Convergence of glucose- and fatty acid-induced abnormal myocardial excitation-contraction coupling and insulin signalling

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Summary

1. Myocardial insulin resistance and abnormal Ca^{2+} regulation are hallmarks of hypertrophic and diabetic hearts, but depletion of energetic substrates does not tell the whole story. Is there a link between the aetiology of these dysfunctions?  
2. Diabetic cardiomyopathy is defined as phenotypic changes in the heart muscle cell independent of associated coronary vascular disease. The cellular consequences of diabetes on excitation-contraction (E-C) coupling and insulin signalling are presented in various models of diabetes in order to set the stage for exploring the pathogenesis of heart disease.  
3. Excess glucose or fatty acids can lead to augmented flux through the hexosamine biosynthesis pathway (HBP). The formation of UDP-hexosamines has been shown to be involved in abnormal E-C coupling and myocardial insulin resistance.  
4. There is growing evidence that O-linked glycosylation (downstream of HBP) may regulate the function of cytosolic and nuclear proteins in a dynamic manner, similar to phosphorylation and perhaps involving reciprocal or synergistic modification of serine/threonine sites.  
5. This review focuses on the question of whether there is a role for the hexosamine biosynthesis pathway and dynamic O-linked glycosylation in the development of myocardial insulin resistance and abnormal E-C coupling. The emerging concept that O-linked glycosylation is a regulatory, post-translational modification of cytosolic/nuclear proteins that interacts with phosphorylation in heart is explored.

Introduction

Diabetes mellitus is the world’s fastest growing disease with high morbidity and mortality rates, predominantly due to heart disease. The epidemic rise in particular of type 2 diabetes is alarming, especially considering the increased incidence of insulin resistance and diabetes in young adults and children. Type 2 diabetes accounts for over 90% of all diabetic cases globally, and is a progressive, multifactorial disease which typically involves comorbidities such as dyslipidemia, obesity, hypertension and insulin resistance. A significant number of diabetic patients exhibit diabetic cardiomyopathy, a clinical presentation distinguished by ventricular dysfunction independent of coronary vascular disease or hypertension, with impaired diastolic function developing first. Several metabolic complications are common to both type 1 and type 2 diabetes. For example, hyperglycemia and elevated triglycerides (and free fatty acids) are accompanied by insulin deficiency or impaired insulin responsiveness. While the time course of developing ventricular dysfunction may be different, the consequences of both types of diabetes are similar. Myocardial insulin resistance associated with abnormal Ca^{2+} regulation in the hypertrophic heart is the subject of a companion paper (Ritchie & Delbridge). The goal of this review is to consider the consequences of diabetes on the heart, with an emphasis on providing insight into how the heart progresses to failure in a number of diseases.

Brownlee has put forth a unifying hypothesis for the development of diabetic complications. He outlines how hyperglycemia induces vascular disease through the induction of reactive oxygen species (ROS), which in turn inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) via upregulation of poly(ADP-ribose) polymerase (PARP), thus increasing flux through four pathways (i.e. polyol, hexosamine, protein kinase C (PKC) and advance glycated endproducts (AGEs)). Each of these pathways has been implicated in the pathogenesis of micro- and macrovascular diseases. ROS generation and subsequent effects on ion channels and cardiac function are considered by other investigators in this issue (Hool and Ritchie & Delbridge). The following discussion will focus primarily on the hexosamine biosynthesis pathway and cytosolic/nuclear O-linked glycosylation as mediators of impaired myocardial excitation-contraction (E-C) coupling and insulin responsiveness.

