

# The pathobiochemical role of the dystrophin-dystroglycan complex and the Ca<sup>2+</sup>-handling apparatus in diabetes-related muscle weakness (Review)

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**Abstract.** Serious diabetic complications affect millions of patients worldwide. Skeletal muscle represents the largest insulin-regulated glucose sink in the body, making insulin resistance and abnormal glucose disposal in muscle fibres a critical aspect of diabetes mellitus. Advances in the biomedical analysis of the molecular mechanisms underlying diabetic complications rely heavily on the study of suitable disease models. The Goto-Kakizaki (GK) rat is an established animal model of non-obese type 2 diabetes. This review discusses the recent finding that expression of the dystrophin-dystroglycan complex is drastically altered in diabetic GK skeletal muscle fibres. In normal muscle, the dystrophin-glycoprotein complex provides a stabilizing connection between the actin membrane cytoskeleton and the extracellular matrix component laminin. A reduction in dystrophin-associated proteins may be associated with a weakening of the fibre periphery, abnormal sarcolemmal signaling and/or a decreased cytoprotective mechanism in diabetic skeletal muscle. Stimulation by insulin might be altered due to impaired linkage between the dystrophin-anchored actin cytoskeleton and the intracellular pool of essential glucose transporters. The diminished recruitment of GLUT4 transporter molecules to the sarcolemma may be a key step in the development of insulin resistance in diabetic skeletal muscles. Thus, analogous to certain forms of muscular dystrophy, altered dystrophin levels

may have pathological effects in type 2 diabetes. In contrast, the dystrophin-glycoprotein complex does not appear to be altered in diabetic cardiac muscle. However, reduced expression of the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase isoform SERCA2 is characteristic of cardiac abnormalities in type 2 diabetes. Reduced Ca<sup>2+</sup> removal from the sarcoplasm may be associated with impaired relaxation kinetics, and could therefore play a pathophysiological role in diabetic cardiomyopathy. Here, the potential impact of these molecular alterations in diabetic muscle tissues is discussed and critically examined with respect to the future design of alternative treatment strategies to counteract diabetes-associated muscle weakness.

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## 1. Introduction

Abnormal glucose metabolism has emerged as a major health problem suffered by millions of diabetic patients worldwide (1). It has been estimated that the prevalence of diabetes in adults will rise to 5.4% by the year 2025, with the majority of novel cases occurring in developed countries (2). While type 1 diabetes is clearly associated with the autoimmune destruction of pancreatic  $\beta$ -cells, type 2 diabetes has as its principal features insulin resistance and impaired  $\beta$ -cell functioning (3). Insulin resistance affects the main insulin target organs, such as the liver, adipose tissue and muscle fibres. Influenced by various genetic factors (4), insulin-resistant type 2 diabetes represents a complex disorder (5), in part caused by the modern lifestyle (6). A classic characteristic of this type of diabetes is the hormonal inability to properly stimulate the peripheral glucose metabolism (7). Since skeletal muscle is the most abundant tissue in the body and is responsible for up to 80% of whole body insulin-stimulated glucose uptake, the

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*Abbreviations:* DG, dystroglycan; Dp427, dystrophin of 427 kDa; GK, Goto-Kakizaki; LAM, laminin; nNOS, neuronal nitric oxide synthase; SERCA, sarcoplasmic or endoplasmic reticulum calcium adenosine triphosphatase

*Key words:* diabetes, dystroglycan, dystrophin, neuronal nitric oxide synthase, Goto-Kakizaki rat, sarcoplasmic or endoplasmic reticulum calcium adenosine triphosphatase 2, syntrophin

study of insulin resistance in muscle fibres is of central importance to the elucidation of the molecular pathogenesis of diabetes mellitus (8). Besides metabolic implications, diabetes also has a direct influence on contractile tissues, causing moderate skeletal muscle weakness in diabetic patients (9) and serious cardiomyopathic complications in the diabetic heart (10).

