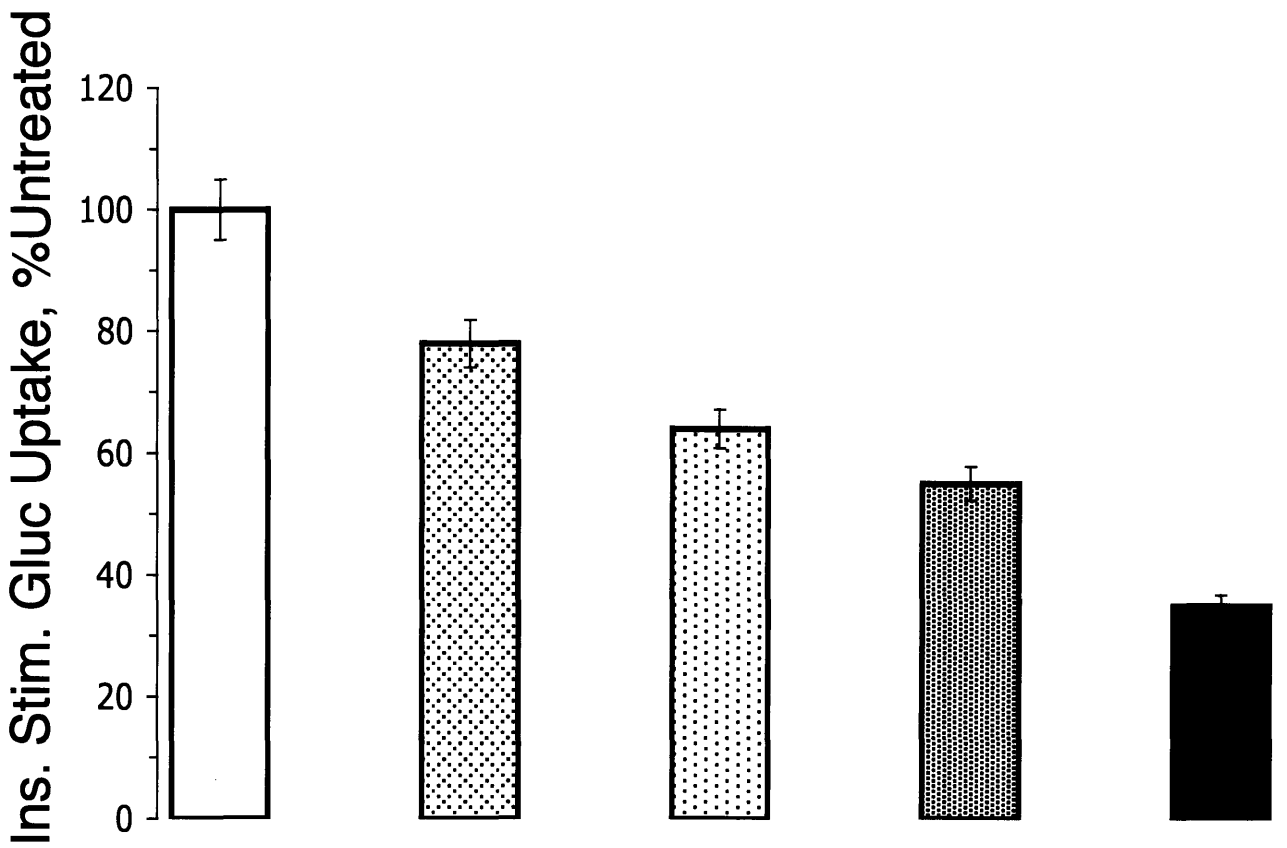
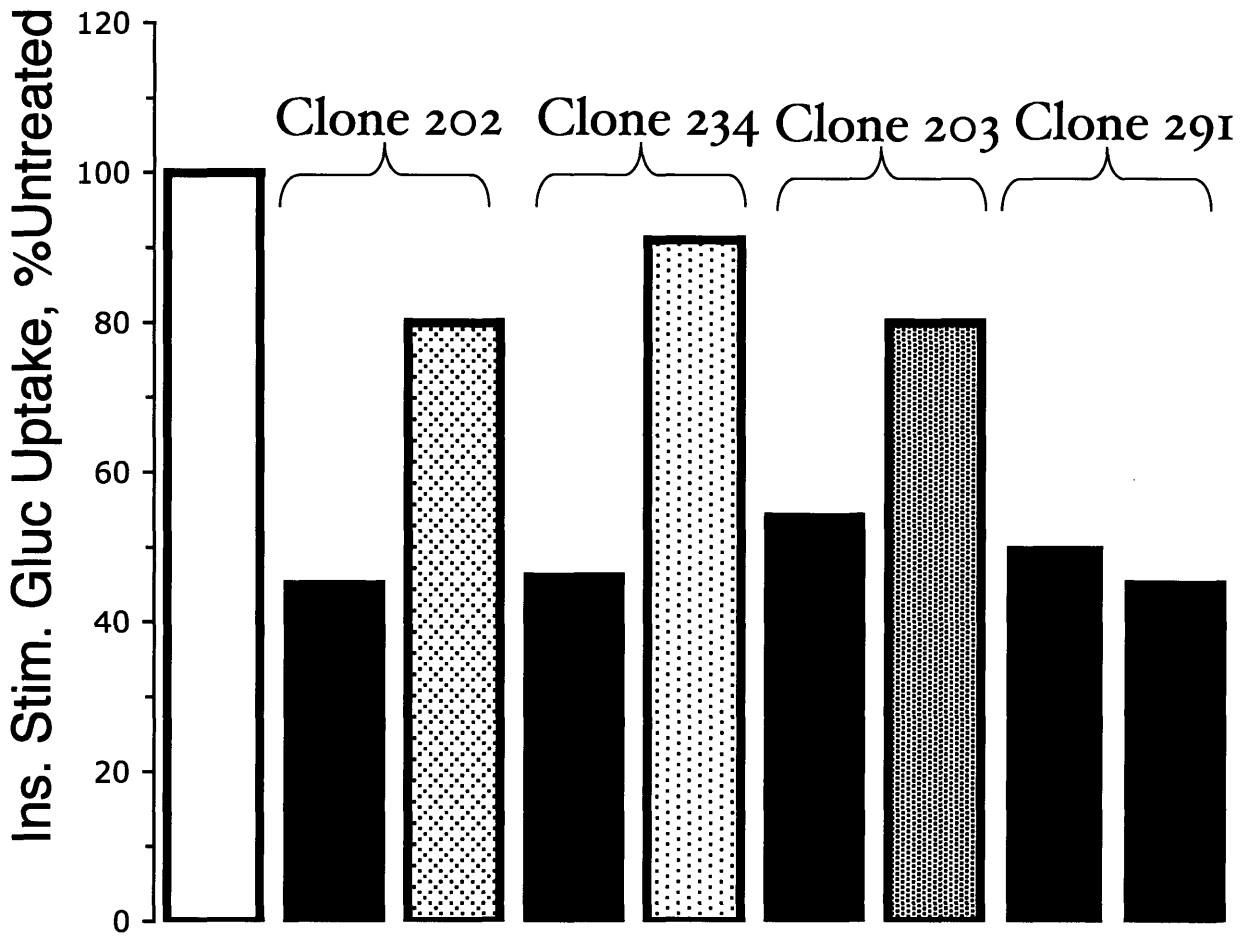

Ins. Stim. Gluc Uptake, %Untreated
120
100
80
60
40
20
0
Serum
ATCC
Hyclone
Whitehead
Sigma
TNF(4ng/ml)
-
+
+
+
+

Figure 3. Effect of 3T3-L1 clone lot on TNF induced insulin resistance and pioglitazone rescue.

Rates of insulin-stimulated glucose transport in TNF-treated cells. Different 3T3-L1 cell lines were untreated, treated with TNF, or treated with TNF plus piogliatzone. Insulin-stimulated rate was calculated as the rate of transport in the presence of insulin minus the basal rate. All values are normalized to the insulin-stimulated rate from untreated cells of the corresponding clone lot.

Fig. 3


Ins. Stim. Gluc Uptake, %Untreated
120
100
80
60
40
20
0
Clone 202
Clone 234
Clone 203
Clone 291
TNF(4ng/ml) - + + + + + + + +
Piog(4uM). - - + - + - + - +

Chapter 3. Gene Expression Analysis and the ROS Hypothesis

3.1 Gene expression analysis

Of the possible state variables that could mediate the actions of TNF and Dex we chose to examine changes in gene expression. Microarray technology allows one to efficiently measure the expression level of virtually all genes and hence be systematic and unbiased in the exploration of mechanism.

mRNA from TNF-treated, Dex-treated and untreated adipocytes was prepared and hybridized to Affymetrix arrays containing probe-sets from 22,690 mouse genes, with experiments performed in triplicate. Below is a brief description of the procedure we used to assess which genes were differentially expressed in response to treatment. For each gene we calculated a score known as the "signal to noise ratio": to compute it one calculates the average expression difference of a gene between two samples and divide this number by a number reflecting the amount of variability in the individual measurements of that gene's expression level (akin to its standard deviation). The result is a score that increases with the overall magnitude of the expression difference but decreases if the measurements are noisy. This is to be contrasted with a "fold" score which simply calculates the ratio of a gene's average expression level in one sample with its average expression in the other. In this case no penalty is incurred for noisy measurements. Once scores have been calculated for all genes they can be ranked from highest to lowest, with the extreme top and bottom of the list representing the most up and downregulated genes respectively. We also desire confidence that genes scoring very high or very low do not do so by chance, i.e. their place on the list reflects biology, not

noise. For simplicity we first divided the up- and downregulated genes into separate lists and considered the "top" of each list to be the genes of most interest. We then determined whether the set of genes ranked higher than a given threshold was significant by calculating a number known as the false discovery rate (FDR). Briefly, the FDR reflects the proportion of genes above a given rank that could be "false positives", i.e. score high due to noise of some kind[1]. It differs from classical measures of confidence in that it does not indicate which specific genes in the group are prone to error. Using the Signifance Analysis of Microarrays software package[1] we determined that the top 350 genes in the up and downregulated gene lists had FDRs of less than 5%. These were the genes we focused on.

We analyzed these results in two ways. Of the genes upregulated by TNF- and Dex-treatment, there were only 34 in common. Similarly of the downregulated genes there were 23 in common. These genes are shown in Table 3 and 4. First we inspected these genes by eye. To make sense of these lists we tried to determine whether they might contain subsets of related genes. We determined that among the upregulated genes about a fifth of them were related to the biology of reactive oxygen species (ROS) (Table 1a) (see Ch.1.5 for background on ROS).

In the second approach, we applied the objective and systematic approach of Gene Set Enrichment Analysis (GSEA)[2,3]. Given a collection of gene sets and the ranked list of gene expression changes resulting from a physiological treatment, GSEA tests whether the members of each set are randomly distributed along the list or clustered near the extremes; the latter indicates that the gene set is regulated by the physiological treatment. The advantage of this approach over our first method can be illustrated by a simple

example: imagine that glycolysis was a pathway activated by both TNF and Dex. But imagine that TNF induced the expression of enzymes 1, 3, and 5 of the pathway whereas Dex induced enzymes 2, 4, and 6. If one were to intersect the lists of TNF- and Dex-induced genes the glycolysis result would be missed entirely, whereas GSEA, with knowledge of the entire gene set, would recognize that it had scored highly in both treatments, and thus identify it.

Using a curated collection of 475 gene sets[3], GSEA identified ROS-related genes as the highest scoring set for both Dex-treatment and TNF-treatment (Table 1b). Moreover, this was the only high-scoring set in common between the two treatments.

We note several additional observations from these results that testify to the utility of GSEA analysis. First it should be appreciated that GSEA can result in false negatives for a number of reasons: a gene set may contain functionally related genes that are simply not transcriptionally co-regulated; a gene set may be transcriptionally coregulated but in response to a particular treatment its regulation is posttranslational; finally, perhaps only a small fraction of the entire set is transcriptionally coregulated resulting in a dilution effect that precludes ever getting a high gene set score. This serves as a warning that despite our best efforts, important results could be missed. That said, in our own data GSEA analysis identified several well known TNF responsive pathways. They included upregulation of a TNF receptor pathway, upregulation of an NF-KappaB pathway, and downregulation of mitochondrial OXPHOS genes. What's more, ROS is well known to be induced by TNF[4], and recently there have been a number of reports that it is induced by Dex[5-8]. So while every method has its limitations, GSEA has empirically given us confidence that it can identify known pathways as well as point out new connections

(ROS induction being common to TNF and Dex) that may not have been previously appreciated.

3.2 ROS pathway validation

The gene expression results raised the possibility that elevated ROS may be a key feature in both models of insulin resistance. Involvement of ROS was inferred from the properties of the genes whose expression was induced in our models. These properties include a gene's known ROS dependent expression, its ability to enzymatically generate ROS, or its ability to prevent oxidative damage. Below we discuss each gene's relation to ROS, corroborative evidence from independent studies that TNF and Dex are known to induce its expression, as well as any data pertaining to its association with diabetes.

Metallothioneins (MT) 1 and 2 are sulfhydryl rich proteins that are capable of binding and sequestering metal ions[9]. A significant number of studies have demonstrated their protective antioxidant function, of which we cite a few results: overexpression of MT1 or MT2 in cardiomyocytes or astrocytes confers resistance to hydrogen peroxide toxicity[10,11]. Knockout of MT1 and MT2 increases the susceptibility of astrocytes to peroxide induced cell death[11]. Finally, cardiomyocytes apoptosis induced by TNF can be blocked by MT2 overexpression, an intervention which also lowers TNF induced reactive oxygen species[12]. There is evidence that metallothioneins can be induced by ROS as demonstrated in lens epithelial cells exposed to tertbutyl-hydroperoxide (an oxidant)[13]. Others have shown that TNF and Dex can induce MT expression in both liver and heart muscle[14,15]. Interestingly, MT2 expression has also been found to be elevated in obese human adipocytes[16].

Cyp1b1 is a cytochrome P450 enzyme appreciated for its ability to activate polyaromatic hydrocarbons. The P450 class of enzymes is well known to be potential sources of superoxide and hydrogen peroxide as byproducts of their catalytic cycles[9]. Cyp1b1, in particular, is both known to be induced by oxidative stress[17] and to be a potential source of ROS[18].

Xanthine dehydrogenase is another enzymatic source of ROS, producing H2O2 or superoxide as a byproduct of converting xanthine to uric acid[19]. It is known to be induced by TNF in primary hepatocytes[20] and by Dex in skeletal muscle[21]. Serum uric acid, the product of XDH catalysis, is also known to be associated with human insulin resistance[22].

Ceruloplasmin is a protein whose metal binding properties are thought to bestow it with antioxidant activity. Ceruloplasmin knockout mice have been found to have increased iron deposition and oxidative stress in brain tissue[23]. Aceruloplasminemic humans have elevated levels of oxidative stress markers in their blood[24]. Our findings concerning its transcriptional sensitivity to TNF and dex find support from previous studies. Rat ceruloplasmin levels have been shown to be elevated in response to TNF injection[14]. It has also been shown to be induced by Dex in hepatocytes[25]. Interestingly its levels are elevated in the blood of obese mice[26] as well as humans with glucose intolerance[27].

Haptoglobin is a hemoglobin binding protein which by sequestering hemoglobin prevents its prooxidant ability to cause lipid peroxidation[9]. Overexpression of haptoglobin confers cellular tolerance to hydrogen peroxide exposure[28]. Mice lacking haptoglobin experience more tissue oxidative damage in response to hemolysis[29]. Its expression is also induced by TNF and Dex in hepatocytes[30,31] and in adipose tissue from

transgenic mice overexpressing TNF receptors[32]. Haptoglobin has been found to be elevated in multiple insulin resistant states: in adipose tissue and serum from obese humans[33], in adipocytes from multiple obese insulin resistant mice including ob/ob, agouti, and db/db mice[32], and finally in serum from mouse models of burn trauma and infection[34,35].

To substantiate the inference that ROS are altered by TNF- and Dex-treatment, we sought to measure ROS levels directly. The direct measurement of ROS is, however, challenging for two reasons. The chief difficulty is that their half-lives are exceedingly short: superoxide on the order of microseconds[9]. To directly measure their levels *in vivo* requires the assay to preserve the integrity of the cells. This is commonly achieved using a membrane permeable chemical probe which reacts with ROS to give a detectable signal. The second problem is the absence of such probes that are specifically oxidized by ROS as opposed to other cellular molecules with oxidizing potential. Consequently the diffusible probe based methods have been interpreted to indicate "redox shift" or "oxidative stress", a more general phenotype.

Another strategy for inferring ROS levels is to detect their oxidative impact on cellular molecules. The advantage of such an indirect method is that ROS leave biochemical footprints that can be much longer lived than the ROS species themselves. Three classes of ROS markers exist: oxidatively modified proteins, lipids, and nucleic acids. Because such markers can persist long after the oxidizing species has degraded, they can reflect the cell's cumulative ROS exposure. In assays of oxidative stress markers it is typical to lyse the cells prior to the detection phase. In such cases careful attention must be paid that the lysis phase not introduce artificial oxidation of the molecules of

interest.

We performed two of the most commonly used ROS assays to validate the gene expression prediction. In the first we measured oxidation of a redox sensitive fluorescent dye, dichlorofluoroscein (DCF), after acutely (30 min.) incubating it with live cells. The resulting signal was higher by 50% and 65%, respectively, in insulin-resistant cells produced by TNF- and Dex-treatment (Fig.1a). We also measured the degree of protein carbonylation in the insulin resistant state. Protein carbonylation is an oxidative modification that results when ROS reacts with amino acids to form carbonyl containing products[9]. Levels of this marker of oxidative stress were elevated 50% and 110% in TNF- and Dex-treated cells. Finally, the correlation between ROS and insulin resistance was strengthened even further when we measured the effect of the clinical insulin sensitizer pioglitazone on oxidative stress. When co-adminstered with TNF and Dex, both DCF activation and protein cabonylation levels were diminished to near control levels (Fig.1b). It should be stated that these assays have no way of telling whether the treatments affected ROS synthesis or ROS destruction, just that the net result is consistent with increased ROS levels. To specifically measure the synthesis or destruction sides of the equilibrium would require the ability to completely inhibit the other side.