Diabetic cardiomyopathy

Diabetic cardiomyopathy has been well characterized in terms of whole heart and cellular dysfunction, including changes in gene expression after prolonged type 1 diabetes. In particular, changes affecting energy substrate utilization and Ca^{2+} regulation clearly contribute to the effects of long-term diabetes. The existence of cardiomyopathy has also been shown in some models of type 2 diabetes, but not in all animal models. Work by many investigators has confirmed that there are changes at the level of the cardiomyocyte which are not solely attributable to impaired coronary blood flow or interstitial fibrosis, including altered functional activity of ion channels and pumps, and changes in gene expression of regulatory and modulatory proteins of E-C coupling. The cellular defects associated with E-C
coupling manifest as prolonged action potentials, slowed cytosolic Ca\textsuperscript{2+} fluxes, and slowed myocyte shortening and lengthening\textsuperscript{17-19}. Some of the molecular changes contributing to these functional abnormalities include (in rat) depressed outward K\textsuperscript{+} currents (particularly I\textsubscript{To}), and depressed expression and function of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX).\textsuperscript{20,21} Depressed phosphorylation of phospholamban (PLB) and β-adrenergic receptor (AR) signalling also contribute to slowed cytosolic Ca\textsuperscript{2+} clearing.\textsuperscript{22,23} Altered myofilament Ca\textsuperscript{2+} sensitivity\textsuperscript{24} and slower crossbridge cycling\textsuperscript{25} also play a role in impaired contractile function. The cardiomyopathy is progressive, as illustrated by sequential changes in mRNA for SERCA, PLB and ryanodine receptors in type 1 diabetic animals.\textsuperscript{26} Proteins modulating E-C coupling are also affected by diabetes (e.g. elevated PKC activity, and isoform distribution\textsuperscript{27}).

Although the cellular consequences of long-term diabetes are well described for heart muscle, little is known about the pathogenesis of diabetic cardiomyopathy. Cellular changes such as prolonged action potential duration and impaired myocyte mechanics appear to occur as early as a few days after induction of type 1 diabetes with streptozotocin (STZ).\textsuperscript{19,28} We have found that a diabetic-like cardiomyopathy is recapitulated by culturing normal adult rat ventricular myocytes in a high glucose medium.\textsuperscript{29,30} After one day of culture in high glucose, the most prominent effects are slowed cytosolic Ca\textsuperscript{2+} removal and prolonged myocyte relengthening, which are not due to acute effects of glucose (<12 hours) or increased osmolarity.\textsuperscript{29} These results are consistent with the early onset of diastolic dysfunction in whole hearts. We have recently shown that depressed SERCA function (but not NCX) is a contributing factor to impaired relaxation in glucose-mediated cardiomyocyte dysfunction. Furthermore, high glucose produces alterations in cytosolic regulatory enzymes (e.g. diminished basal PKA and increased PKC activity) without changes in expression of the Ca\textsuperscript{2+} regulating proteins SERCA, PLB or NCX.\textsuperscript{31,32}

In contrast to type 1 diabetic animals, which are hyperglycemic and insulinopenic, rats with systemic insulin resistance (induced by sucrose feeding), present with euglycemia and hyperinsulinemia, and yet the same abnormal cardiac Ca\textsuperscript{2+} regulation is observed\textsuperscript{14}. Myocyte relaxation is slowed, and SERCA function is impaired, without changes in protein content. When whole body insulin resistance is either prevented or reversed, myocyte mechanical function is normal.\textsuperscript{33} Furthermore, we have preliminary evidence that elevated fatty acids in vitro can produce similar mechanical dysfunctions as those of high glucose or diabetes (unpublished). As discussed below, both high glucose and fatty acid also produce blunted insulin-stimulated glucose uptake.