The coordinated functioning of signaling pathways is critical if skeletal muscles are to respond to changing physiological demands (11). Therefore, alterations in regulatory events that influence the metabolism may result in serious pathological side effects as, for example, observed in metabolic syndrome and diabetes (12,13). The signaling pathway of insulin action is controlled by phosphorylation steps that couple the activation of the surface insulin receptor to the translocation of glucose transporter units (14). Hormone binding to the  $\alpha$ -subunits of the insulin receptor results in the autophosphorylation of the  $\beta$ -subunits, which activates the external tyrosine kinase activity of the receptor complex. The insulin receptor substrate IRS1 is a key intracellular target for phosphorylation, functioning as a docking molecule for signaling molecules such as Grb2. The subsequent insulin receptor pathway involves the activation of IRS1-associated phosphoinositide-3-kinase activity, Akt kinase serine phosphorylation and, finally, the recruitment of glucose transporters (15). Within this highly complex regulatory network, pathophysiological resistance to the initial hormonal signaling cascade triggers impaired activation of essential downstream substrates within the insulin pathway (16). The main glucose transportation step at the level of the skeletal muscle surface membrane, provided by the insulin-sensitive type 4 glucose transporter GLUT4, has been demonstrated to be the rate-limiting step in the glucose metabolism (17).

Interestingly, the membrane cytoskeletal protein dystrophin and GLUT4 co-localize in skeletal muscle fibres (18), and the dystrophin-associated proteins  $\alpha$ -dystroglycan,  $\beta$ -dystroglycan and syntrophin are closely linked to an adapter protein of the insulin signaling pathway, the growth factor receptor-bound protein Grb2 (19). It is therefore not surprising that, recently, expression of the dystrophin isoform Dp427 and of its associated proteins,  $\alpha$ -dystroglycan,  $\beta$ -dystroglycan, syntrophin and nitric oxide synthase, was shown to be altered in diabetic skeletal muscle fibres (20). This finding provides important insight into the potential mechanisms underlying insulin resistance in muscle tissue, and is discussed in this review. Both dystrophin and members of the dystrophin-associated glycoprotein complex are involved in various muscle pathologies, suggesting common molecular or cellular abnormalities in a variety of neuromuscular disorders (21-23). To explore this idea in more detail, a brief overview of the proposed structure and function of these muscle proteins is provided. The potential impact of a reduced abundance of the dystrophin-dystroglycan complex and the syntrophin-nNOS complex in diabetic fibres is then discussed with respect to sarcolemmal integrity, plasma membrane signaling and cytoprotection in diabetic muscle. Importantly, altered dystrophin expression in diabetes may explain the abnormal subcellular location of the glucose transporter GLUT4 in skeletal muscle (20). Reduced levels of the dystrophin-dystroglycan complex may cause impaired anchoring of the actin membrane cytoskeleton, which

in turn might interfere with the proper recruitment of GLUT4 units to the plasmalemma. This important aspect of insulin resistance in diabetic skeletal muscles is critically examined in this review.

## 2. The dystrophin-glycoprotein complex

Following the molecular genetic identification of dystrophin, sequence homology analyses and biochemical studies strongly indicated that the Dp427 isoform of the expressed muscle protein functions as a cytoskeletal element (24). Internal domains of full-length dystrophin exhibit extensive sequence similarities with spectrin, suggesting a spectrin-like role in the membrane cytoskeleton of muscle fibres. Similar Dp427 isoforms exist in both skeletal and cardiac muscle, although with a slightly different subcellular localization. In skeletal muscle, Dp427-M and its associated surface proteins are restricted to the sarcolemma (23,25) while, in cardiac fibres, Dp427-C is also found in the transverse-tubular region of the surface membrane (26,27). The gigantic 2.4-Mb dystrophin gene with a coding sequence of 79 exons was shown to correspond to a full-length 14-kb mRNA message. Seven different promoters, which are located throughout the dystrophin gene in the chromosomal Xp21 region, are responsible for the tissue-specific distribution of the various dystrophin isoforms. The complex domain composition of the various dystrophin isoforms and dystrophin-related proteins is described in notable reviews by Muntoni *et al* (28) and Ahn and Kunkel (29).