These results can be corroborated by indirect evidence from different cellular systems treated with Dex or TNF. Hepatocytes treated with Dex show increased oxidative stress in the DCF assay[6]. Rats treated with Dex have increased muscle carbonyl formation[36]. TNF has been shown to cause oxidative stress in many systems, including muscle[37]. With respect to specific ROS assays: TNF increases glutathione oxidation in primary hepatocytes (see below)[38] and increases DCF oxidation in fibroblasts[39]. Primary

skeletal muscle from mice exposed to TNF also shows increased levels of DCF oxidation[40]. Moreover, in mice implanted with a tumor (a model of TNF treatment) gene expression markers of ROS were found to be elevated[41].

Of all the effects individually exerted by TNF or Dex, our gene expression data point to ROS as a common response. Moreover amid the long history of studies on Dex and TNF one can find separate accounts of their ability to elevate ROS levels, further strengthening the plausibility of our observations. There may also be other common pathways that we did not detect due to the limitations of gene expression as a state description or limitations in our methods of analysis. Moreover, there are likely to exist pathways unique to TNF or Dex that contribute to insulin resistance. In what follows we focus on the question of whether the common elevation of ROS levels in states of insulin resistance is in fact causal.

Methods

Reagents. Pioglitazone (Alexis Biochemicals). Dexamethasone (Biomol). TNF (Cell Sciences). "Complete mini" protease inhibitors, (Roche Biochemicals). Mannitol, Insulin, Isobutylmethylxanthine, Digitonin (Sigma). Trizol, DMEM (Invitrogen). CM-DCF (Molecular Probes). Oxyblot (Serologicals). Fetal Bovine Serum, Bovine Calf Serum (HyClone). 3T3-L1 preadipocytes were obtained from ATCC.

Cell Culture. Early passage 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium supplemented with Glutamax, 10% bovine calf serum, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were maintained at 37°C, 10% CO_2 and mycoplasma-free. Differentiation medium was added to pre-adipocytes two days after reaching confluence (Day 0). Differentiation medium consisted of DMEM supplemented with 10% Fetal Bovine Serum (FBS), to which was added a cocktail containing 0.11 mg/ml isobutylmethylxanthine, 1ug/ml insulin, 250nM dexamethasone and 1.5uM pioglitazone. On Day 2, the medium was replaced with DMEM/10%FBS containing 1ug/ml insulin and 1.5uM pioglitazone. From Day 4 onward, cells were maintained in DMEM plus 10%FBS with a media change every other day until experimental treatments were initiated. Batches of pre-adipocytes and FBS were screened for their ability to give rise to adipocytes satisfying the positive control behavior depicted in Fig.1a,b(Ch.2).

Induction of insulin resistance. Dex (20 nM) or TNF (4 ng/ml) treatment was initiated with mature adipocytes anywhere from day 8 to day 14 of differentiation. Media was changed daily for TNF for a total incubation time of 4 days. Dex media was changed every other day for a total of 8 days. For experiments in which transgene expression was induced, doxycycline was added to cells on day 4 of differentiation and TNF or Dex treatment was always initiated on day 8. For washout experiments cells were made insulin resistant as above at which point media absent TNF or Dex was added and refreshed every two days for a total of 8 days.

mRNA isolation, target preparation and hybridization. RNA was harvested from cells that were grown and differentiated in 10cm dishes. Cells were lysed in Trizol and RNA was prepared according to the manufacturer's instructions. We prepared target as previously described[1] and hybridized it to Affymetrix M430A arrays.

Data scaling and expression analysis. For each treatment group, a reference data set was generated by averaging the expression of each gene over all three replicate hybridizations. Each data set was then fit to its reference set, using a least-squares fit. Finally, each data set was scaled to have a mean expression of 1. To assess differential expression, we used the software package SAM, creating a list of genes ordered according to a modified signal-to-noise metric[2]. We used the GSEA software package and the MSigDB v1.0 database[3] to identify functional gene sets (the "c2" subdatabase) whose expression was enriched in TNF or Dex treated cells. See http://www.broad.mit.edu/gsea/msigdb/msigdb_index.html for further information regarding MsigDB.

DCF assay. Cells were washed twice in KRP buffer, incubated in prewarmed KRP containing 25mM glucose and 5uM CM-DCF, and placed at 37°C. After 30 min., cells were washed once with KRP and flourescence was immediately measured in a plate reader with an excitation/emission frequency of 485/515. DCF values were calculated after subtracting background fluorescence levels, measured under identical conditions but without DCF.

Protein Carbonylation. Carbonyl levels were determined in 10ug of adipocyte protein lysate using a commercial kit (Oxyblot), according to manufacturer's instructions.

Western Blots. Cells were scraped into protein lysis buffer and flash frozen. Thawed lysates were spun at 10,000 x g for 30min at 4°C. A total of 20ug of clarified lysate per lane was separated by SDS-PAGE, followed by transfer to a nitrocellulose membrane. Individual proteins were detected with the specified antibodies and visualized by blotting with horseradish peroxidase-linked secondary antibodies.

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Figure 1. Direct evidence for ROS elevation in the insulin resistant state

(a) Measurement of cellular redox status. Bars show rates of DCF oxidation in untreated cells, cells treated with TNF alone, Dex alone, pioglitazone alone, TNF plus pioglitazone and Dex plus pioglitazone. Results are mean +/- s.e.m. Asterisks: $p<0.05$ vs. untreated, t-test. Plus sign: $p<0.05$ vs. TNF or Dex alone, t-test.

(b) Measurement of chronic oxidative stress. Immunoblots show total protein carbonylation. In the left panel, lanes 1-3 were loaded with an equal amount of protein from cells that were untreated, treated with TNF alone and treated with TNF plus pioglitazone. The right panel is analogous with Dex replacing TNF.

Fig. 1

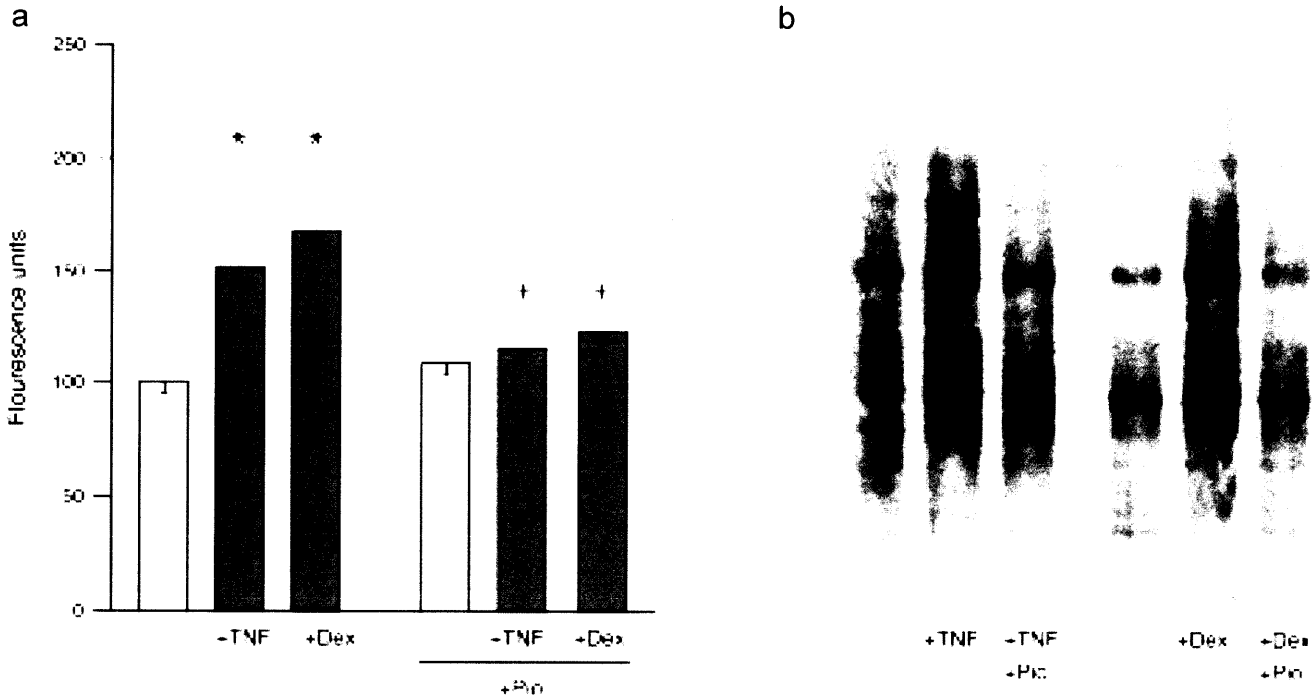
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Table 1 Analysis of gene expression induced by TNF- and Dex-treatment.

1a. ROS-related genes induced by Dex and TNF.*

Gene	Fold Change	
	TNF	Dex
Metallothionein 1	1.6	3.8
Metallothionein 2	4.9	4.6
Cyptochrome P450, 1b1	4.1	2.5
Xanthine Dehydrogenase	2.6	4.2
Haptoglobin	4.5	2.7
Ceruloplasmin	7.1	3.4

*The intersection of the top 350 genes induced by Dex and the top 350 genes induced by TNF contains 34 genes, of which 6 are related to ROS biology. The top 350 genes correspond roughly to those with a false-discovery rate of less than 5%.

1b. Gene sets showing positive association with Dex treatment and TNF treatment*

TNF			Dex		
Gene Set	N	NES	Gene Set	N	NES
ROS	36	2.78	ROS	36	4.02
TNFR2 pathway	28	2.68	Electron Transport Chain	154	3.84
TNF pathway	50	2.65	OXPHOS (Broad)	138	3.76
Death receptor	52	2.54	Krebs-TCA Cycle	36	3.34
Interferon gamma	31	2.46	OXPHOS (GenMAPP)	36	3.33
G-alpha-13	54	2.46	Growth Hormone / AKT	37	3.11
Cell Cycle (G1/S)	27	2.45	Proteasome pathway	35	3.04

* Gene sets determined by GSEA method[3] from among 475 sets. N, number of genes in set. NES, normalized enrichment statistic. These sets all have false-discovery rates < 0.001, based on permutation of gene labels.

Table 2. Analysis of gene expression down-regulated by TNF- and Dex-treatment. *

TNF			Dex		
Gene Set	N	NES	Gene Set	N	NES
OXPHOS	138	3.28	Rhodopsin-like GPCRs	135	2.22
Electron Transport Chain	154	3.25	Ctla4 pathway	37	2.11
Krebs-TCA Cycle (Broad)	36	2.93	Inflammation pathway	31	2.06
Glycolysis Gluconeogenesis	66	2.67	IL-12 pathway	31	1.66
TCA Cycle (GenMAPP)	29	2.56	Wnt calcium/cGMP pathway	30	1.63
Fatty Acid Processing	32	2.57	S1P signaling	36	1.58
Pyruvate Metabolism	51	2.55	Calcineurin pathway	41	1.52

*Gene sets showing a negative association with Dex treatment and TNF treatment, determined by GSEA method from among 475 sets. N, number of genes in set. NES, normalized enrichment statistic.

Table 3. Individual genes upregulated by TNF- and Dex-treatment.*

Upregulated Genes	Fold Change	
	TNF	Dex
Cytochrome P450, 2f2	9.0	2.1
LR8	7.2	3.3
tetraspanin 4	2.6	1.5
inter alpha-trypsin inhibitor, heavy chain 4	4.2	2.0
kidney expressed gene 2	7.4	3.5
complement component 3	2.4	3.6
angiopoietin-like 4	2.6	2.3
carboxy-terminal domain, RNA polII, small phosphatase 2	1.7	1.5
A20-binding inhibitor of NF-kappa B	3.8	1.6
ras homolog gene family, member U	1.8	2.0
CD8 antigen, beta chain	1.2	1.6
ribosomal protein L13a	1.3	3.6
NFkappaB light chain gene inhibitor, alpha	3.7	1.7
Slit-like 2 (Drosophila)	1.9	1.4
nucleosome assembly protein 1-like 1	1.8	2.1
S100 calcium binding protein A10	1.5	2.6
G protein-coupled receptor 175	2.5	1.6
small nuclear ribonucleoprotein D2	1.2	1.5
complement component 1, r subcomponent	4.6	1.3
gelsolin	1.5	2.8
p65/RelA subunit of NF-kappaB	1.8	1.8
baculoviral IAP repeat-containing 3	2.1	1.8
thrombospondin 2	2.7	2.1
expressed sequence AA959742	1.5	1.7
ribosomal protein S2	1.3	1.7
granulin	1.8	2.1
growth hormone receptor	2.1	2.6
ADP-ribosylation factor 3	1.4	1.8

*Genes unrelated to ROS that are upregulated by Dex and TNF. The intersection of the top 350 genes induced by Dex and the top 350 genes induced by TNF contains 34 genes, of which 28 are not obviously related to ROS biology.

Table 4. Genes downregulated by TNF- and Dex-treatment.*

Downregulated Genes	Fold Change	
	TNF	Dex
carbonic anhydrase 3	4.6	8.9
G0G1 switch gene 2	4.5	1.9
electron transferring flavoprotein, beta	2.0	1.8
mitochondrial adenine nucleotide translocator	2.3	1.6
monoglyceride lipase	2.2	1.7
pyruvate carboxylase	2.6	1.4
laminin, beta 3	2.3	2.8
glycerol phosphate dehydrogenase 1	2.2	1.5
heme-binding protein	1.8	1.9
nitric oxide synthase 3	1.9	2.1
CD1d1 antigen	2.2	2.0
farnesyl diphosphate farnesyl transferase 1	1.7	1.9
adenylate kinase 2	1.8	1.4
2-hydroxyphytanoyl-CoA lyase	1.7	1.6
type XV collagen	2.1	2.2
adrenergic receptor, beta 3	2.4	2.0
vanin 3	1.5	1.5
guanine nucleotide binding protein, beta 5	2.0	1.5
OTU domain, ubiquitin aldehyde binding 2	1.6	1.8
ILS ATP citrate lyase	1.9	2.1
procollagen, type VI, alpha 2	1.8	1.9
Rab3d, member RAS oncogene family	1.6	1.3
phosphofructokinase, liver, B-type	1.7	1.7

*Genes downregulated by Dex and TNF. The intersection of the top 350 genes downregulated by Dex and the top 350 genes downregulated by TNF contains 23 genes.

Chapter 4. Evidence that ROS Causes Cellular Insulin Resistance

We sought to test whether ROS plays a causal role in insulin resistance by assessing whether a variety of treatments chosen specifically as suppressors of ROS would also act as insulin sensitizers. Here we discuss the properties of putative antioxidants that justify their use as ROS scavengers. Then we report the results of six different antioxidant treatments on TNF and Dex induced insulin resistance.

4.1 Properties of antioxidants

Antioxidants molecules are ubiquitous having been found in everything from coffee to orange juice to red wine, but exactly what properties warrant the term "antioxidant"? There are at least five typical criteria; satisfaction of any given one has been used as grounds for the antioxidant label. The first is the most fundamental: can the molecule inhibit an in vitro reaction between an oxidant and a target molecule? Second, in cells exposed to an exogenous oxidant can it prevent some measure of oxidative stress? Third, in these same cells can it prevent a functional effect of the oxidant (e.g. cell death)? Fourth, in cells with an endogenous rise in oxidant levels due to a specific deficiency in a ROS scavenging gene can the molecule prevent some measure of oxidative stress? Finally, can it prevent a functional consequence of such a deficiency? There is also a criterion for the special case of a putative antioxidant that is also an endogenous cellular factor: do depressed levels of this factor result in oxidative stress? Notice that these criteria often do not specify the nature of the oxidant nor the particular

measure of oxidative stress. This reflects the variety of oxidant agents and the paucity of specific, direct assays of ROS; the end result is liberal standards that accommodate a wide variety of "antioxidants". The fourth and fifth criteria are arguably the most stringent for judging a molecule's *in vivo* specificity for hydrogen peroxide or superoxide: specificity is supported by both demanding that it complement a ROS specific phenotype and by requiring it to reverse some measure of elevated ROS, thereby minimizing the probability that complementation is downstream of the intrinsic ROS scavenging deficit. One goal of this discussion is to recognize that ROS studies need to be interpreted in the context of the detailed properties of the antioxidants used. Otherwise a proliferation of positive and negative results obtained with different antioxidants can lead to confusion, as has been the case in the field of insulin resistance.

To illustrate this range of properties we evaluate four representative antioxidants: vitamin E, lipoic acid, N-acetylcysteine, and MnTBAP. Vitamin E is an antioxidant obtained from the diet whose in vitro activity is to prevent oxidative damage to lipids, in particular lipid peroxidation. It does not scavenge ROS per se, it simply repairs the damage they inflict on lipids[1]. Tissues from vitamin E deficient animals show evidence of lipid peroxidation and they are more sensitive to the toxic effects of pure oxygen. In settings where lipid peroxidation is the mechanism by which ROS exert their effect, vitamin E could be an effective treatment.

Lipoic acid is an endogenous molecule used as a cofactor in the enzymatic decarboxylation of alpha-keto acids. Its antioxidant activity in vitro is to scavenge hydroxyl radicals. It also has the ability to bind metals such as copper and iron, preventing them from participating in radical generating reactions[1]. Lipoic acid can block

oxidative stress as assayed by DCF and glutathione oxidation in epithelial cells exposed to tert-butylhydroperoxide (an oxidant)[2]. It can also protect muscle and fat cells in culture against the insulin resistance inducing effects of hydrogen peroxide[3,4]. But there is no evidence describing its effects on oxidative stress produced endogenously as from a genetic deficiency of ROS scavenging. This importance of this last point is emphasized the following observation: if a molecule has in vitro antioxidant activity then it is not much of a stretch to imagine that when coincubated with an exogenous oxidant it will prevent that oxidant's effects on cells.

The most widely used antioxidant in the literature is N-acetylcysteine (NAC). It can directly scavenge H_2O_2 as well as hydroxyl radical and has also been shown to stimulate glutathione synthesis[5]. It can reduce oxidative stress in cells exposed to hydrogen peroxide as assayed by DCF and glutathione oxidation[6]. NAC prevents exogenous hydrogen peroxide's effect on primary cardiac muscle gene expression[7] as well its effects on adiponectin secretion from adipocytes[8]. Finally, the survival of SOD1 deficient yeast was shown to be significantly improved by NAC treatment[9].

Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), like NAC has been shown to satisfy four out of the five criteria. It has both catalase and superoxide dismutase activity in vitro[10,11]. It can prevent cell injury and oxidative DNA damage in cells exposed to hydrogen peroxide[10,12]. And it complements both SOD-/- bacteria[11], SOD2-/- primary cultured neurons[13], and SOD2-/- mice[14], extending their survival in each case. That MnTBAP has this effect in organisms as evolutionarily distant as bacteria and mice strengthens the case for its *in vivo* ROS scavenging activity as opposed to the ability to rescue some downstream function.

4.2 Small Molecule Antioxidants Prevent Cellular Insulin Resistance

We began our investigation of the causal role of ROS in insulin resistance by evaluating the two small anti-oxidant molecules, NAC and MnTBAP. NAC and MnTBAP were applied to adipocytes concomitantly with TNF or Dex. Both anti-oxidants showed dose-dependent ability to suppress insulin resistance induced by either treatment, preventing 25-65% of the defect in insulin- mediated glucose uptake (Fig.1a,b); importantly, neither compound increased insulin action on its own (data not shown). Furthermore, MnTBAP largely prevented the increase in protein carbonylation (Fig.5b). We also briefly explored the effect of serum lot on TNF induced ROS formation and MnTBAP rescue. Over three different serum lots we found a tight correlation between the extent of insulin resistance induced by TNF, the concomitant degree of oxidative stress as assayed by DCF (Fig.2), and the extent of IMGU rescue by MnTBAP (Fig.3). It will be important to perform similar experiments with Dex. These results add yet further evidence for the causal importance of ROS to insulin resistance in our model.

In addition to its effect on glucose uptake we also examined the effect of MnTBAP on various parameters of insulin signaling. Treatment with TNF and Dex decreased levels of insulin stimulated serine phosphorylation on Akt and p70S6 kinase (p70S6K), while co-treatment with MnTBAP largely prevented this decrease (Fig.4). As Akt participates in the acute insulin to Glut4 signaling cascade, its improved activation could explain how MnTBAP exerts its insulin sensitizing effect. On the other hand p70S6K is known to be unimportant for the acute signal transmission from insulin to Glut4. In this case, the result

could be viewed as an independent metric reflecting improved insulin signaling. Note that impaired insulin dependent p70S6K phosphorylation is not a metric limited to our system but has also been shown to depressed in insulin resistant Pima Indians[15] and a mouse model of burn injury[16]. The Akt result can be viewed in a similar light if the stronger causal interpretation of its activation is false.

Elevated ROS levels are known to stimulate threonine phosphorylation of JNK[17], a kinase notable for its previous links to insulin resistance[18,19]. TNF and Dex-treatment increased phosphorylation of JNK, an event confirmed to be ROS dependent as MnTBAP diminished it to nearly baseline levels (Fig.5a). JNK therefore becomes an attractive candidate for mediating the effects of ROS on insulin resistance, a possibility we discuss further in Ch.6.

Previous data that bear on the causality of ROS in cellular insulin resistance have demonstrated that exposure to high dose hydrogen peroxide is sufficient to reduce insulin stimulated glucose uptake in 3T3-L1 adipocytes[20] and muscle cells[3]. However, such findings do not imply that ROS plays a causal role in any physiological model of insulin resistance. This requires showing that insulin resistance can be prevented to some degree by blocking the elevation of ROS levels. Our small molecule antioxidant data provide a first line of evidence that ROS elevation is in fact necessary for TNF and Dex to exert their effects on IMGU.

There exist numerous examples of indirect support for our findings. In particular, there are multiple reports that TNF and Dex create oxidative stress in other cell types, as we discussed in Ch.3. More importantly, NAC and MnTBAP have been found to block functional effects of TNF and Dex such as the induction of apoptosis or muscle

weakness[21-24]. Finally, it has been commonly observed that ROS activates JNK, an effect which it is now known can prevented by MnTBAP[25]. A persistent theme of this chapter is that the ability to demonstrate a causal role for ROS in insulin resistance or any other phenotype hinges on the appropriate choice of antioxidant.

4.3 ROS Scavenging Gene Expression Prevents Cellular Insulin Resistance

We further explored the causal relationship between ROS and insulin resistance by constructing four 3T3-L1 cell lines carrying transgenes encoding ROS scavenging enzymes, including CuZnSOD, MnSOD, a form of catalase with its peroxisomal localization signal removed ('cytocat'), and catalase targeted specifically to the mitochondrion ('mitocat') (see Methods). The transgenes were delivered by retroviral transduction with expression directed by an inducible promoter (Tet-on system), so as to minimize any effects of transgene expression on adipocyte differentiation. The transgenes were induced prior to treatment with TNF or Dex, resulting in a 3-5-fold increase in enzyme activity above endogenous levels (Fig.7). Mitocat and cytocat were most potent, preventing up to 65% of the reduction in insulin-stimulated glucose uptake; CuZnSOD and MnSOD prevented 50% and 25%, respectively (Fig.6). Neither the transgene inducer (doxycycline) nor the inducible expression system itself had any effect on glucose transport (data not shown).

As with the antioxidants NAC and MnTBAP we looked for evidence that ROS scavenging genes could reverse the effects of TNF and Dex in other experimental

settings. Catalase overexpression suppresses dex induced thymocyte apoptosis[26]. CuZnSOD overexpression can protect myeloid cells from growth inhibition by TNF[27]. MnSOD overexpression has been shown in numerous settings to block TNF induced apoptosis[28].

What is the cellular source of ROS in our models? While the literature brims with connections between Dex, Tnf and mitochondria, our own data do not permit a definitive connection. Cytoplasmic versus mitochondrial localization of catalase did not make a significant difference in their insulin sensitizing effect. SOD1 is found both in the mitochondrial intermembrane space and in the cytoplasm so its effect is not decisive either. SOD2 is mitochondrial, but its sensitizing effect was the weakest; however it would be difficult to exclude mitochondria as a significant ROS source based on this. An alternative enzymatic ROS source that has received much attention is the NADPH oxidase complex[8,29]. Further studies will be needed to thoroughly vet this candidate in addition to a putative mitochondrial source.

With the identification of ROS as a common response to TNF and Dex treated adipocytes and the demonstration that this response is required for their full effect on insulin resistance we accomplished the first goal of the strategy outlined in Ch.2. Our next goal was to determine whether our efforts to carefully setup the cellular model would translate into a successful prediction of a causal role for ROS *in vivo*.

Methods

Reagents. MnTBAP was custom synthesized by Frontier Scientific. Pioglitazone (Alexis Biochemicals). Rosiglitazone KCl (Cayman Chemical). Puromycin, Dexamethasone (Biomol). TNF (Cell Sciences). Antibodies to AKT, Phospho-AKT(Ser473), p70S6K, Phospho-p70S6K(Thr389), JNK, Phospho-JNK(Thr183/Tyr185) were all obtained from Cell Signaling. Doxycycline (Clontech). "Complete mini" protease inhibitors, Hygromycin (Roche Biochemicals). N-Acetylcysteine, Mannitol, Insulin, Isobutylmethylxanthine, Digitonin (Sigma). Trizol, DMEM (Invitrogen). CM-DCF (Molecular Probes). Oxyblot (Serologicals). Fetal Bovine Serum, Bovine Calf Serum (HyClone). 3T3-L1 preadipocytes were obtained from ATCC.

Cell Culture. Early passage 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium supplemented with Glutamax, 10% bovine calf serum, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were maintained at 37°C, 10% CO_2 and mycoplasma-free. Differentiation medium was added to pre-adipocytes two days after reaching confluence (Day 0). Differentiation medium consisted of DMEM supplemented with 10% Fetal Bovine Serum (FBS), to which was added a cocktail containing 0.11 mg/ml isobutylmethylxanthine, 1ug/ml insulin, 250nM dexamethasone and 1.5uM pioglitazone. On Day 2, the medium was replaced with DMEM/10%FBS containing 1ug/ml insulin and 1.5uM pioglitazone. From Day 4 onward, cells were maintained in DMEM plus 10%FBS with a media change every other day until experimental treatments were initiated. Batches of pre-adipocytes and FBS were screened for their ability to give rise to adipocytes satisfying the positive control behavior depicted in Fig.1 (Ch.2).

Induction of insulin resistance. Dex (20 nM) or TNF (4 ng/ml) treatment was initiated with mature adipocytes anywhere from day 8 to day 14 of differentiation. Media was changed daily for TNF for a total incubation time of 4 days. Dex media was changed every other day for a total of 8 days. For experiments in which transgene expression was induced, doxycycline was added to cells on day 4 of differentiation and TNF or Dex treatment was always initiated on day 8. For washout experiments cells were made

insulin resistant as above at which point media absent TNF or Dex was added and refreshed every two days for a total of 8 days.

Glucose Uptake Assay. Cells in 12 well dishes were washed twice with KRP (127mM NaCl, 4.7mM KCl, 0.9mM $MgSO_4$, 10mM $NaPO_4$, 0.9mM $CaCl_2$) and incubated with pre-warmed KRP (670ul) containing 0.2% fatty acid free BSA and the appropriate concentration of insulin (10nM insulin as a maximum stimulus). The dish was then allowed to float in a 37°C water bath for 30min. After this period, tritiated 2-deoxyglucose (2DG) and unlabeled 2DG (total vol. 75ul) were dispensed into each well for a final concentration of 1uCi/ml and 0.1mM respectively. Cells were incubated for an additional 5 min. at 37°C and the reaction was stopped by immersing the entire dish in a reservoir of ice cold PBS. While submerged, the plate was gently swirled for 30 sec. The plate was then dried, excess PBS was aspirated, and 740ul of digitonin release buffer (100mg/ml Mannitol, 1mg/ml digitonin) was applied to each well. After 10 min. at room temperature, 665ul from each well was counted in a scintillation counter. Fresh media was always added to cells 24 h prior to assay.

Western Blots. Cells were scraped into protein lysis buffer and flash frozen. Thawed lysates were spun at 10,000 x g for 30min at 4°C. A total of 20ug of clarified lysate per lane was separated by SDS-PAGE, followed by transfer to a nitrocellulose membrane. Individual proteins were detected with the specified antibodies and visualized by blotting with horseradish peroxidase-linked secondary antibodies.

Construction of transgenic cell lines. cDNA clones of *MnSOD*, *CuZnSOD* were gifts from Dr. L. Oberley (Univ. of Iowa) cDNA clones of *mitocat* and *cytocat* were gifts from Dr. A. Agarwal (Univ. of Florida) and Dr. J.A. Melendez (Albany Med. Center). The cDNA clone for *sc-rttaM2* was kindly provided by Dr. W. Hillen (Univ. of Erlangen, Germany). The *sc-rttaM2* gene was amplified by PCR and cloned into the vector pMSCV-puro (Clontech). To minimize leaky expression, we modified the retroviral vector pRevTRE (Clontech) by exchanging its TRE with that derived from pTRETight (Clontech) to create pRevTREtight. *CuZnSOD* was subcloned from pOberley1 into

pRevTRETight. *MnSOD* was subcloned from pOberley2 into pRevTRETight. *Mitocat* and *Cytocat* were PCR amplified and ligated into pRevTRETight. Stable preadipocyte lines were created by retroviral transduction as previously described[4]. The Tet-on system was introduced in two steps. First, the construct that drives expression of the tetracycline-sensitive transcription factor, sc-rttaM2, was integrated and selected with puromycin (2ug/ml). Second, the construct containing a tetracycline response element (TRE) that drives expression of the transgene of interest was integrated and selected with hygromycin (0.175mg/ml).

Catalase activity. For catalase assays, adipocytes were harvested in 0.1% Triton-X100, 50mM $NaPO_4$ buffer, pH 7 with protease inhibitors. Cells were lysed by flash-freeze thawing 3 times, followed by centrifugation at 10,000 x g for 30 min at 4°C. A total of 1-5ul of lysate was diluted in 500ul of 50mM $NaPO_4$, pH7.0 containing 10mM H_2O_2. The decomposition of H_2O_2 was then followed spectrophotometrically by measuring the rate of decline in absorbance at 240nm over a 1 min. interval.

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Figure 1. Effect of anti-oxidants on insulin resistance.

(a) Partial restoration of TNF or Dex induced insulin resistance by MnTBAP. Bars show insulin-dependent glucose transport. Bars on left indicate untreated cells (white) or TNF-treated cells (grey) co-treated with indicated levels of MnTBAP. Bars on right are analogous for Dex-treated cells. Results are mean +/- s.e.m. Asterisks indicate $p<0.05$ versus TNF or Dex alone, t-test.

(b) Partial restoration of TNF or Dex induced insulin resistance by NAC. Data are analogous to (a).

Fig. 1

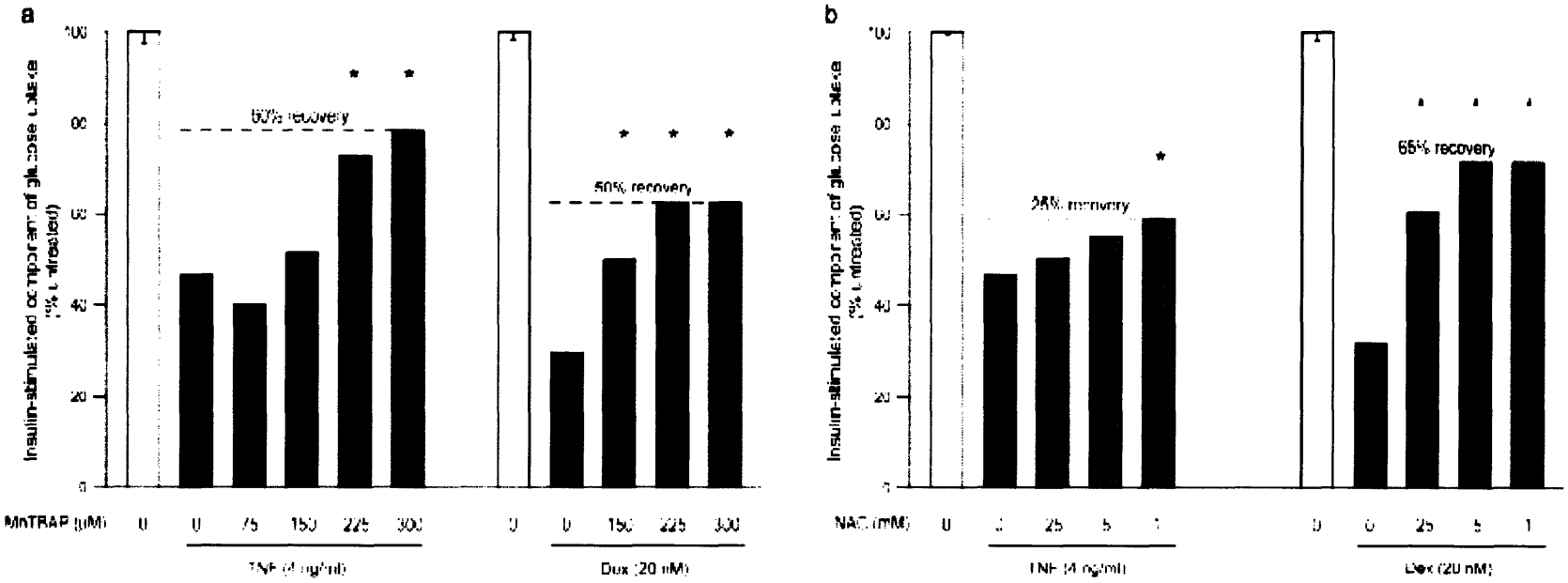

a
b
MnTRAP (μM)
NAC (mM)
TNF (4 ng/ml)
Dex (20 nM)
50% recovery
65% recovery

Figure 2. Serum effect on TNF induced DCF oxidation and insulin resistance.

The effect of different serums on TNF's ability to repress insulin stimulated glucose uptake (y-axis) in relation to TNF induced DCF oxidation (x-axis). TNF mediated insulin resistance is calculated as the % depression in insulin stimulated glucose uptake (total uptake minus basal uptake) relative to vehicle treatment. Each point represents a serum from a different manufacturer. From left to right the serum sources are ATCC, Whitehead Institute, Cellgro, and Sigma. Results are mean +/- s.e.m.