Myocardial insulin resistance in type 1 and type 2 diabetes

Myocardial insulin resistance develops in animal models of both type 1 and type 2 diabetes.\textsuperscript{34} Although studies to date indicate multiple sites of impaired insulin signalling in various animal models, the findings clearly support the existence of myocardial insulin resistance, as seen clinically.\textsuperscript{34} Insulin-stimulated glucose uptake, protein synthesis and glycogen synthesis have been shown to be depressed in the diabetic rat heart and cultured cardiomyocytes.\textsuperscript{35,36} We have recently shown that myocytes isolated from type 1 diabetic rats are insulin resistant (as measured by insulin-stimulated glucose uptake) within days after STZ-induction of the disease.\textsuperscript{31}

Insulin stimulates metabolic, mitogenic, and anti-apoptotic pathways, beginning with autophosphorylation of tyrosine sites on the β subunit of the insulin receptor (IR), which promotes receptor kinase activity and facilitates interactions between the receptor and its substrates (for review see Zierath & Wallberg-Henriksson\textsuperscript{37}). Figure 1 illustrates one aspect of the IR signalling cascade which involves tyrosine phosphorylation of insulin receptor substrate (IRS) and subsequent activation of phosphatidylinositol (P)3-kinase (PI3K). Downstream effectors of PI3K may include phosphoinositide-dependent kinase (PDK) and atypical PKC isoforms (e.g. PKCζ), ultimately leading to translocation of GLUT4-containing vesicles to the sarclemma.\textsuperscript{38} Akt (also known as PKB) recruitment to these vesicles may also be involved in insulin-stimulated glucose uptake.\textsuperscript{38,39}

One potential mechanism underlying insulin resistance is phosphorylation of serine/threonine residues on IRS molecules in both skeletal muscle and heart (for review see Gual et al\textsuperscript{40}), leading to a reduction in IRS interactions with PI3K. However, insulin-stimulated phosphorylation of PI3K is significantly elevated in cardiomyocytes from short-term alloxan-induced (type 1) diabetic animals, although glycogen synthase activity is impaired.\textsuperscript{41} Hearts taken from rats 6 weeks post-STZ injection (type 1 diabetes model) show a number of alterations including elevated phosphorylation of IR and IRS-2 (but not IRS-1), higher levels of β85 subunit and PI3K activity associated with IRS-2, and elevated phosphorylation of Thr-308 (but not Ser-473) on Akt. The latter may be the basis for impaired Akt activity (i.e. reduced phosphorylation of glycogen synthase kinase, GSK) and depressed glycogen synthesis in the diabetic heart.\textsuperscript{36} There is also evidence that increased glycosylation of proteins can lead to skeletal muscle insulin resistance independent of its effects on Akt and GSK isoforms\textsuperscript{42} (see discussion below).

It is well established that elevated glucose can induce skeletal muscle insulin resistance,\textsuperscript{43} but the effects on cardiac muscle are poorly described. We have recently shown that adult ventricular myocytes exposed to high extracellular glucose are insulin resistant, as measured by impaired glucose uptake, and exhibit contractile dysfunction similar to that seen in diabetes.\textsuperscript{31} In the same high glucose model, Ren and colleagues showed that myocytes become resistant to both the positive inotropic effects of insulin-like growth factor (IGF-1) and its stimulation of Akt phosphorylation.\textsuperscript{44} Whether there is a link between myocardial insulin resistance and abnormal E-
Figure 1: A simplified representation of insulin receptor signalling pathways, and potential inhibitory serine/threonine sites associated with diabetes. Serine/threonine phosphorylation by PKC or glycosylation (not shown) of IRS1/2 in heart muscle may impair insulin signalling. Solid line indicates stimulatory activity, and dashed line indicates inhibitory activity. Insulin stimulated glucose uptake is the focus of this discussion, although other known pathways are represented.

C coupling remains to be substantiated. In support of this concept, Shimoni and colleagues have recently shown that in a cardiomyocyte-specific insulin receptor knockout mouse (i.e. without systemic insulin resistance), the action potentials are prolonged45 (for review see Abel34).