As shown in Fig. 1A, the structure of full-length muscle dystrophin consists of the following main regions: i) the carboxy-terminal region with an  $\alpha$ -helical domain, followed by ii) a  $Zn^{2+}$ -dependent zinc finger domain, iii) a cysteine-rich domain with  $Ca^{2+}$ -dependent twin EF-hand motifs, iv) a PPxY-binding WW motif, v) a spectrin-like triple-helical repeat domain forming the central structure of dystrophin, vi) proline-rich hinge regions partitioning the rod domain, and vii) the amino-terminal region consisting of calponin homology regions. While the amino-terminal and rod domains exhibit actin-binding sites, the carboxy-terminal region interacts directly with  $\beta$ -dystroglycan, syntrophins and dystrobrevins (30,31). The structure of all dystrophins includes the WW domain, the EF-hand motif and the carboxy-terminal helix region, but shorter isoforms lack the amino-terminal actin-binding domains (28). Hence, only the full-length Dp427 isoform appears to function as an indirect linker between the cortical actin network and extracellular laminin. In skeletal muscle, an important autosomal homologue of Dp427 exists, the dystrophin-related protein named utrophin. Its Up395 isoform interacts with the dystroglycan sub-complex, agrin and rapsyn at the neuromuscular junction, and one of its main functions is probably the clustering of nicotinic acetylcholine receptor units opposite the synaptic junction (32,33).

Dp427 does not exist in isolation at the muscle surface, but is closely associated with several glycoproteins (34). In skeletal muscle, dystrophin-associated proteins with a wide range of molecular masses and biochemical properties were identified that localize to cytoskeletal, extracellular, cytosolic and integral domains (23). The diagrammatic presentation in Fig. 1B outlines the basic arrangement of the dystrophin-