Fig. 2

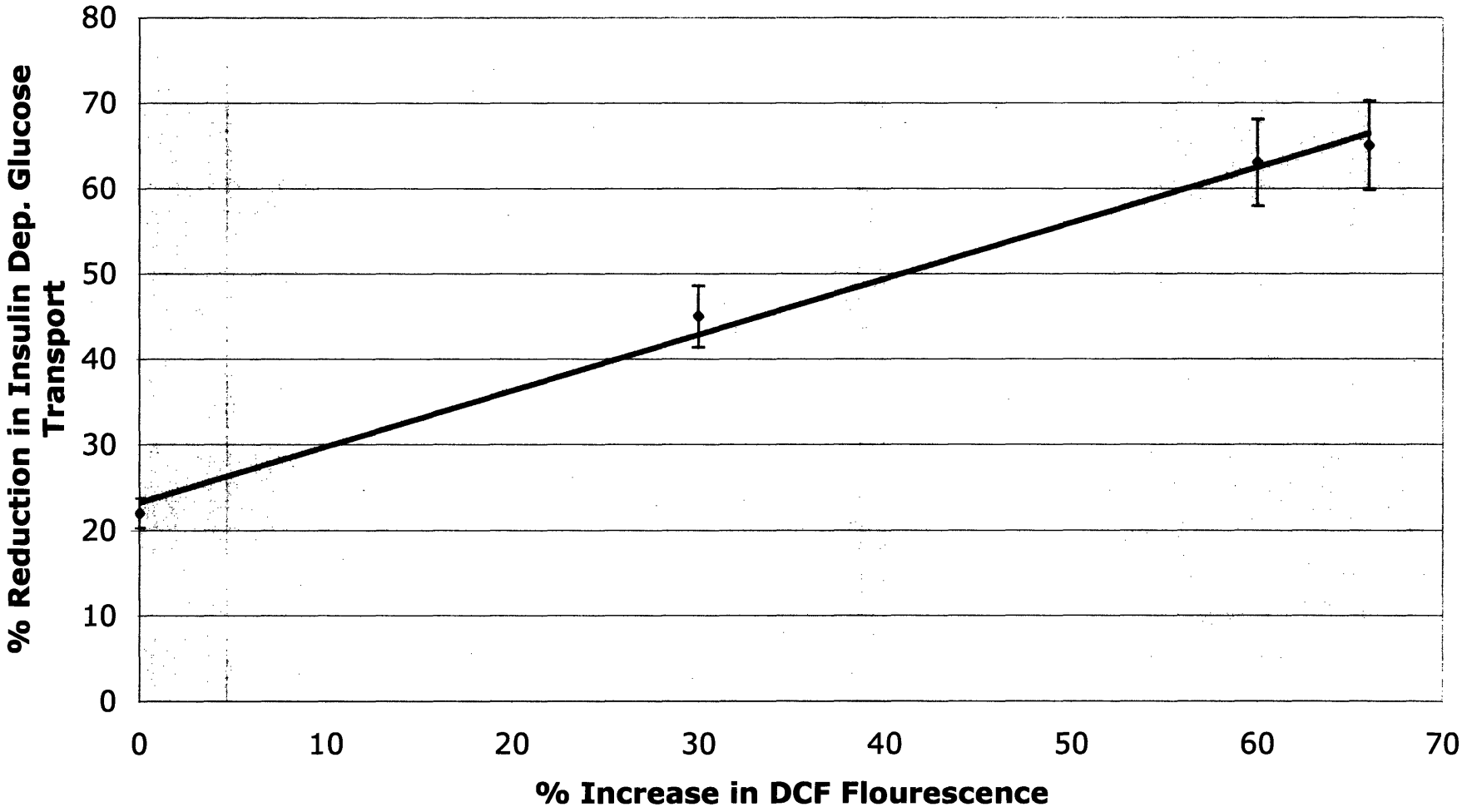
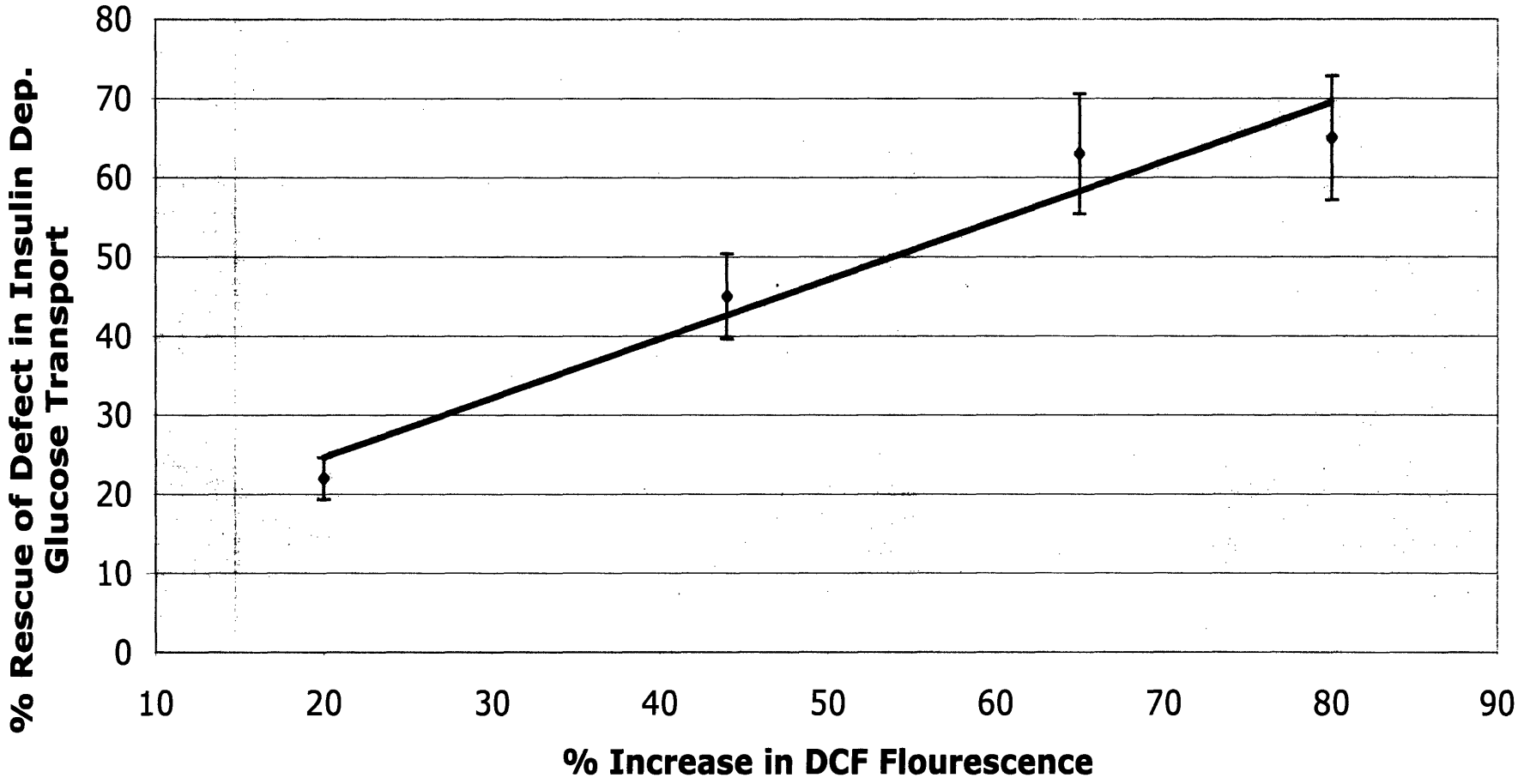

% Reduction in Insulin Dep. Glucose Transport
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
% Increase in DCF Flourescence

Figure 3. Serum effect on TNF induced DCF oxidation and MnTBAP mediated rescue of glucose uptake.

The effect of different serum lots on MnTBAP's ability to prevent TNF mediated insulin resistance (y-axis) in relation to TNF mediated DCF oxidation (x-axis). MnTBAP's effect is calculated as the % recovery of the defect in insulin mediated glucose uptake caused by TNF. Each point represents a serum from a different manufacturer. From left to right the serum sources are ATCC, Whitehead Institute, Cellgro, and Sigma. Results are mean +/- s.e.m.

Fig.3


% Rescue of Defect in Insulin Dep. Glucose Transport
80
70
60
50
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30
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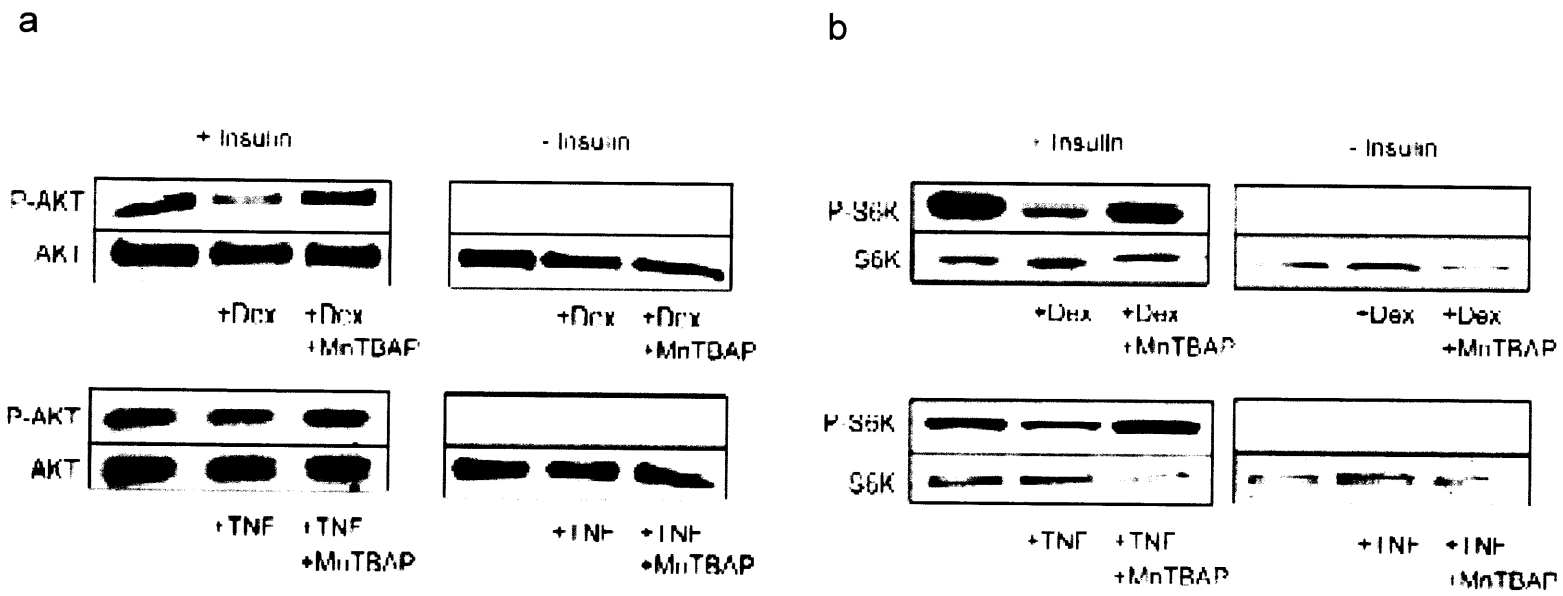
10
0
10
20
30
40
50
60
70
80
90
% Increase in DCF Flourescence

Figure 4. Effect of anti-oxidants on insulin resistant signal transduction.

(a) Effect of MnTBAP on measures of insulin signal transduction in cells with TNF or Dex-induced insulin resistance. Immunoblots show phospho-AKT levels upon acute stimulation of cells with insulin (lanes 1-3) or in the absence of insulin (lanes 4-6). In the top panels lysates from untreated cells were loaded in lanes 1 and 4, lysates from Dex-treated cells in lanes 2 and 5, and lysates from Dex plus 250uM MnTBAP-treated cells in lanes 3 and 6. Total AKT protein levels are shown in the panel immediately below its phosphorylated counterpart. The bottom panels are arranged similarly with cells made insulin resistant by TNF. Signals were quantitated by densitometry and normalized to total protein. The level of insulin stimulated phospho-AKT decreases by 40% and 60%, respectively, in TNF and Dex treated cells. MnTBAP treatment recovers phospho-AKT levels to within 5% (TNF) and 30%(Dex) of that found in untreated cells.

(b) Effect of MnTBAP on measures of insulin signal transduction in cells with TNF or Dex-induced insulin resistance. Stimulation of S6K phosphorylation by insulin is assayed with data arranged as in c). Signals were quantitated by densitometry and normalized to total protein. The level of insulin stimulated phospho-S6K decreases by 35% and 65%, respectively, in TNF and Dex treated cells. MnTBAP treatment recovers phospho-S6K levels to within 25% (both TNF and Dex) of that found in untreated cells.

Fig. 4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- Insulin
P-AKT
AKT
+Dex
+Dex +MnTBAP
+TNF
+TNF +MnTBAP
b
+ Insulin
- Insulin
P-S6K
S6K
+Dex
+Dex +MnTBAP
-Dex
+Dex +MnTBAP
+TNF
+TNF +MnTBAP
+TNF
+TNF +MnTBAP

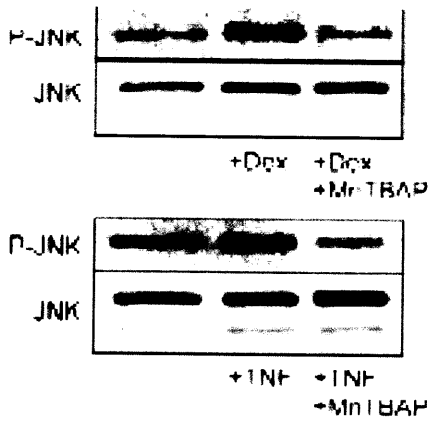
Figure 5. Effect of MnTBAP on JNK activation and marker of oxidative stress.

(a) JNK phosphorylation status in cells treated with Dex, TNF, or co-treated with 250uM MnTBAP. Immunoblot shows phospho-JNK levels. In the top panels lanes 1-3 contain lysates from untreated cells, cells treated with TNF alone, or TNF plus MnTBAP. In the bottom panels lanes 1-3 contain lysates from untreated cells, cells treated with Dex alone, or Dex plus MnTBAP. Total JNK levels are shown in the panel immediately below that of phospho-JNK. Signals were quantitated by densitometry and normalized to total protein. The level of phospho-JNK increases by 25% and 80%, respectively, in TNF and Dex treated cells. MnTBAP treatment restores phospho-JNK to untreated levels.

b) Measurement of chronic oxidative stress. Immunoblots show total protein carbonylation. In the left panel, lanes 1-3 were loaded with an equal amount of protein from cells that were untreated, treated with TNF alone, and treated with TNF plus 250uM MnTBAP. The right panel is analogous with Dex replacing TNF.

Fig. 5

a


P-JNK
JNK
+Dex
+Dex
+MnTBAP
P-JNK
JNK
+TNF
+TNF
+MnTBAP

b

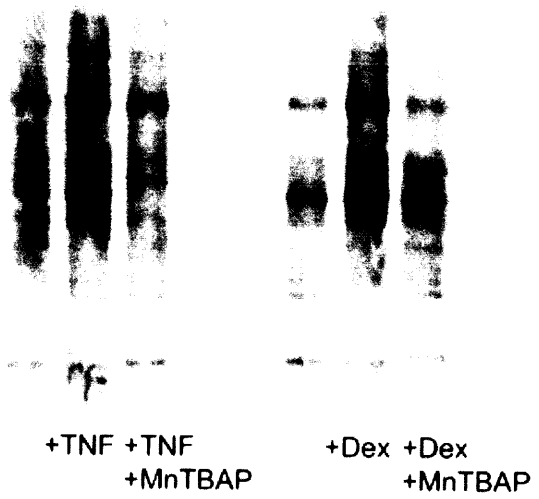

+TNF
+TNF
+MnTBAP
+Dex
+Dex
+MnTBAP

Figure 6. Effects of transgenes on insulin resistance.

(a) Effect of cytocat expression on TNF and Dex induced insulin resistance. Bars show insulin dependent rates of glucose transport in untreated cells with or without cytocat induction (white), TNF treated cells with or without cytocat induction (grey,left), or Dex treated cells with or without cytocat induction (grey, right).

(b) Effect of mitocat expression on TNF and Dex induced insulin resistance.

(c) Effect of CuZnSOD expression on TNF and Dex induced insulin resistance.

(d) Effect of MnSOD expression on TNF and Dex induced insulin resistance.

Results are mean +/- s.e.m. Asterisks indicate $p<.05$ versus TNF or Dex alone, t-test.

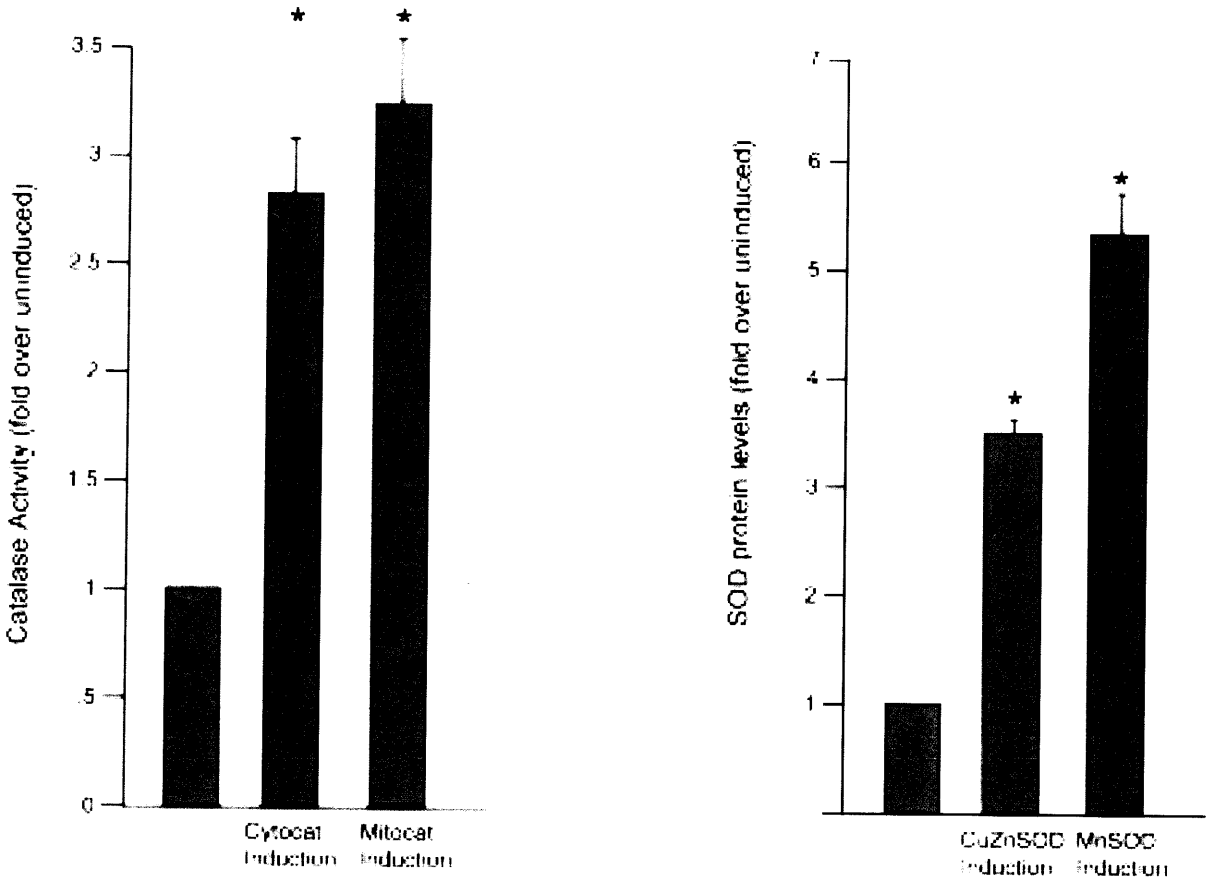
Fig. 6

Figure 7. Characterization of Products of Antioxidant Transgenes

a) Catalase activity. Catalase activity was measured from transgenic adipocytes that had been stimulated for 4 days with doxycycline (2ug/ml). Results are mean +/- s.e.m.

b) SOD levels. SOD protein levels were quantitated by densitometry of an immunoblot of total protein from transgenic adipocytes that had been stimulated for 4 days with doxycycline (2ug/ml). Results are mean +/- s.e.m.