Elevated plasma levels of free fatty acids have been explored as key factors in generating insulin resistance in a variety of tissues (for review see Gual et al.40), with saturated fatty acids having a more pronounced ability compared with unsaturated fatty acids, in both adipose and skeletal muscle.46,47 There is growing evidence that fatty acid-induced insulin resistance is not due exclusively to a shift in energetics (i.e. inhibiting glycolysis and glucose oxidation), but rather to changes in the kinetics and interactions among signalling molecules of the IR. Lipid perfusion of isolated rat hearts can block insulin-stimulated glucose uptake and glycogen synthesis, in association with reduced activities of IRS, PI3K, Akt and GSK.48 The saturated fatty acid palmitate can reduce insulin-stimulated Akt phosphorylation and activity, correlated with decreased glucose uptake, in both perfused working rat heart and cultured mouse cardiac cells.49 Recently, PKCθ has been implicated in lipid-induced skeletal muscle insulin resistance.50

Hexosamine biosynthesis pathway

The hexosamine biosynthesis pathway (HBP) leads to the formation of UDP-N-acetylgalcosamine (UDP-GlcNAc, Figure 2). There is considerable evidence from adipose tissue and skeletal muscle to support the hypothesis that elevated extracellular glucose leads to excess flux through the HBP (for review see Whalen & Hart51). The HBP gained prominence in the field of diabetes when Marshall et al.52 found that by inhibiting the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT), they could reverse glucose-induced cellular insulin resistance. It has been shown by a number of investigators that glucosamine (which is taken up by cells via glucose transporters) induces insulin resistance (distal to GFAT). McClain and colleagues demonstrated that overexpression of GFAT leads to skeletal muscle and adipocyte insulin resistance (for review see Cooksey & McClain53).

The HBP is also implicated in lipid-induced insulin resistance. Impairment of insulin-stimulated glucose uptake in skeletal muscle is characteristic of type 2 diabetes and can be induced by prolonged exposure to elevated levels of plasma free fatty acids,54 with an associated accumulation of end products of the HBP.55 Hawkins and colleagues55 demonstrated that this effect can be reproduced by elevating muscle levels of UDP-GlcNAc. A recent study using cultured human myotubes demonstrated that saturated fatty
Diabetic cardiomyopathy and O-linked glycosylation

Figure 2: Hexosamine biosynthesis pathway and glycosylation pathways. Hexosamine biosynthesis pathway is controlled, in part, by the rate limited enzyme GFAT. ER and Golgi body N- and O-linked glycosylation occur on nascent proteins. O-linked glycosylation can also occur on cytosolic and nuclear proteins. Cytosolic/nuclear O-linked glycosylation is catalyzed by a single enzyme, OGT, whereas deglycosylation is driven by a single enzyme, O-GlcNAcase. Reagents used to inhibit enzymes are indicated by dashed lines. It should be noted that alloxan, BAG and STZ are non-specific inhibitors.

acids (palmitate and stearate) activate the HBP by upregulating GFAT expression (rather than simply inhibiting enzymes involved in glucose oxidation), whereas di- and mono-unsaturated fatty acids have weak or no effect, respectively.56

We have shown that glucosamine mimics the adverse effects of glucose on cardiomyocyte E-C coupling,29 and that myocyte insulin resistance accompanies these changes.31 We also have preliminary data showing that fatty acids (e.g. oleate) induce cardiomyocyte mechanical dysfunction and impair insulin-stimulated glucose uptake (unpublished data). These observations led us to consider whether there is a direct link between myocardial insulin resistance and abnormal E-C coupling found in the diabetic heart. Alternatively, these two pathologies could develop in parallel as outlined by Brownlee.7 It is noteworthy that the diabetic heart and hypertrophic/failing heart share many of the same characteristics of E-C coupling, and that the hypertrophic heart is known to be insulin resistant.6,34

Cytosolic/nuclear O-linked glycosylation

The process of post-translational modification of proteins through N- and O-linked glycosylation has been extensively studied. What is relatively unexplored (in terms of heart disease) is the process of O-linked glycosylation by a single sugar moiety (N-acetylglucosamine). Hart and colleagues have worked over the past 20 years to develop reagents (e.g. antibodies) and elucidate the process of this ubiquitous form of glycosylation. Two reviews by Hart and colleagues51,57 describe dynamic glycosylation modification of many modulators, highlight how signal transduction is altered by this process, and discuss strategies for investigating glycosylation-mediated regulation. The following is a brief introduction to this pathway.