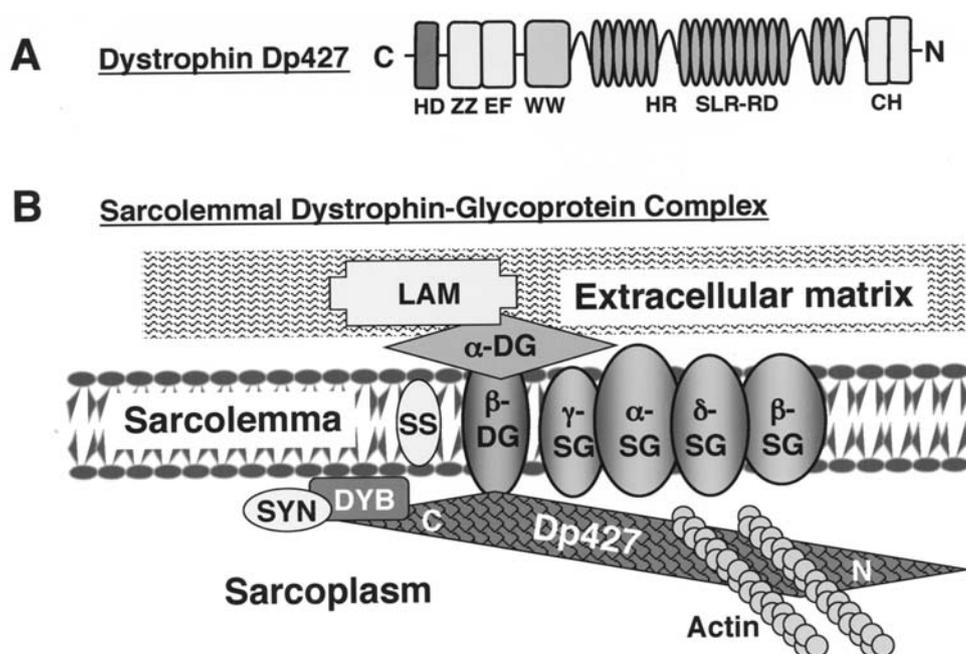


Figure 1. Domain composition of full-length muscle dystrophin and composition of the dystrophin-glycoprotein complex. (A) Domain structure of the Dp427 isoform of dystrophin. (B) Arrangement of the central elements of the dystrophin-associated glycoprotein complex from muscle fibres. Full-length muscle dystrophin consists of the carboxy-terminal region with an  $\alpha$ -helical domain (HD), a  $Zn^{2+}$ -dependent zinc finger domain (ZZ), a cysteine-rich domain with  $Ca^{2+}$ -dependent twin EF-hand motifs (EF), a PPxY-binding WW motif (WW), a spectrin-like triple-helical repeat domain forming the central rod structure of dystrophin (SLR-RD), proline-rich hinge regions partitioning the rod domain (HR) and the amino-terminal region consisting of calponin homology (CH) regions. The supramolecular dystrophin complex from muscle consists of the Dp427 isoform of dystrophin, the integral glycoprotein  $\beta$ -dystroglycan ( $\beta$ -DG), the extracellular receptor protein  $\alpha$ -dystroglycan ( $\alpha$ -DG), the sarcoglycan isoforms  $\alpha$ -SG,  $\beta$ -SG,  $\delta$ -SG and  $\gamma$ -SG, the extracellular matrix component laminin (LAM), the tetraspan-like protein sarcospan (SS), syntrophin (SYN), dystrobrevin (DYB) and cortical actin.

glycoprotein complex from skeletal muscle. The dystroglycan sub-complex, consisting of the integral glycoprotein  $\beta$ -dystroglycan of 43 kDa and the extracellular laminin-binding protein  $\alpha$ -dystroglycan of 156 kDa (35), can be considered the central backbone of this supramolecular membrane assembly (36). Both dystroglycans are encoded by a single mRNA (35). The proper trafficking and processing of dystroglycans is essential for sarcolemmal stability (37), and post-translational modifications such as glycosylation are critical to the functioning of the dystrophin-associated glycoprotein complex (38). Cytoplasmic dystrophin-dystroglycan interactions appear to be regulated by tyrosine phosphorylation (39), whereby the WW domain of Dp427 plays a vital role in protein-protein interactions (40). The  $Ca^{2+}$ -dependent coupling between the extracellular G domain of laminin and  $\alpha$ -dystroglycan provides, via its link to integral  $\beta$ -dystroglycan, an indirect bridge to the sub-sarcolemmal dystrophin network (36). Consequently, the dystrophin-dystroglycan complex forms a molecular anchor for the actin membrane cytoskeleton on the inside of a fibre and structural stabilization of the basal lamina on the outside of the muscle (23).

In contrast to  $\beta$ -dystroglycan, the other main group of integral dystrophin-associated proteins in muscle, the  $\alpha\beta\gamma\delta$  sarcoglycans of 25-50 kDa, are indirectly linked to Dp427 (41). In addition, a small integral dystrophin-associated element is the tetraspan-like protein called sarcospan (42) whose membrane targeting appears to be dependent on the assembly of the complete sarcoglycan complex (43). The muscle Dp427 isoform is also a key binding protein for several sarcoplasmic classes of proteins, including syntrophins and

dystrobrevins (44,45). These dystrophin-associated components in turn anchor key regulatory proteins to the sarcolemma, such as the neuronal nNOS isoform of nitric oxide synthase (46), the intermediate filament component syncoilin (47), the voltage-dependent  $Na^{+}$ -channel (48) and the membrane water channel aquaporin-4 (49). Especially interesting is the linkage between the dystrophin-glycoprotein complex and the enzyme nNOS. Nitric oxide synthases intracellularly produce this readily diffusible and short-lived signaling molecule, which was shown to mediate diverse functions in skeletal muscle related to myogenesis, metabolism, fibre contraction and blood flow (50). Since it was demonstrated that syntrophin-linked nNOS is reduced in diabetic skeletal muscle (20), abnormalities in the dystrophin-glycoprotein complex may also be relevant for impaired nitric oxide-dependent signaling events in type 2 diabetes.