Fig. 7


Catalase Activity (fold over uninduced)
3.5
3
2.5
2
1.5
1
.5
0
*
*
Cytocat
Mitocat
SOD protein levels (fold over uninduced)
6
5
4
3
2
1
*
*
CuZnSOC
MnSOC

Chapter 5. Evidence that ROS causes insulin resistance *in vivo*

5.1 Experimental support for causality in a mouse model of obesity

In this chapter we give an account of the evidence supporting a causal contribution of ROS to insulin resistance in mice and humans. Earlier we formulated a strategy for identifying causal factors in insulin resistance based on the observation that it was present in many different clinical conditions. The clinical observations were reduced to a question about two contrasting cellular models, our investigation of which identified ROS as a shared causal factor. Here we report the results of testing the ROS hypothesis in a third model of insulin resistance, the leptin-deficient ob/ob mouse. We also discuss evidence from the literature supporting a causal role for ROS in rodent models of obesity and close with a critical analysis of the evidence linking ROS to insulin resistance in humans.

Does obesity lead to oxidative stress in mice? Several different ROS assays and rodent models of obesity in have been used to address this question. Cardiac muscle (which supports IMGU) from ob/ob mice display increased protein carbonyls, increased oxidized glutathione, and increased levels of malondialdehyde(MDA), a marker of lipid peroxidation[1]. Both leptin deficient fa/fa rats and high fat diet fed rats also show evidence of lipid peroxidation (TBARS) in cardiac muscle[2]. Furthermore fa/fa rats show increased levels of protein carbonylation in skeletal muscle[3]. In primary fat cells isolated from high fat died fed mice, oxidative stress was demonstrated by the DCF assay[4]. Adipose tissue from KKAy mice, another obese mouse model, displayed oxidative stress as assayed by increased H2O2 production and increased levels of lipid peroxidation in the TBARS assay[5]. Finally, liver mitochondria from ob/ob mice produce H2O2 at twice the normal

rate[6,7]. Furthermore they display elevated levels of malondialdehyde in both whole liver homogenates and purified liver mitochondria[7].

We sought to extend the evidence of a causal role for ROS from the cellular models to an *in vivo* model of insulin resistance, ob/ob mice. These mice become extremely obese, developing significant insulin resistance and glucose intolerance by eight weeks of age. We tested whether chronic delivery of MnTBAP could improve glucose homeostasis. Male ob/ob mice received MnTBAP (2.5, 5, or 10 mg/kg), the TZD rosiglitazone (3 mg/kg), or vehicle alone. Treatments were administered daily, beginning at 8 weeks of age and continuing for a period of 12 weeks. These doses of MnTBAP are similar to or lower than doses shown to improve longevity in Sod2 knockout mice[ref].

MnTBAP produced a dose-dependent improvement in several measures of glucose homeostasis without having an effect on body weight over the duration of the treatment period (Fig. 1). For example, mice receiving the highest dose of MnTBAP showed nearly normal glucose levels in the fed state (~15% above levels from lean C57BL/6 mice in MnTBAP treated vs. ~50% in vehicle treated) (Fig. 1). Interestingly, rosiglitazone lowered fed glucose below the level of lean mice. This likely reflects its ability to stimulate basal glucose uptake (as we also observed in the 3T3-L1 model)[8-11]. MnTBAP also improved glucose tolerance and insulin sensitivity (at 5 and 10 mg/kg), with a maximum effect comparable to that of animals treated with rosiglitazone (Fig.1,2). There was no significant effect on serum insulin levels in the fed or fasted state (data not shown).

On what tissues does MnTBAP act to improve glucose homeostasis? Since the insulin tolerance test assesses the direct effect of insulin on glucose lowering, and since

muscle is the principal sink under such conditions, our results imply that MnTBAP has an insulin sensitizing effect on muscle. However, as MnTBAP was administered systemically the effect could be mediated indirectly by a signal coming from a different tissue such as adipose or liver. The results of the glucose tolerance test (GTT) reflect not only MnTBAP's effect on muscle insulin sensitivity, but also its effect on the insulin secretory response. Given the wealth of evidence for a deleterious effect of ROS on beta-cell insulin secretion[12], it is plausible that MnTBAP treatment led to greater insulin release contributing to an improved GTT response. Tissue specific transgenic mouse experiments will be needed to determine which tissues mediate the effects of ROS on whole body insulin sensitivity.

NAC treatment might also be expected to have a similar insulin sensitizing effect. However, the cell culture data indicated that it was less potent than MnTBAP. It is also known to have a very short half-life in the blood[13], necessitating constant infusion by pumps to maintain adequate blood levels. For these biological and technical reasons we did not pursue studies with NAC.

What other evidence in rodent models of obesity bears on the question of ROS causality? Two different antioxidants have been used to assess this question: lipoic acid and apocynin. Lipoic acid (Ch.4) treatment of obese rats leads to improved IMGU in ex vivo assays of muscle[14]. This observation is difficult to interpret given that lipoic acid can stimulate glucose transport in the absence of insulin[15,16]. Moreover, lipoic acid can act as both a prooxidant and an antioxidant[17,18]. Finally, just as with our studies of MnTBAP, treating whole mice with an agent does not provide evidence for a direct on muscle. In a second study apocynin, a putative antioxidant, was shown to reduce glucose and insulin

levels in obese insulin resistant KKAy mice. These results are difficult to interpret for two reasons. First, insulin sensitivity per se was not measured as the mice were never stimulated with insulin. Secondly, apocynin is a poorly characterized agent whose claims to antioxidant activity stem from its putative inhibition of NADPH oxidase, a ROS generating enzyme system. In all, these are suggestive data but the lack of an *in vivo* assay of insulin sensitivity or a proven antioxidant leave open the question of ROS causality.

In addition to the two cellular models and the obesity model of insulin resistance, hyperglycemia is yet a fourth model of insulin resistance for which there exists evidence consistent with a ROS dependent mechanism. Recent important work in the field of diabetic complications has reinforced the thesis that cells exposed to hyperglycemia experience oxidative stress[19]. Hyperglycemia can also induce insulin resistance as demonstrated by the following observations. Mice with a muscle specific knockout of Glut4 are hyperglycemic, and clamp studies demonstrated that their adipose tissue displays reduced IMGU. To prove that this effect depended on hyperglycemia, the mice were treated with phlorizin, a kidney glucose reuptake inhibitor that promotes glucose excretion in the urine. With the establishment of euglycemia, adipose tissue insulin sensitivity returned[20]. It is also known that poorly controlled type 1 diabetics with hyperglycemia are insulin resistant[21] and that upon treatment induced euglycemia their insulin sensitivity returns. These two facts demonstrate a correlation between states of hyperglycemia, oxidative stress, and insulin resistance. There are two studies that explicitly connect hyperglycemia to insulin resistance through ROS. Rats made hyperglycemic by glucose infusion for 6 hours become insulin resistant, as assayed by the

hyperinsulinemic euglycemic clamp. Moreover, under these conditions skeletal muscle was found to be oxidatively stressed, as indicated by increased protein carbonyl levels. Critically, coinfusion of the antioxidant N-acetylcysteine both prevented carbonyl formation as well as insulin resistance[22]. Complementing these studies in whole animals, a recent study examined the effect of hyperglycemia on cellular insulin resistance. 3T3-L1 adipocytes cultured under hyperglycemic conditions became insulin resistant, an effect that was reported to be ROS dependent[23]. It remains to be determined whether these properties hold in cultured muscle. Furthermore it will be important to evaluate whether the insulin resistance effect of the hyperglycemia-ROS axis in whole animals is due to a direct effect on muscle.

5.2 Clinical observations supporting a causal role for ROS in humans

Two of the most important questions this work stimulates are (1) Is there evidence for oxidative stress in insulin resistant humans? And (2) Does ROS cause insulin resistance in humans?

Measurement of ROS in humans presents special challenges as tissue biopsies are rarely available. As a result a special collaborative effort has been undertaken to identify a reasonable biomarker of oxidative stress[24]. The animal model used for these studies was carbon tetrachloride (CCl_4) treated mice. CCl_4 is activated by the liver to a highly oxidizing molecule known as trichloromethyl radical which then inflicts oxidative damage on protein, lipids, and DNA. Among the molecules assayed in the blood and urine of treated mice, F2 isoprotanes were identified as the most sensitive and dose responsive markers. F2 isoprostanes are prostaglandin-like compounds derived from the

peroxidation of arachidonic acid and are now becoming widely used assays of oxidative stress in both human and mouse studies. Two of the animal models of obesity in which tissue markers of oxidative stress were observed also display evidence of increased plasma and/or urinary F2 isoprotanes: the ob/ob mouse[25] and the fa/fa rat[26]. What's more, a model of glucose toxicity, the streptozotocin treated mouse, also displays elevated urinary isoprostanes[27]. That this biomarker is elevated in mouse models for which tissue specific evidence of oxidative stress exists helps validate its predictive value.

There are now several human studies reporting oxidative stress in obesity induced insulin resistance or type 2 diabetes[28] as measured by increased plasma or urinary F2 isoprostane levels. These studies distinguish between obesity in the absence of T2D[5,29], T2D in the absence of complications[30], and T2D in the presence of complications[30,31]. Since hyperglycemia is well known to cause oxidative stress, observing F2 isoprostanes in fulminant diabetes may simply reflect an effect rather than a cause of insulin resistance. But the finding of oxidative stress markers in obese individuals without T2D supports the possibility that oxidative stress may be a cause. Moreover, weight loss leads to a fall in plasma F2 isoprostane levels[32].

In another study of the association between oxidative stress and human obesity it has been observed that intramyocellular lipid from obese individuals is significantly oxidized relative to that found in lean individuals[33]. Moreover, the lipid that accumulates in the muscle cells of endurance trained athletes is also significantly less oxidized, even less than in lean muscle. These observations are striking because they may explain a paradox: it has been widely suggested that intramyocellular lipid buildup is a cause of

insulin resistance, yet endurance trained athletes are highly insulin sensitive. It may be that the effect of lipid accumulation is greater if it is first oxidized.

Is there any evidence that ROS play a causal role in human insulin resistance? As we discussed in chapter 1.4, evidence of ROS causality can come in two forms: either a primary ROS imbalance is sufficient to cause insulin resistance, or scavenging it can prevent insulin resistance brought on by some condition or treatment. Human trials testing the antioxidants lipoic acid and vitamin E in a setting of insulin resistance have been small and had mixed results[34-36]. In the absence of trials with an agent that possesses many of the antioxidant properties we outlined in chapter 3 (such as MnTBAP), the question of causal necessity remains open.

Interestingly, evidence for causal sufficiency can be found in four rare human diseases whose primary defect is thought to lead to ROS imbalance: familial amyotrophic lateral sclerosis (FALS), Friedrich's ataxia, ataxia telengectasia, and acatalasia. FALS patients harbor a mutation in SOD1 that results in a gain of function phenotype which may lead to ROS generation[37]. In the tissues that have been examined, namely spinal cord and brain, FALS patients display elevated levels of protein carbonylation[38]. There is a spontaneous form of the disease as well, whose etiology is not known, but in which patients also display excess protein carbonylation in spinal cord[39]. Interestingly, abnormal glucose metabolism has been repeatedly observed in ALS patients[40]. In at least one study, a group of ALS patients (mixed FALS and SALS) without diabetes was found to exhibit insulin resistance as assayed by the hyperinsulinemic euglycemic clamp technique[40].

Friedrich's ataxia (FA) patients harbor mutations in the gene frataxin, which is involved in mitochondrial iron regulation. As we discussed in chapter 3, iron can

participate in the Fenton reaction to generate the highly damaging hydroxyl radical. In FA skeletal and heart muscle biopsies displayed a decrease in the levels of aconitase activity, a protein whose inactivation by oxidative stress has been used as a ROS assay[41,42]. Other oxidative stress markers elevated in FA include plasma lipid peroxidation (MDA)[43] and urinary levels of oxidized nucleotides (8DOG)[44]. FA patients are also insulin resistant as assayed by the insulin tolerance test[45].

Mutations in ATM gene underly the disease ataxia telengectasia. ATM has long been known to be involved in the repair of DNA that has been damaged by oxidative stress. But recently evidence has accumulated that it may directly control cellular ROS levels[46]. In ATM knockout mice, DCF assays show elevated ROS levels in hematopoietic stem cells[47]. Brain and testes from these mice also show elevated levels of nitrotyrosinated proteins, another marker of oxidative stress[48]. In AT patients, plasma/urine levels of oxidized nucleotides as well as peroxidated lipids have been observed[42]. The rate of insulin resistance among AT patients has been observed to be as high as 60% when assayed by the insulin tolerance test[49]. This is compared to 7% in the general population.

Finally it has been recently recognized that the rate of diabetes among patients exhibiting low levels of catalase is nearly twice as high as that among the general hospital population[50]. That said, it has not yet determined whether these patients are in fact insulin resistant.

Taken together these four disease examples suggest that conditions that affect ROS balance can lead to insulin resistance.

The comparative nature of our strategy for identifying a common cause of insulin resistance suggests a number of predictions. In particular, one might predict that a range of clinical conditions associated with insulin resistance will also show evidence of increased ROS levels. There is in fact evidence for this association in tumour cachexia, burn injury, sepsis, starvation, acromegaly, and ageing. Tumor bearing rats display elevated skeletal muscle protein carbonyls, and tumor bearing mice have skeletal muscle gene expression signatures indicative of elevated ROS levels[51]. A rat model of burn injury exhibits a rise in levels of skeletal muscle protein carbonyl as well as oxidized glutathione[52,53]. Human burn patients experience a rise in plasma lipid peroxidation (MDA)[54]. Similarly septic rats also have increased skeletal muscle protein carbonyls[55] and septic humans have increased plasma levels of protein carbonyls[56,57]. Starving mice display a similar gene expression signature of ROS[51]. Food deprived rats experience oxidative damage to the liver in the form of increased protein carbonyls and increased mitochondrial ROS production[58,59]; this effect is not limited to the liver as cardiac muscle is also oxidatively stressed as assayed by increased levels of oxidized glutathione[60]. Human acromegalics have high levels of plasma lipid peroxidation (TBARS). In these patients suppression of growth hormone levels (the cause of insulin resistance) with octreotide also suppresses the oxidative stress[61]. The final condition that exposes a correlation between oxidative stress and insulin resistance is aging. Surprisingly, this correlation is strengthened by the inclusion of centenarians who are comparatively insulin sensitive and in whom oxidative stress, as measured by plasma lipid peroxidation (TBARS), is simultaneously reduced[62]. In total it's remarkable how many cases of insulin resistant conditions have been independently discovered to be consistent with the ROS

hypothesis. The looming open question is whether these associations are due to a causal relationship.

We discuss one more example of a striking prediction suggested by our findings. We tested the antioxidant potential of pioglitazone – it was found to decrease DCF oxidation and protein carbonylation in response to TNF and Dex. Others have also noted antioxidant effects of TZDs[63,64]. If true, one might predict pioglitazone could be of therapeutic value in a disease thought to be due to ROS imbalance. One such disease we have discussed is FALS. In two recent studies this prediction was confirmed: pioglitazone was found to extend the survival of mouse models of FALS[65,66]. It is tempting to speculate that such a powerful demonstration of antioxidant activity could also explain a component of its insulin sensitizing effect.

Methods

Reagents. MnTBAP was custom synthesized by Frontier Scientific. Rosiglitazone KCl (Cayman Chemical). One touch ultra glucose strips (Cardinal Health).

Animals. Male *ob/ob* mice were purchased from Jackson Laboratories and randomly assigned to treatment group, so as to ensure each group had an equal average weight. Drugs were resuspended in PBS and each mouse received daily 300 uL subcutaneous injections. Mice were weighed weekly.

Glucose and insulin tolerance tests. For the glucose tolerance test, mice were fasted for 12 h and then injected subcutaneously with glucose (1.0 g/kg body weight). Blood samples were taken at regular time points (0-140 min), and blood glucose levels were determined with a portable glucose meter. For insulin tolerance tests, mice were fasted for 4 h and handled 30 min prior to performing the test. We then injected human regular insulin (2.0 U/kg body weight) subcutaneously. Blood samples were taken at regular intervals (0-120 min) and blood glucose was measured as described above.

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Figure 1. Effects of chronic treatment with MnTBAP or rosiglitazone on obese mice.

(a) Effect on body weight. Bars show weights of animals following 12 weeks of treatment with daily subcutaneous injection of MnTBAP or rosiglitazone. Weights were determined in animals treated with 2.5 mg/kg (n=7), 5mg/kg (n=8), or 10mg/kg(n=8) MnTBAP, 3mg/kg rosiglitazone (n=8), or vehicle (phosphate-buffered saline) (n=8).

(b) Effect on fed glucose levels. Bars indicate mean fed glucose levels averaged over 18 days during the last eight weeks of treatment. Glucose levels were determined at random times during the day and on random days during this period.

(c) Effect on insulin sensitivity. Insulin tolerance tests were performed on animals from each treatment group. Lines indicate the time-course of glucose levels following subcutaneous injection of human insulin (2U/kg). Diamonds indicate vehicle treated, squares indicate 5mg/kg MnTBAP, and circles indicate rosiglitazone.

(d) Effect on glucose tolerance. Glucose tolerance tests were performed, with lines indicating the time-course of glucose excursion following subcutaneous injection of glucose (1g/kg). Diamonds indicate vehicle treated, squares indicate 10mg/kg MnTBAP, and circles indicate rosiglitazone.

Results are mean +/- sem. Astersisks indicate $p<0.05$ versus vehicle treated animals, t-test. Glucose and insulin tolerance test data from additional doses of MnTBAP can be found in the supplementary information.