O-linked β-N-acetylgalactosamine (O-GlcNAc; pronounced Oh glick nack) is enzymatically attached to the hydroxyl side chain of either a serine or threonine residue. It is a post-translational modification similar to O-linked glycosylation, in that there is a dynamic modification of these residues that is enzymatically controlled, both in terms of glycosylation and deglycosylation. Unlike kinases and phosphatases (of which there are many isoforms), there is a single enzyme identified that drives the glycosylation using UDP-GlcNAc, known as O-GlcNAc transferase (OGT), and a single enzyme that catalyses the deglycosylation, known as β-D-N-acetylgalactosaminidase (O-GlcNAcase; pronounced Oh glick na case). A simplified scheme is presented in Figure 2, which depicts the hexosamine biosynthesis pathway branching off of fructose-6-phosphate, and two arms of glycosylation. The bottom left of Figure 2 illustrates the well characterized N- and O-linked glycosylation which occurs in the ER and
Golgi bodies, whereas the bottom right illustrates the cytosolic/nuclear O-linked glycosylation.

O-GlcNAc modification of proteins has been found in most tissues studied to date, and occurs rapidly, on a timescale similar to that of phosphorylation. The modification of serine/threonine may be on a single residue or multiple sites of a protein. It may compete with phosphorylation or work synergistically. The process is extremely labile. While there are no specific inhibitors of OGT (although alloxan is used as a nonspecific inhibitor), an inhibitor of O-GlcNAcase, O-(2-acetamido-2-deoxy-D-glycopyranosylidene) amino-N-phenylcarbamate (more affectionately known as PUGNAc), is available commercially. The list of proteins on which this type of modification occurs is growing rapidly, and it includes transcription factors, enzymes, chaperones, hormone receptors, transporters, and cytoskeletal proteins. OGT itself is modified by both O-GlcNAc and O-phosphate. The transcription factor c-Myc is an example of synergistic regulation between O-GlcNAc and O-phosphate, whereas the following are examples of the competitive nature between these modifications.

Some of the cytosolic proteins found to be O-GlcNAcylated include key regulatory molecules and enzymes involved in insulin signalling and glucose metabolism. IRS-1 and IRS-2 are glycosylated in skeletal muscle treated with elevated levels of glucosamine, and a similar finding has been reported for human coronary artery endothelium. These endothelial cells also exhibited glycosylated PI3K (p85 subunit) in response to glucose or glucosamine treatment, and an attenuation of insulin-stimulated phosphorylation of IR, IRS-1 and IRS-2, and their interactions with PI3K. There was also reduced insulin-stimulated Akt phosphorylation and subsequent impairment of eNOS activity. Dillmann and colleagues had similarly shown that endothelial NO synthase (eNOS) activity was inhibited by high glucose or glucosamine through O-GlcNAc modification of eNOS near one of the Akt sites. Furthermore, elevated levels of O-GlcNAc in 3T3-L1 adipocytes cause insulin resistance through defective Akt activation. O-GlcNAc modification may be dynamic, as outlined above, or it may be a chronic modification. For example, pancreatic β-cell death and impaired insulin secretion are associated with augmented O-GlcNAc accumulation. As discussed below, the diabetic heart also appears to have elevated UDP-GlcNAc and O-GlcNAc levels.

### Cardiac E-C coupling and cytosolic/nuclear O-linked glycosylation

Very little is known regarding the effects of O-linked glycosylation in heart. As mentioned above, we found that glucosamine induces abnormal myocyte mechanics as seen in our high glucose model and in cells from diabetic animals. Marchase and colleagues demonstrated that glucose-induced changes in capacitative Ca2+ currents in neonatal cardiomyocytes were attributable to modifications through the HBP, since the effects were blocked with azaserine. While the role of this current in cardiomyocytes is not well understood, the authors suggest that it may be involved in cellular responses to stress as described in other cell types. This is supported by a recent study showing that O-GlcNAcation directly influences intracellular Ca2+ homeostasis.