### 3. Dystrophin and diabetic muscle weakness

Deficiency in the dystrophin isoform Dp427 triggers a similar pathobiochemical response in both skeletal and cardiac muscle fibres, suggesting that the complications observed in both types of muscle in X-linked muscular dystrophy are initiated by the loss of the dystrophin-associated surface glycoprotein complex (51). It is assumed that in dystrophin-deficient fibres the reduction in dystrophin-associated glycoproteins results in the loss of a proper linkage between the sub-sarcolemmal membrane cytoskeleton and the extracellular matrix (21-23). The impaired integrity of the muscle plasma membrane then renders fibres more susceptible to the micro-rupturing of the surface mem-

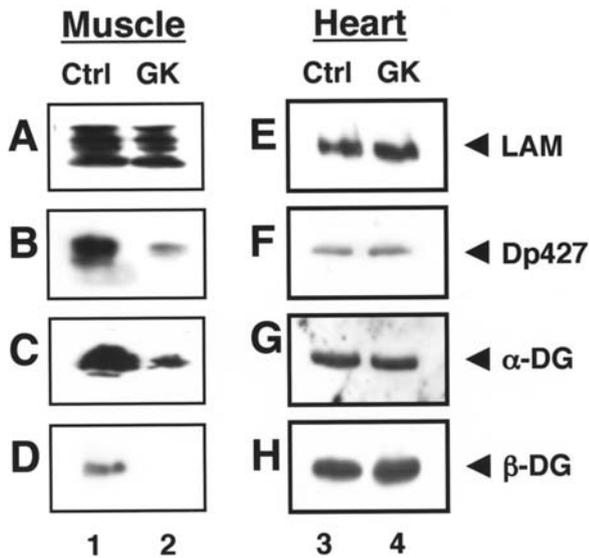


Figure 2. Immunoblot analysis of the dystrophin-dystroglycan complex in diabetic GK skeletal muscle and heart. (A-H) Immunoblots labeled with antibodies to laminin (LAM; A and E), the Dp427 isoform of dystrophin (B and F),  $\alpha$ -dystroglycan ( $\alpha$ -DG; C and G) and  $\beta$ -dystroglycan ( $\beta$ -DG; D and H). For comparative studies, the crude microsomal fraction was isolated from normal and GK skeletal muscle and heart homogenates by an established subcellular fractionation method (20). Gel electrophoretic separation of proteins and immunoblot analysis were carried out by standard procedures, as previously optimized by our laboratory (20). Lanes 1 and 2, membranes from normal control and diabetic GK skeletal muscle, respectively; lanes 3 and 4, membranes from normal control and diabetic GK heart, respectively. Arrowheads mark the positions of immuno-decorated proteins.

brane, which initiates a natural re-sealing process at the sites of sarcolemmal disintegration (52). The newly-introduced membrane patches contain  $\text{Ca}^{2+}$ -leak channels that trigger an influx of  $\text{Ca}^{2+}$  ions into dystrophic fibres, which in turn activate  $\text{Ca}^{2+}$ -dependent proteases (53). This cycle of membrane rupturing, abnormal ion fluxes and subsequent degradation of muscle proteins probably plays a central role in the downstream events eventually leading to the characteristic muscle wasting observed in dystrophinopathies (54). Analogously, since members of the dystrophin-glycoprotein complex interact with proteins involved in the insulin signaling cascade (18,19), it was of interest to determine whether a causal link may also exist between diabetic side effects in contractile fibres and alterations in the dystrophin-associated network of muscle surface proteins (20).

As a rule, inbred animal strains show genetically fewer inter-individual differences than human patients. This has led to the extensive usage of spontaneous and genetically engineered animal models of human diseases for determining basic pathological mechanisms and for evaluating new therapeutic strategies. This also includes rodent models of common neuromuscular diseases, as recently reviewed by Doran *et al.* (55). Most importantly, considerably lower experimental repeats are capable of producing meaningful biochemical data when animal models are employed, as compared to testing human specimens. In the case of type 2 diabetes, an established model system is represented by the spontaneously diabetic Goto-Kakizaki (GK) rat (56). Since the GK rat is non-obese, potential alterations in diabetic muscle fibres can be studied without potential complicating factors due to obesity. The

diabetic GK phenotype manifests itself by increased blood glucose levels without alterations in body weight or non-fasting plasma insulin levels (57), whereby diabetic skeletal muscles show a reduced percentage of oxidative fibres and decreased IRS1 and PI3K activity in oxidative muscles. The insulin signaling pathway is chronically altered in the GK rat, including: i) inhibition of insulin receptor autophosphorylation, ii) decreased levels of IRS1 tyrosine phosphorylation and iii) impaired downstream activation of PI3K, PKB/Akt and ERK (58-60).