Fig. 1

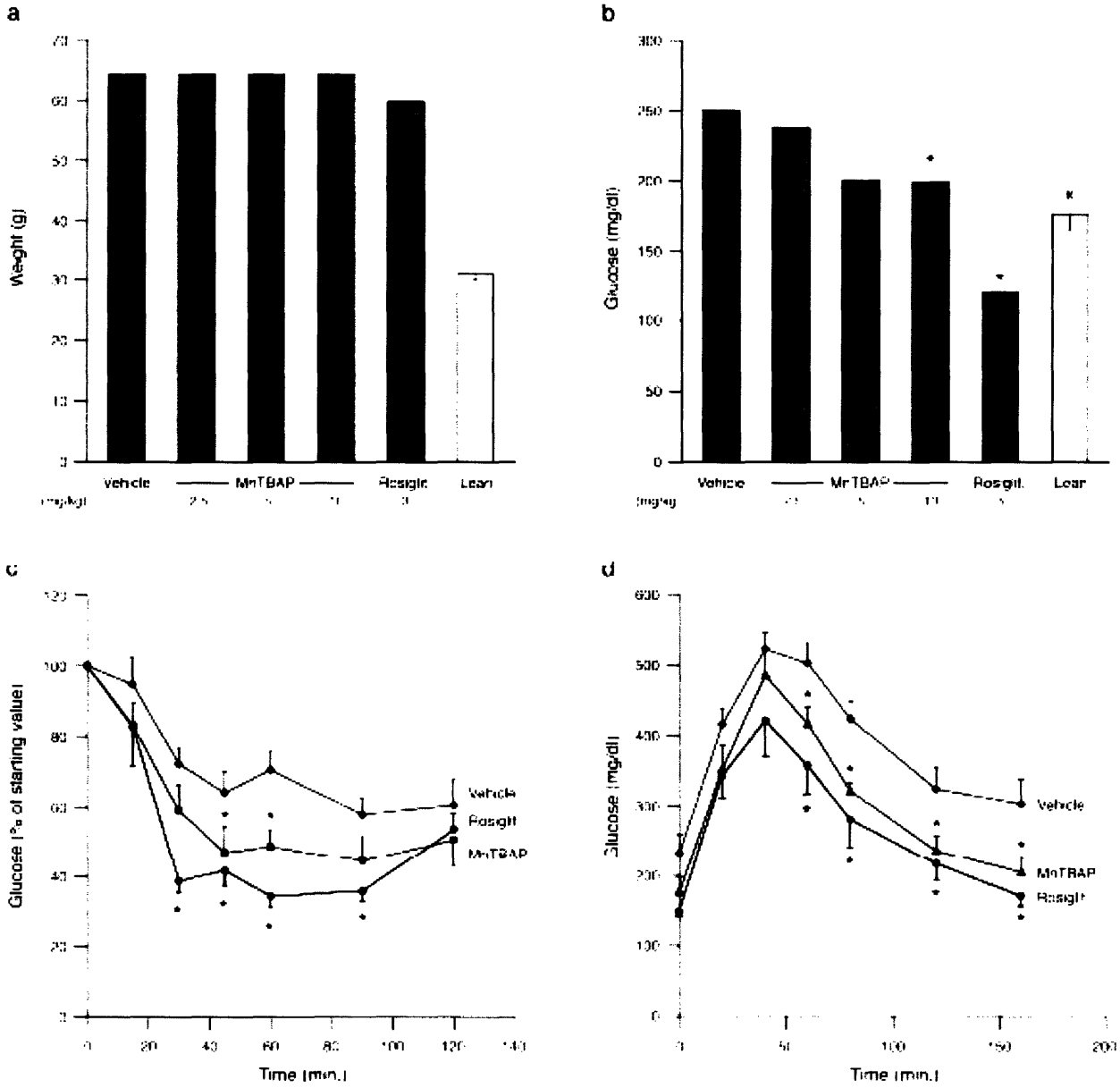

a
Weight (g)
Vehicle
MnTBAP
Rosigli.
Lean
b
Glucose (mg/dl)
Vehicle
MnTBAP
Rosigli.
Lean
c
Glucose (% of starting value)
Vehicle
Rosigli
MnTBAP
Time (min.)
d
Glucose (mg/dl)
Vehicle
MnTBAP
Rosigli
Time (min.)

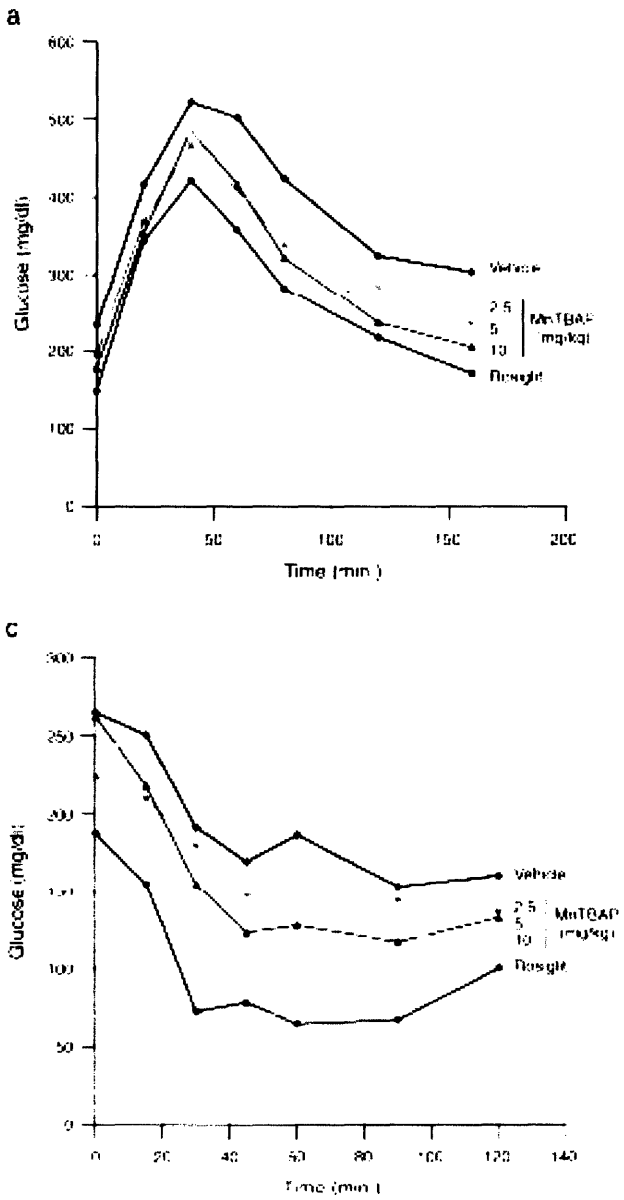
Figure 2. Additional characterization of obese mice chronically treated with MnTBAP or rosiglitazone

(a) Effect on glucose tolerance for each dose of MnTBAP (elaborates on Fig.4 which shows only 10mg/kg dose). Glucose tolerance tests were performed, with lines indicating the time-course of glucose excursion following subcutaneous injection of glucose (1g/kg). Blue indicates vehicle treated, black indicates 10mg/kg MnTBAP, green indicates 5mg/kg, cyan indicaties 2.5mg/kg, red indicates rosiglitazone.

(b) Area Under the Curve (AUC) calculated from the glucose tolerance test in (a). Results are mean +/- s.e.m. Asterisk indicates $p<0.05$ vs. PBS treated (t-test).

(c) Effect on insulin sensitivity for each dose of MnTBAP (elaborates on Fig.4). Insulin tolerance tests were performed on animals from each treatment group. Lines indicate the time-course of glucose levels following subcutaneous injection of human insulin (2U/kg). Blue indicates vehicle treated, cyan indicates 10mg/kg MnTBAP, black indicates 5mg/kg, green indicates 2.5mg/kg, red indicates rosiglitazone.

Fig. 2


a
Glucose (mg/dl)
Time (min)
Vehicle
2.5
5
10
MnTBAP
mg/kg
Rosiglit
b
Area Under the Curve (g*min/dl)
Vehicle
MnTBAP
Rosiglit
c
Glucose (mg/dl)
Time (min)
Vehicle
MnTBAP
Rosiglit

Chapter 6. Conclusions and Future Directions

In the final chapter of this thesis we assess the strengths and weaknesses of our results, describe two models of insulin resistance that synthesize our observations with other proposed mechanisms, map out future directions prompted by this work, and finally present some new preliminary data, including the identification of two novel insulin sensitizers.

6.1 Assessment of our results

This work was motivated by the observation that there are multiple clinical settings of insulin resistance whose pathophysiologic relationship to the insulin resistance of diabetes is unknown. We conjectured that if the underlying cellular pathways were similar then a comparative analysis would be a powerful way to reveal them. A systematic and unbiased approach based on the analysis of expression profiles suggested the hypothesis that ROS could be part of such a common pathway. Subsequent data supported a causal role for ROS in three models of insulin resistance. Moreover the literature contains evidence for a significant association between ROS and multiple other insulin resistant conditions including starvation, trauma, cancer cachexia, and sepsis. This raises the possibility that ROS are a common component of many different forms of insulin resistance. Clearly more work will be needed to clarify its role, particularly its contribution to cellular insulin sensitivity *in vivo* (see section 6.3). Given that interventions aimed at increasing ROS scavenging could not completely suppress insulin resistance, it is also likely that there are parallel pathways contributing to insulin

resistance some of which may be unique to particular clinical conditions. Furthermore, the insulin resistance pathway to which ROS does belong is undoubtedly composed of multiple steps (see section 6.2), many of which are unknown. With respect to the problem of insulin resistance in its entirety, it is inescapable that ROS is but one piece of the puzzle, and there is much more to be done.

It is worth going over our results in the context of the framework we proposed in Ch1.4, beginning with the question of a ROS state change in insulin resistance. First, by two different assays, we found that ROS levels were elevated in TNF and Dex treated adipocytes. A scan of the literature turned up studies demonstrating that TNF and Dex stimulate ROS formation in muscles cells as well. Furthermore, markers of oxidative stress had been discovered in virtually every insulin resistant condition we first considered, their levels raised both in mouse models and in humans. Care should be taken when interpreting this data given that many different markers of ROS have been assayed, and it is not always clear what these markers are a measure of. In the future it will be important to restrict assays to those for which there is some consensus, such as F2 isoprostanes or protein carbonylation (see Ch.5).

The second question pertains to causality. Earlier work had demonstrated that exposing cells to exogenous ROS was sufficient to cause insulin resistance. We provided evidence with six proven antioxidants that ROS was also necessary for 3T3-L1 adipocyte insulin resistance caused by Dex and TNF. Interestingly, others had also demonstrated a ROS mediated mode of action for Dex and TNF in muscle cells, albeit for phenotypes other than insulin resistance. We used one of these antioxidants, MnTBAP, to test the causal role of ROS *in vivo*. It improved insulin sensitivity as assayed by the insulin

tolerance test (ITT). Reassuringly, its slightly weaker potency relative to rosiglitazone mirrored what we had observed in our cellular models. While the ITT is known to be trustworthy measure of muscle insulin sensitivity[1], it is not the gold standard. In the future, the use of hyperinsulinemic euglycemic clamps together with tracers should provide a definitive answer.

Does ROS mediate its effect directly on cellular insulin sensitivity or does it mediate its effects indirectly, through other tissues? Our data from cellular models support the plausibility of a direct mechanism. However the *in vivo* experiments are simply consistent with a direct effect. Elevated ROS in a state of obesity has been observed in all of the tissues known to modulate glucose metabolism, including the liver, fat, and the beta cell. Treating mice with MnTBAP may have affected one or more of these tissues, which may have lead to improved muscle insulin sensitivity by indirect means. As we discuss in section 6.3, tissue specific genetic interventions will be critical to assessing whether the contribution of ROS *in vivo* is direct or indirect.

With respect to the consideration of signaling versus glucose uptake, our cell culture data addressed both. We demonstrated ROS mediated effects on glucose uptake as well as insulin signaling, as reflected by Akt-pS and p70S6K-pS. The next step would be to measure additional signaling events, including IR-pY, IRS1-pY, IRS2-pY, PI3K activation, and AS160-pS. Measuring these variables *in vivo* would help solidify the identity of the ROS dependent steps.

Finally, what evidence confounds the ROS hypothesis? In our evaluation of current molecular hypotheses from Ch.1 the most common confounding factor was the normal function of insulin, be it by stimulating IRS1 serine phosphorylation, JNK

activation, or ATP formation (it also stimulates NF-kB activation[2]). Since hyperinsulinemia is present in insulin resistant conditions, factors that are normally stimulated by insulin might appear to be candidate causes when in fact they might simply be markers. In a surprising coincidence, there is some evidence that insulin also stimulates ROS formation[3]. As a result, the effect of hyperinsulinemia will have to be accounted for when evaluating the role of ROS *in vivo*.

6.2 Synthesis

To put our results in perspective we present two complementary models of insulin resistance that feature a role for ROS. The two models differ in scale, the first being molecular and the second being functional. But in both cases the aim is to provide a simple scheme that organizes many seemingly independent observations. Perhaps more importantly they provide a basis for new predictions, which we address with preliminary data in the next section.

6.2.1 Molecular Model

First, we describe the chain of molecular and cellular events by which obesity could lead to ROS production and subsequently insulin resistance. For now we only consider the case of obesity-induced insulin resistance, attempting to highlight the connection of ROS to a number of previously studied insulin resistance pathways. The model begins by identifying obesity induced changes in the extracellular environment and then asks how they in turn lead to intracellular changes in skeletal muscle, including ROS production and its effects on other pathways. In addition, the direct effects of

obesity on muscle are treated separately from effects that are indirect, mediated through changes in liver and adipose tissue function.

What extracellular changes induced by obesity might lead to intracellular changes in muscle ROS levels? At least two changes in the extracellular environment of skeletal muscle might do this: increased levels of free fatty acids (FFA), and increased paracrine stimulation by TNF from fat pad macrophages[4]. The paracrine effects in the latter mechanism depend on the observed spatial proximity of muscle and fat cells in states of obesity. Furthermore, the basis for suggesting that these mechanisms cause ROS production in obesity derives from observations that in isolation these stimuli are capable of increasing ROS levels. An important experiment that remains is to show that reversing these changes in obesity ameliorates the associated oxidative stress.

The extracellular stimuli that cause oxidative stress may do so through a common intracellular pathway. One such candidate is the ceramide pathway. It has been observed that both TNF and Dex stimulation lead to the generation of the sphingolipid ceramide or ceramide metabolites[5]. Additionally, fatty acid accumulation in muscle can serve as the substrate for de novo ceramide synthesis. Ceramide is known to cause ROS formation by direct interaction with mitochondrial membranes[6]. Moreover, addition of ceramide to adipocytes and muscle cells is known to cause insulin resistance[7]. It has also been shown that fatty acid induced insulin resistance in cultured muscle is suppressible by expression of the ceramide degrading enzyme, acid ceramidase[8]. Finally, *in vivo* ceramide has been found to accumulate in insulin resistant muscle from both humans and rodents[5].

It is worth considering at least two pathways leading to ROS generation whose activation is not necessarily shared by the extracellular stimuli we considered. First,

excess oxidation of intracellular FFA could directly lead to ROS by saturating the mitochondrial electron transport chain with reducing equivalents. Second, macrophages are well known to generate extracellular superoxide using the NADPH oxidase system. Their infiltration of fat pads and adjacent muscle could lead to direct release of superoxide on muscle cell membranes.

What is the mechanism by which elevated muscle ROS might eventually influence the insulin signal transduction cascade? Several intracellular factors that are altered in obesity and thought to promote insulin resistance include JNK, IRS-1, NFkB, AKT, and OXPHOS. Evidence from independent studies has demonstrated a causal relation between ROS and these factors. One commonly cited activator of JNK is exogenous ROS[9]. In addition to our work, others have previously demonstrated that TNF's ability to activate JNK is in part dependent on ROS[10]. Serine phosphorylation of IRS-1 has been suggested as a mechanism involved in insulin resistance, and JNK is thought to be one its upstream serine kinases. It is thus possible to link ROS to IRS-1 through JNK activation. NFkB is another protein that has frequently been shown to display ROS dependent activation[11]. Like JNK, TNF induced NFkB activation has in some settings, including 3T3-L1 adipocytes[12], also been shown to be ROS dependent. As we discussed in Ch.1, AKT is an important signaling molecule in the insulin signal transduction cascade. Recently it has been shown that oxidative stress accelerates S-nitrosylation of AKT[13], thereby decreasing its activity. Taken together, several signaling alterations associated with insulin resistance could be explained in part by oxidative stress. Finally there is a long history of work connecting ROS formation with mitochondrial dysfunction. Elevated ROS levels are known to inhibit OXPHOS[14] and

inhibitors of OXPHOS are in turn capable of generating ROS, placing mitochondria squarely in this pathway. The recent accumulation of evidence supporting decreased mitochondrial function in insulin resistant muscle adds yet another observation that would be accounted for by a ROS related mechanism.

Obesity also leads to changes in liver and adipose tissue that may indirectly contribute to muscle insulin resistance. Candidate mechanisms by which adipocytes may influence muscle include their ability to attract TNF-producing macrophages, and their secretion of adiponectin, which is an insulin sensitizing factor. Obesity-induced reduction in adiponectin secretion has recently been shown to be attributable in part to adipocyte oxidative stress[15]. A model that accounts for these facts would contend that in a state of obesity adipocytes experience autocrine glucocorticoid stimulation and paracrine TNF stimulation, which then leads to oxidative stress. This in turn reduces adiponectin secretion. Such a mechanism is supported by recent experiments demonstrating a ROS dependent mechanism through which TNF decreases adiponectin secretion in 3T3-L1 adipocytes[12]. In the case of liver, one of the many effects of obesity is the accumulation of intrahepatic fat (steatosis). Moreover it has been observed that steatosis is associated with insulin resistance in multiple pathological states. That this association may be causal in nature is supported by the observation that perturbations resulting in steatosis often cause insulin resistance in muscle. Interestingly, obesity is also known to induce oxidative stress in liver. Furthermore, when obese mice are treated with a ROS scavenger, MnTBAP, the steatosis improves significantly[16,17]. Thus obesity induced hepatic oxidative stress could contribute to steatosis, which in turn may be an indirect factor leading to muscle insulin resistance.

6.2.2 Functional model

Having described a model focused on obesity-induced insulin resistance, we now put forward a second model which encompasses the full complement of insulin resistant conditions. Its goal is of a different character than the first model in that it advances a hypothesis to explain how these different conditions have come to share multiple functional and molecular features. Specifically it attempts to answer two questions: (1) why should insulin resistance be a phenotype of such an array of clinical conditions, and (2) why might the insulin resistance of these conditions be expected to share a common cellular pathway of which ROS is a component? Our model derives from an evolutionary argument that tries to explain the genesis of the insulin resistant response. One of its utilities is that it can be exploited to make predictions about novel insulin sensitizing factors. We demonstrate this in the next section when we describe the identification of just such a factor.