Studies conducted by Dillmann and colleagues suggest that nuclear O-linked glycosylation of the Sp1 transcription factor may contribute to reduced expression of SERCA2a in neonatal cardiomyocytes cultured in high glucose. The same study also demonstrated elevated levels of UDP-GlcNAc in hearts of STZ-induced diabetic adult mice, supporting the idea that increased glucose flux through the HBP occurs in type 1 diabetes. In further support of this concept, when an adenovirus expressing O-GlcNAcase (enzyme that removes the sugar moiety) was introduced into hearts of type 1 diabetic mice, it significantly reduced the myocardial content of O-GlcNAc, and improved the diabetic phenotype of prolonged Ca2+ transients and myocyte mechanics, and whole heart ventricular dysfunction. Although total PLB content was reduced, there was a significant increase in SERCA2a protein levels and phosphorylated PLB, thus providing cellular mechanisms for improved myocardial relaxation.

High glucose, fatty acids, angiotensin II (AngII) and PKC are among a number of factors which modulate both cardiac E-C coupling and insulin signalling (for reviews see Malhotra et al. and Fares, and references therein). While the role of this current in cardiomyocytes is not well understood, the authors suggest that it may be involved in cellular responses to stress as described in other cell types. This is supported by a recent study showing that O-GlcNAc alteration directly influences intracellular Ca2+ homeostasis.

We have recently shown that myocytes cultured in high glucose or isolated from diabetic animals are insulin resistant, and this can be prevented or reversed (respectively) by inhibiting PKC.

High glucose can cause isolated cardiomyocytes to produce and release AngII, which then acts as an autocrine signal to increase PKC activity. AngII has also been implicated in the attenuation of outward K+ currents in ventricular myocytes from diabetic animals, leading to prolonged action potentials. While there are conflicting data as to whether high glucose is cardioprotective or pro-apoptotic, in the context of the HBP, high glucose...
attenuates AngII-induced hypertrophy in neonatal rat ventricular myocytes, which is blocked by azaserine (a non-selective inhibitor of GFAT) and mimicked by glucosamine.65 Conversely, high glucose has also been shown to induce apoptosis through increased synthesis of AngII, which appears to involve O-linked glycosylation of a transcription factor for AngII (i.e. p53, as described by Fiordaliso et al.25).

There is a host of other regulators of myocardial E-C coupling which may be influenced by O-GlcNAc modification. For example, a decrease in eNOS activity relating to O-GlcNAcylation as shown in endothelial cells,60 could potentially modulate the complicated balance of adrenergic and cholinergic regulation in the heart (for review see Massion & Balligand66). Diabetes and high glucose impair inotropic responses to α-adrenergic agonists, which is dependent on the HBP.64 It would be intriguing if these effects were influenced by changes in eNOS activity. There is evidence that flux through the HBP enhances the activity of specific PKC isoforms (e.g. PKCδ1 and PKCδ8) which were not accompanied by membrane translocation.77 Whether PKC activity can be modified by O-GlcNAcylation (as it is by O-phosphorylation) has not been demonstrated. Whether ion channels and pumps are modified by O-GlcNAc also remains to be established.

Conclusions

To date, there is insufficient evidence to demonstrate a direct link between the aetiologies of abnormal myocardial E-C coupling and that of impaired insulin signalling; however, these processes seem to be inextricable. Studying the pathogenesis of these dysfunctions in a variety of models (e.g. diabetes and hypertension) may lead to the identification of convergence of the cellular responses to excess glucose and fatty acids. The hexosamine biosynthesis pathway and post-translational modification by cytosolic/nuclear O-linked glycosylation may provide the key elements to elucidating the connection.

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