The first step to recognizing the relationship between insulin resistant conditions is to divide them into two groups: those in which insulin resistance has a physiologic role and those in which it is a pathology. It is thought that in pregnancy, sepsis, trauma, and starvation insulin resistance has an adaptive role, namely to prevent glucose uptake by muscle and fat and to redirect it to a different target organ. In pregnancy the target is the fetus; in sepsis the immune system is a target; in trauma the wound is a target; and in starvation glucose is saved for the brain. We classify these conditions as examples of "physiologic insulin resistance". In contrast, we classify conditions such as obesity, the

metabolic syndrome, type 2 diabetes, and tumor cachexia as examples of "pathologic insulin resistance", because in these settings it has no known adaptive function.

Fuel metabolism in conditions of physiologic insulin resistance has a common character. In addition to decreased insulin mediated glucose uptake, these conditions all exhibit many of the following responses: increased adipocyte lipolysis[18-20], increased skeletal muscle proteolysis[19,21-24], increased hepatic gluconeogenesis[19,25,26], decreased skeletal muscle protein translation[27-29], and oxidative phosphorylation (OXPHOS) [30-32]. When viewed in aggregate, these pathways fall into two functional units: one that promotes the release of stored energy (from muscle or fat) and one that acts to conserve glucose. The logic of these large-scale metabolic shifts is easiest to comprehend for the state of starvation. In starvation the brain becomes prioritized as a glucose sink; to meet this need energy stores such as fat and amino acids are converted to glucose by the liver. This requires lipolysis of adipocyte lipid reserves. When these sources are exhausted, they are supplanted by skeletal muscle protein reserves released by proteolysis. At the same time glucose and protein consumption by muscle and fat is restricted by a depression in protein translation, glucose transport, and OXPHOS flux. Furthermore, since insulin stimulates consumption/storage and inhibits release of stored energy, insulin resistance becomes yet another way to restrain and promote these respective processes. Insulin resistant conditions other than starvation reflect variations on the same theme, the difference being the target organ to which nutrients are diverted. In this broader view, insulin resistance is just one component of a set of physiologic responses aimed at redirecting nutrient flow in the organism.

The metabolic pathways that mediate nutrient redirection in physiologic insulin resistance are in many cases known to be sensitive to ROS. Importantly, all of these conditions cause oxidative stress in skeletal muscle, with the exception of pregnancy (for which we know of no data). Moreover, as discussed in Ch.2, these conditions are also associated with increased circulating levels of glucocorticoids and/or TNF. This leads to the prediction that oxidative stress is present in adipose tissue as well. In fact, for the case of starvation, this has been confirmed[33]. Causal involvement of ROS is supported by several observations. First, cells that are oxidatively stressed exhibit reduced glycolysis[34], OXPHOS[14,35], and protein translation[36]. Oxidative stress can also directly promote muscle proteolysis[37]. Moreover, TNF mediated muscle proteolysis has been shown to be ROS dependent[38]. Our own data demonstrate a similar phenomenon in adipocytes, namely that both TNF and Dex cause proteolysis that is ROS dependent (data not shown). In addition our preliminary observations indicate that adipocyte lipolysis stimulated by TNF or Dex is ROS dependent (data not shown). We do not wish to imply that ROS is the sole mediator of these responses, and clearly there remain many ROS dependencies that have not been demonstrated. But we do believe there is enough data from independent studies to warrant consideration of a model in which ROS is a causal component of the nutrient redirection program.

The starvation response has a direct analog in microorganisms, which exhibit remarkably similar metabolic adaptations including the involvement of ROS. The microorganism starvation response involves increased proteolysis, membrane autophagy (perhaps the analog of lipolysis), and decreases in translation, and OXPHOS[39], all of which help prolong the cell's survival in a nutrient poor environment. Interestingly, both

yeast and bacteria experience oxidative stress as they become deprived of nutrients. Moreover, just as in the mammals, the oxidative stress is thought to play a causal role in these metabolic pathways. It leads to increased proteolysis, decreased translation and decreased OXPHOS[14,40-42]; many factors are involved and ROS is only one contributor. When insulin resistant states are viewed coarsely as a shift from nutrient consumption and storage to nutrient release, they appear very similar to the starvation state of bacteria and yeast. This similarity likely reflects a common evolutionary origin.

We thus hypothesize that the common association of ROS with different insulin resistant states, rather than being a coincidence, results from the evolutionary conservation of a cellular program originating in bacteria. The central goal of this program is nutrient redirection. With the advent of multicellularity, the program acquired the insulin resistance response, which was placed under similar molecular control as the rest of the program, control that was sensitive to ROS. Furthermore, this program became decoupled from direct activation by nutrient deprivation (note that mammalian insulin resistant states do not have depleted blood levels of nutrients). Instead starvation became one of several conditions that would lead to the release of specific activating hormones such as glucocorticoids or inflammatory cytokines. Finally, the pathologic insulin resistant states represent conditions that inappropriately activate this physiologic response. They do this by tapping into one or more steps of the physiologic pathway: increasing the levels of activating hormones such as in tumor cachexia ("cachectin"/TNF) or Cushing's syndrome(glucocorticoids), increasing the intracellular levels of ROS (fatty acid mediated ceramide production for example), or any of the other many steps that contribute to insulin resistance.

The evolutionary argument is an attractive way to compactly explain many of the coincident similarities between insulin resistant states, their functional roles, and their molecular control. A prediction worth highlighting because it may explain the success of our experimental design regards the relation between adipocyte and muscle insulin resistance. We decided to study adipocytes for technical reasons with the hope that it would provide an accurate model for insulin action in muscle. But according to the relation between insulin resistance and fuel metabolism outlined here, adipocytes and muscle cells serve nearly identical functions. They both harbor a reserve fuel with insulin promoting its storage, and insulin resistance promoting its release. So it may not be surprising that their molecular control would be similar. This might explain why the insulin sensitizing action of ROS scavenging in adipocytes should translate to an animal model where muscle is the predominant insulin dependent glucose sink.

The evolutionary model may help one to recognize primitive forms of "insulin resistance" in organisms that are biochemically and genetically tractable. In particular, powerful techniques can be used to identify factors controlling the starvation response in bacteria and yeast. If such methods identify evolutionary conserved factors besides ROS, they could be candidates for controlling insulin sensitivity in mammals.

The goal of the two models we presented is to explain and predict. The ROS hypothesis provides a concise way to reconcile many observations ranging from functional similarities with ancient pathways to connections among known insulin resistance pathways. In the next section, we demonstrate the models' predictive powers by showing how they were used to identity two novel insulin sensitizers.

6.3 Future Directions

We now focus our attention on two important questions motivated by our work: (1) what additional evidence would further strengthen the ROS hypothesis? (2) what processes in the cell lead to increased ROS levels and what factors lie downstream of ROS and mediate the decrease in insulin sensitivity? We present new preliminary data addressing each question. In particular, we highlight the importance of the models described above in our efforts to answer the second question.

The evidence supporting the ROS hypothesis could be reinforced in several ways. The first would be to identify additional potent antioxidants with insulin sensitizing properties. Since we began our work using MnTBAP and NAC, additional related compounds with improved properties have been described. In particular, new catalytic metalloporphyrin antioxidants with greater potency than MnTBAP have been discovered[43], and NAC variants with improved pharmacokinetics and pharmacodynamics have been identified[44]. Their effects on adipocyte insulin resistance warrant testing.

The most significant data needed to strengthen the hypothesis will come from animal experiments. It will be important to evaluate the effect of MnTBAP or its variants on additional *in vivo* models of insulin resistance, in particular diet induced obesity (DIO). The effects of transgenic interventions in ROS scavenging genes (both gain and loss of function) on *in vivo* insulin sensitivity will need to be tested. In particular, muscle specific overexpression of ROS scavenging genes such as catalase or SOD1 will shed considerable light regarding the ROS dependence of cellular insulin mediated glucose uptake (IMGU). In preliminary experiments, we obtained SOD1 heterozygous knockout from a collaborator and have begun breeding them to generate mouse cohorts for use in

evaluating SOD1's impact on insulin sensitivity. We have some preliminary data from a pilot experiment in these mice demonstrating that a pooled group of four SOD1 heterozygote and two homozygote null mice are glucose intolerant relative to their wild type littermates (Fig.1). While not direct evidence for insulin resistance, it is consistent. Once the breeding is completed, we will be able to answer the question more definitely.

The origin of ROS in insulin resistant states is also an important issue that deserves attention. As discussed earlier, ceramide and its metabolites are likely candidates for this function. Just as ceramidase expression is able to block FFA induced insulin resistance in cultured muscle, it will be important to determine whether similar interventions inhibit TNF or Dex induced insulin resistance in cell culture and ultimately, in in vivo models of insulin resistance. Moreover, it will be interesting to determine whether these interventions block ROS formation.

6.3.1 OXPHOS

Finally, we discuss and present evidence for two pathways that might mediate the downstream effects of ROS. The first is OXPHOS. The decision to explore the role of OXPHOS in insulin sensitivity was motivated primarily by the fact that OXPHOS is known to be a direct molecular target of ROS. This property is illustrated by several facts. Many mitochondrial OXPHOS proteins possess superoxide sensitive cofactors (so called iron-sulfur centers) which, when oxidized, inactivate the protein's enzymatic activity[14]. Purifed mitochondria exposed to elevated levels of exogenous ROS display decreased oxygen consumption and ATP production[14]. Finally, in a screen of yeast

mutants for hypersensitivity to hydrogen peroxide toxicity the category of mutant genes overwhelmingly overrepresented was the OXPHOS pathway[45].

To explore the role of mitochondrial OXPHOS in insulin resistance and in particular its relation to ROS, three types of data must be collected: (1) measurement of OXPHOS function in states of insulin resistance, such as TNF or Dex treatment; (2) manipulation of OXPHOS in the presence or absence of TNF and Dex, together with measures of insulin sensitivity, ROS and OXPHOS; and (3) manipulation of ROS in the presence or absence of TNF and Dex, together with measures of insulin sensitivity, ROS and OXPHOS. We are in the early stages of testing this hypothesis but have collected some interesting preliminary data addressing the second point. In Figure 2a, we show that low nanomolar concentrations of rotenone and antimycin A, inhibitors of OXPHOS complexes I and III respectively, are sufficient to induce profound insulin resistance in 3T3-L1 adipocytes. This fulfills the criteria for causal sufficiency.

Having shown that perturbations which decrease OXPHOS can cause insulin resistance, we attempted to answer the next logical question: can increasing the output of OXPHOS in the setting of TNF or Dex treatment restore insulin sensitivity? There are at least two requirements to achieve increased OXPHOS output: (1) a high energy, cell permeable substrate that can serve as an electron donor, and (2) increased expression or activity of some component of OXPHOS to accept these electrons and pass them down the chain. Unfortunately, the endogenous electron donors, NADH and FADH2, are not membrane permeable. The problem with glucose or pyruvate as substrates is that one must rely on the activity of endogenous enzymes to convert them to NADH, a variable which may be difficult to predict or control. With respect to the second requirement, the

challenge is that OXPHOS complexes are multisubunit molecular machines that do not lend themselves to an obvious genetic strategy for boosting their activity. One approach that could conceivably work is to overexpress transcription factors such as PGC-1alpha that are capable of inducing the entire program of mitochondrial biogenesis. The drawback is that many other genes will be induced whose influence may confound the effect on OXPHOS that we are trying to isolate. To solve both of these problems, we turned to an enzyme found in bacteria and yeast known as mandelate dehydrogenase(MDH). This enzyme extracts high-energy electrons from the cell permeable compound mandelic acid and transfers them directly to cytochrome C, an electron carrier protein that lies between complex III and complex IV. Yeast expressing MDH are able to survive and proliferate with mandelate as their sole energy source[46]. We attempted to increase OXPHOS capacity by transgenically expressing yeast MDH in 3T3-L1 adipocytes in the presence or absence of mandelate supplemented media. MDH expression together with mandelate was found to suppress up to 75% of the defect in IMGU induced by TNF or Dex treatment(Fig.3a,b). This intriguing result suggests we may be on the right path, but it is just the first of a sequence of experiments needed to establish a necessary role for OXPHOS in insulin resistance. Additional results that will be needed include: (1) validation of the presumed function of yeast MDH in adipocytes, by demonstrating that it localizes to the mitochondrial intermembrane space and increases membrane potential in the presence of mandelate; (2) evidence that TNF and Dex lower membrane potential; and (3) elucidation of the causal relation between membrane potential and ROS levels.

6.3.2 Frat1

A second insulin sensitizing factor was identified by reasoning from the functional model. As we discussed earlier, insulin resistance is one component of a coordinated response at nutrient redirection. A primitive version of this program is present in yeast and operates under conditions of nutrient deprivation. We therefore asked which proteins are known to be critical for executing the different metabolic adaptations to starvation. The most prominent protein candidate which is also conserved up to mammals is the TOR kinase. In particular, nutrient depletion leads to decreased TOR activity and this contributes to decreased protein translation among other responses.

Is there evidence to support the functional model's prediction that mTOR could modulate insulin sensitivity? One way to perturb mTOR is with the small molecule inhibitor rapamycin, which induces a starvation-like phenotype in yeast and mammalian cells despite the presence of adequate nutrients. The first indication that it may also affect insulin sensitivity is that its clinical use is known to be associated with diabetic-like symptoms[Sabatini, personal comm]. In a direct test of the hypothesis we found that treating 3T3-L1 adipocytes with rapamycin for fourteen days led to insulin resistance (Fig.2b). This result has been observed by others and published[47]. The more interesting question is whether increasing mTOR activity can promote insulin sensitivity. Unfortunately, despite the wealth of treatments that indirectly reduce mTOR activity, there are very few factors known to increase its activity (akin to the paucity of insulin sensitizers). In fact, when we began this study there were no known factors that could constitutively activate mTOR. We therefore loosened the criteria and asked whether there existed factors that could complement decreased mTOR activity. The protein, frat1,

possesses this property. When overexpressed, it suppresses rapamycin's growth inhibitory effects on lymphocytes[Sabatini, personal comm.]. We generated 3T3-L1 preadipocytes stably expressing frat1 under a tetracycline inducible promoter and found that when its expression was induced in mature adipocytes, it was an extremely potent insulin sensitizer, preventing 90-100% of the defect in IMGU caused by TNF or Dex(Fig.4a,b).

Frat1 is an insulin sensitizing protein discovered by virtue of its ability to complement mTOR inhibition. But what is its physiologic function? A search of the literature revealed that frat1 inhibits the activity of the kinase Gsk3beta towards specific substrates[48]. Moreover, Gsk3beta is a protein previously implicated as a cause of insulin resistance[49]. Additional known results reveal intriguing connections to our own work. In particular Gsk3beta has been observed to be activated by oxidative stress, TNF, or Dex[50-52]. Moreover, another commonly used inhibitor of Gsk3beta, lithium, has been shown to possess insulin sensitizing properties in diabetic rats[53]. We subsequently were able to demonstrate that lithium could prevent up to 50% of the defect in IMGU caused by Dex(Fig.4c). Experiments with TNF are still pending. We would like to flesh out the role of Gsk3beta further as well as analyze its connection to ROS. Possible experiments include measuring insulin sensitivity, gsk3beta activity, and ROS levels under conditions of (1) Gsk3beta inhibition with RNA interference or by the expression of dominant negative mutants (2) Gsk3beta activation through expression of constitutively active mutants, and (3) increased ROS scavenging either with MnTBAP or catalase overexpression. That a question about yeast nutrient sensing could result in the discovery of a novel insulin sensitizer is a testament to the power of these ROS-based models.

In conclusion, the ROS hypothesis explains many clinical observations about insulin resistance, it ties together multiple mechanistic hypotheses, and it makes novel predictions for which we continue to find experimental support. We suspect it will ultimately fulfill its potential as an important component of the pathways undergirding many forms of insulin resistance.

Methods

Reagents. Pioglitazone (Alexis Biochemicals). Dexamethasone (Biomol). TNF (Cell Sciences). Rapamycin (Calbiochem). Mandelate, Mannitol, Insulin, Isobutylmethylxanthine, Digitonin (Sigma). DMEM (Invitrogen). Fetal Bovine Serum, Bovine Calf Serum (HyClone). Doxycycline (BD Bioscience). 3T3-L1 preadipocytes were obtained from ATCC.

Cell Culture. Early passage 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagle Medium supplemented with Glutamax, 10% bovine calf serum, 100U/ml penicillin and 0.1mg/ml streptomycin. Cells were maintained at 37°C, 10% CO_2 and mycoplasma-free. Differentiation medium was added to pre-adipocytes two days after reaching confluence (Day 0). Differentiation medium consisted of DMEM supplemented with 10% Fetal Bovine Serum (FBS), to which was added a cocktail containing 0.11 mg/ml isobutylmethylxanthine, 1ug/ml insulin, 250nM dexamethasone and 1.5uM pioglitazone. On Day 2, the medium was replaced with DMEM/10%FBS containing 1ug/ml insulin and 1.5uM pioglitazone. From Day 4 onward, cells were maintained in DMEM plus 10%FBS with a media change every other day until experimental treatments were initiated. Batches of pre-adipocytes and FBS were screened for their ability to give rise to adipocytes satisfying the positive control behavior depicted in Fig.1a,b.

Induction of insulin resistance. Dex (20 nM) or TNF (4 ng/ml) treatment was initiated with mature adipocytes anywhere from day 8 to day 14 of differentiation. Media was changed daily for TNF for a total incubation time of 4 days. Dex media was changed every other day for a total of 8 days. Rapamycin media was changed every other day for a total of 14 days. Rotenone and Antimycin supplemented media was changed every day for 2 days. For experiments in which transgene expression was induced, doxycycline was added to cells on day 4 of differentiation and TNF or Dex treatment was always initiated on day 8. For washout experiments cells were made insulin resistant as above at which point media absent TNF or Dex was added and refreshed every two days for a total of 8 days.

Glucose Uptake Assay: Cells in 12 well dishes were washed twice with KRP (127mM NaCl, 4.7mM KCl, 0.9mM $MgSO_4$, 10mM $NaPO_4$, 0.9mM $CaCl_2$) and incubated with pre-warmed KRP (670ul) containing 0.2% fatty acid free BSA and the appropriate concentration of insulin (10nM insulin as a maximum stimulus). The dish was then allowed to float in a 37°C water bath for 30min. After this period, tritiated 2-deoxyglucose (2DG) and unlabeled 2DG (total vol. 75ul) were dispensed into each well for a final concentration of 1uCi/ml and 0.1mM respectively. Cells were incubated for an additional 5 min. at 37°C and the reaction was stopped by immersing the entire dish in a reservoir of ice cold PBS. While submerged, the plate was gently swirled for 30 sec. The plate was then dried, excess PBS was aspirated, and 740ul of digitonin release buffer (100mg/ml Mannitol, 1mg/ml digitonin) was applied to each well. After 10 min. at room temperature, 665ul from each well was counted in a scintillation counter. Fresh media was always added to cells 24 h prior to assay.

Animals. Male *sod1*+/- and +/+ mice were obtained from Holley VanRemmen.

Glucose tolerance tests. For the glucose tolerance test, mice were fasted for 12 h and then injected subcutaneously with glucose (2.0 g/kg body weight). Blood samples were taken at regular time points (0-140 min), and blood glucose levels were determined with a portable glucose meter.

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Figure 1. Effect of SOD1 deficiency on glucose tolerance

Effect on glucose tolerance. Glucose tolerance tests were performed, with lines indicating the time-course of glucose excursion following subcutaneous injection of glucose (2g/kg). The blue line indicates wildtype(n=8), the violet line indicates SOD1 deficient mice(n=6, 4 +/-,2 -/-). Results are mean +/- sem. Astersisks indicate $p<0.05$ versus wildtype animals, t-test.

Fig. 1

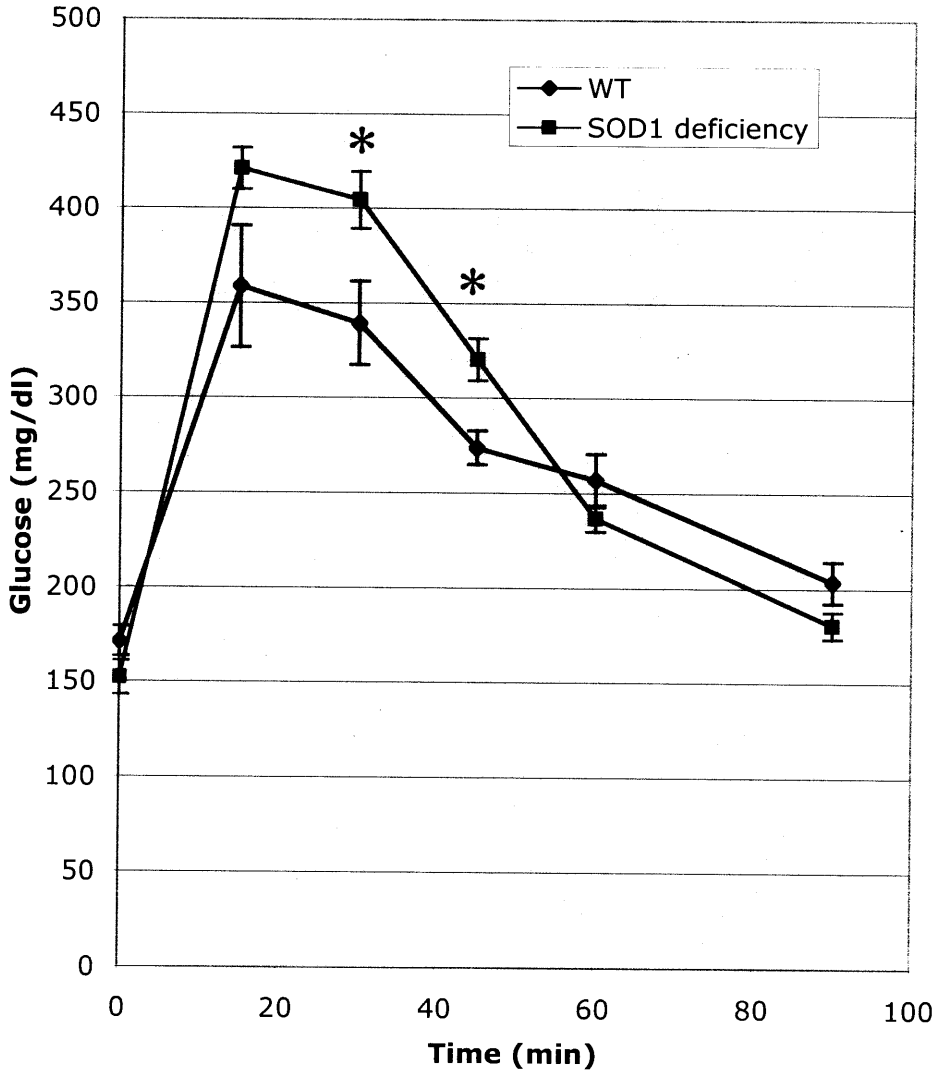

500
450
400
350
300
250
200
150
100
50
0
Glucose (mg/dl)
0
20
40
60
80
100
Time (min)
WT
SOD1 deficiency
*
*

Figure. 2 Additional models of insulin resistance

(a) Rates of insulin stimulated glucose transport in Antimycin A or rotenone treated cells. Insulin-stimulated rate was calculated as the rate of transport in the presence of insulin minus the basal rate. All values are normalized to the insulin-stimulated rate from untreated cells. Results are mean +/- s.e.m.

(b) Rates of insulin stimulated glucose transport in rapamycin treated cells. Data are analogous to a)

Fig. 2

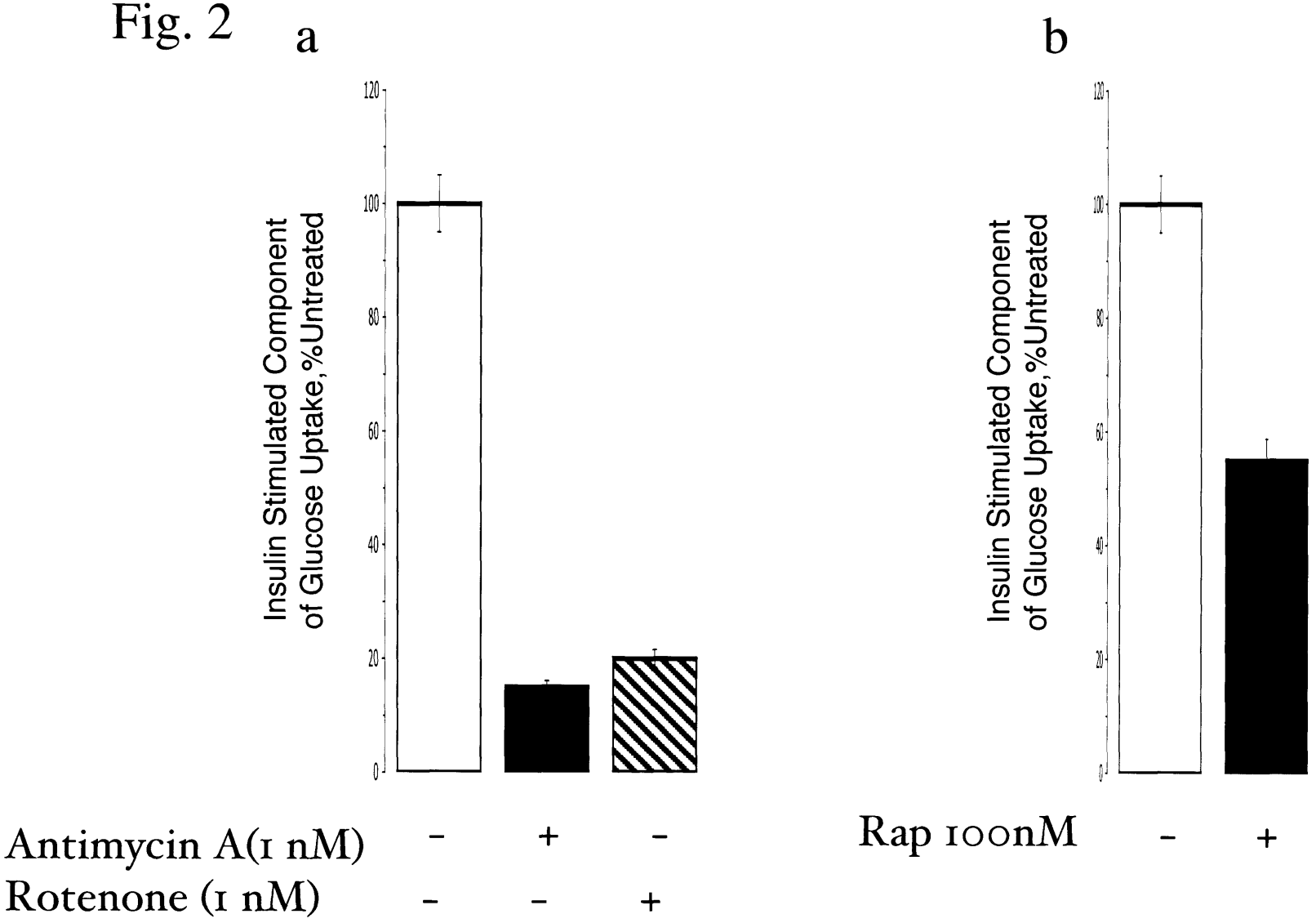

a
b
Insulin Stimulated Component of Glucose Uptake, %Untreated
120
100
80
60
40
20
0
Antimycin A(1 nM) - + -
Rotenone (1 nM) - - +
Rap 100nM - +

Figure 3. Effects of Mandelate Dehydrogenase expression on insulin resistance.

(a) Effect of Mandelate Dehydrogenase expression on TNF induced insulin resistance. Bars show insulin dependent and basal rates of glucose transport in untreated cells with or without MDH induction, mandelate treatment, and both MDH induction together with mandelate.

(b) Effect of Mandelate Dehydrogenase expression on dex induced insulin resistance. Data are analogous to (a).

Fig. 3

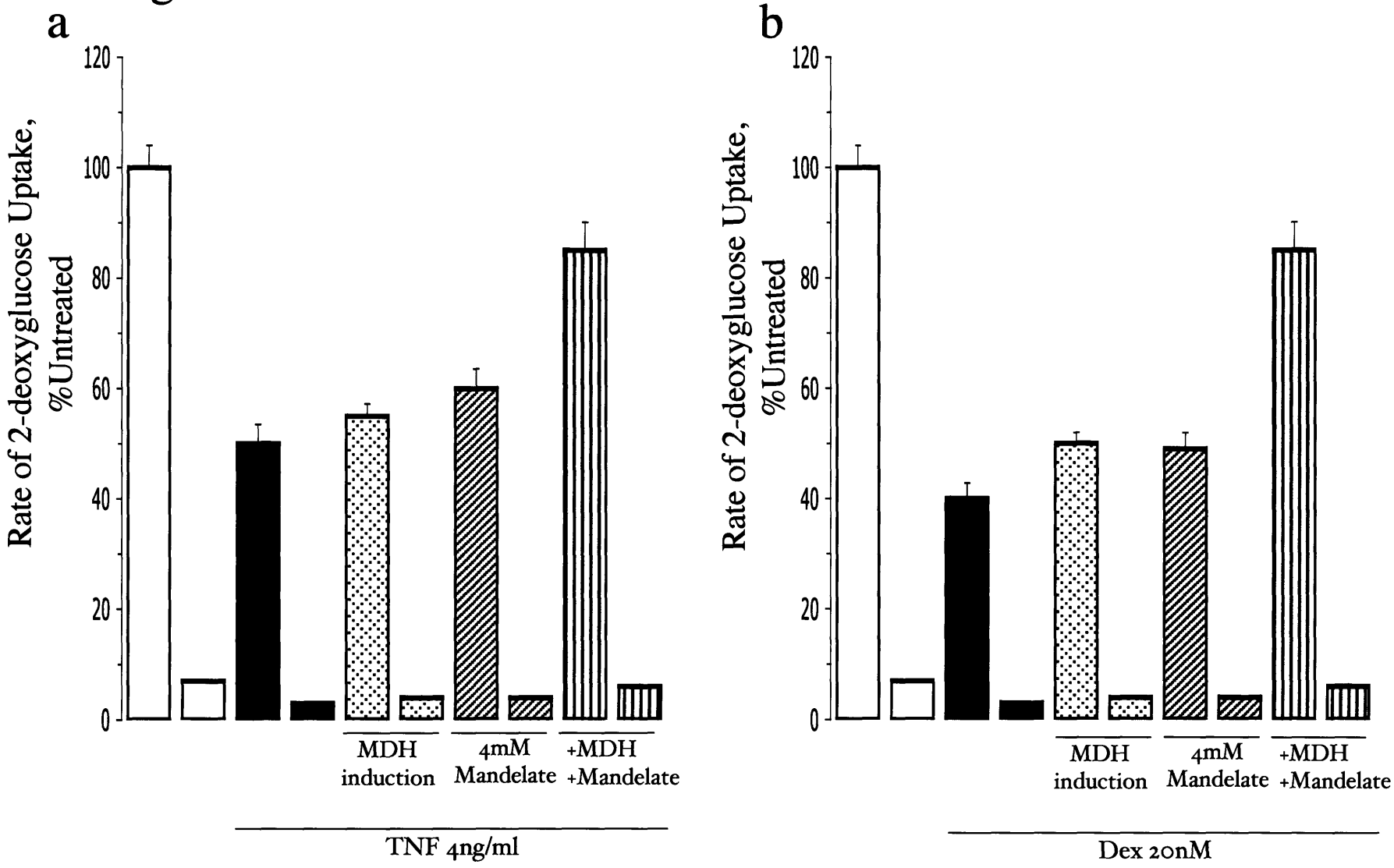

a
b
Rate of 2-deoxyglucose Uptake, %Untreated
120
100
80
60
40
20
0
MDH induction
4mM Mandelate
+MDH +Mandelate
TNF 4ng/ml
Dex 20nM

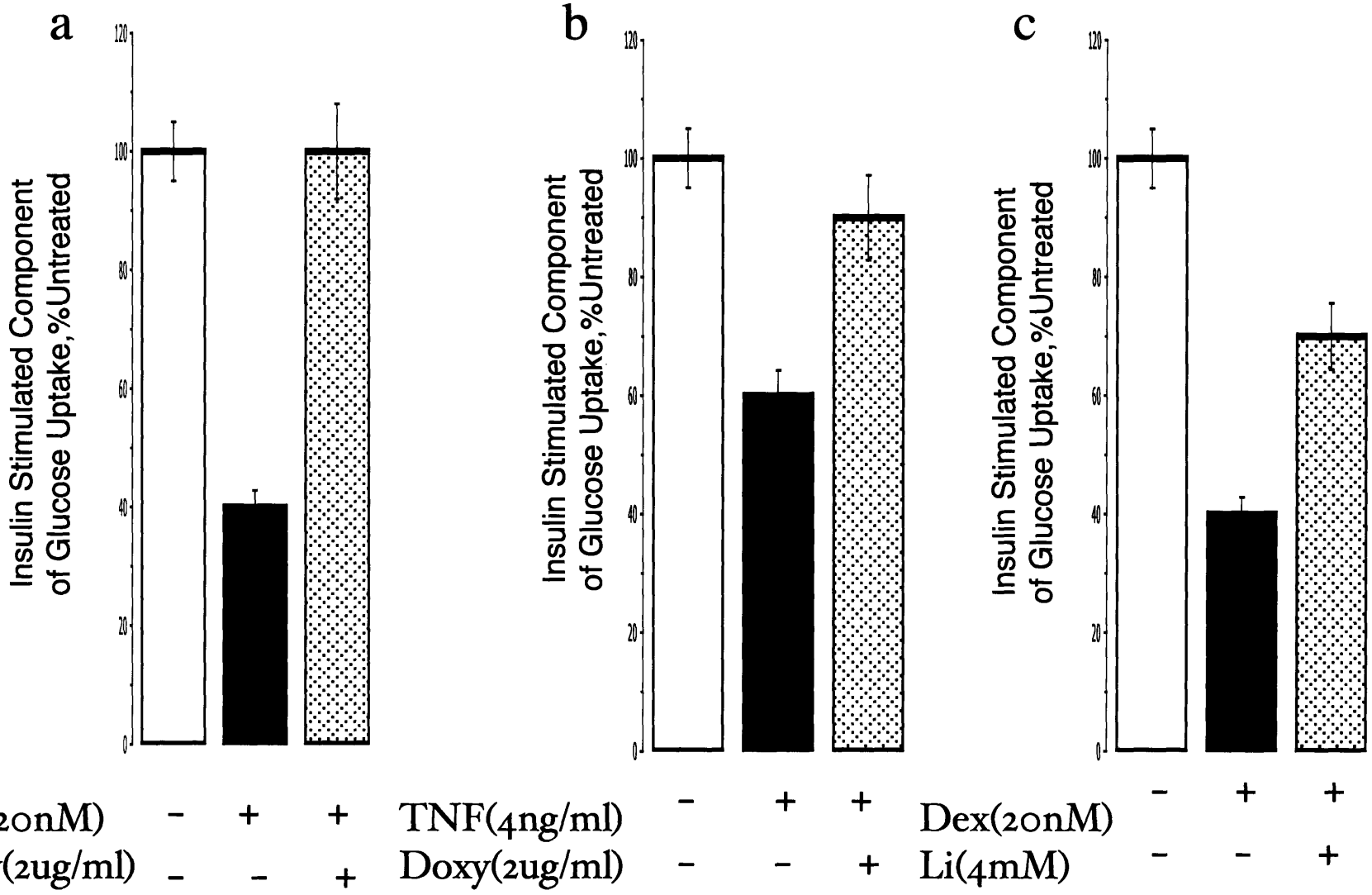
Figure 4. Effects of frat1 expression or lithium on insulin resistance.

(a) Effect of frat1 expression on dex induced insulin resistance. Bars show insulin dependent rates of glucose transport in untreated cells with or without frat1 induction. Insulin-stimulated rate was calculated as the rate of transport in the presence of insulin minus the basal rate. All values are normalized to the insulin-stimulated rate from untreated cells. Results are mean +/- s.e.m.

(b) Effect of frat1 expression on TNF induced insulin resistance. Data are analogous to a).

(c) Effect of Lithium on dex induced insulin resistance. Bars show insulin dependent and basal rates of glucose transport in untreated cells, dex-treated, or treated with both dex and lithium.

Fig. 4


a
b
c
Insulin Stimulated Component of Glucose Uptake,%Untreated
120
100
80
60
40
20
0
Dex(20nM) - + +
Doxy(2ug/ml) - - +
TNF(4ng/ml) - + +
Doxy(2ug/ml) - - +
Dex(20nM) - + +
Li(4mM) - - +