Immunoblot analysis (Fig. 2) was carried out with hind limb skeletal muscle and heart specimens from 12-week-old normal control Wistar rats versus age-matched GK rats. This is a suitable age range for studying diabetic complications, since key symptoms of type 2 diabetes, such as defects in insulin secretion and peripheral insulin resistance, occur by 4 weeks of age in the GK rat model (61,62). Immunodecoration revealed a comparable density of extracellular matrix protein laminin in normal and GK samples in both skeletal muscle and the heart (Fig. 2A and E). In stark contrast, the Dp427 isoform of skeletal muscle dystrophin and the  $\alpha\beta$ -dystroglycan sub-complex were found to be drastically reduced (Fig. 2B-D). The cardiac equivalent of the dystrophin-dystroglycan complex was, however, not affected in the diabetic GK rat (Fig. 2F-H). Besides laminin, sarcoglycans and dystrobrevin were also shown to be unaltered in GK skeletal muscle preparations (20). Hence, in diabetic skeletal muscle fibres, but not in heart tissue, the structural backbone of the trans-sarcolemmal linkage between laminin and cortical actin is impaired. Findings from the Western blot analysis were confirmed by immunofluorescence microscopy, which showed a reduction in Dp427,  $\alpha$ -dystroglycan and  $\beta$ -dystroglycan in a selected fibre population from GK muscle (20). This effect was not fibre-type specific, and altered expression of the dystrophin-dystroglycan complex did not appear to induce obvious signs of general muscle pathology. Subcellular fractionation studies using sucrose gradient centrifugation confirmed the abnormal location of the main muscle glucose transporter (20), suggesting a possible link between abnormal dystrophin expression and decreased GLUT4 mobility in type 2 diabetes.

Since the disintegration of the dystrophin-glycoprotein complex has clearly been established as a key event leading to muscle wasting in various neuromuscular diseases (21), it can be assumed that the altered density in dystrophin and the two core dystroglycan proteins may contribute to insulin resistance. As sarcoglycans and laminin are indirectly associated with dystrophin, it is not surprising that a selective reduction in dystroglycans is present in diabetic skeletal muscle. Based on the current findings, it is difficult to make a clear connection between impairments in individual insulin signaling steps and primary defects in type 2 diabetes. Detailed functional studies of the dystrophin-dystroglycan complex are now necessary to determine the exact mechanism that underlies the loss of this supramolecular surface complex in selected diabetic myofibres. However, since dystrophin forms the central molecular anchor of the sarcolemma, which is crucial for membrane stabilization during contractile activity, signal transduction events and receptor clustering (63), its altered abundance or effect on the function of binding partners may render diabetic muscle fibres more susceptible to cellular degeneration. In this respect, the



on that the expression of  $\alpha$ -syntrophin and its associated nNOS is drastically reduced in diabetic skeletal muscle fibres (20) suggests a potential role for disturbed dystrophin expression in abnormal insulin signaling and impaired cellular protection in diabetic muscle. Reduced nitric oxide synthase activity is an established pathobiochemical symptom in skeletal muscle from type 2 diabetic patients (64) and X-linked muscular dystrophy (65). Since nitric oxide is an important cytoprotective and anti-inflammatory factor (66), reduced nNOS expression in GK muscle indicates an involvement of the dystrophin complex in diabetic muscle weakness. Thus, a reduction in the dystrophin-dystroglycan complex and the syntrophin-nNOS complex in diabetic skeletal muscle may induce structural weakening of the fibre periphery, impaired insulin signaling and decreased cytoprotection. Possibly, faulty anchoring of the cortical actin network due to reduced levels of the dystrophin-dystroglycan complex impairs the recruitment of sufficient numbers of glucose transporter molecules from the intra-cellular pool to the sarcolemma. Therefore, the stimulatory effect of insulin or muscle contraction is not properly converted into downstream signaling events and molecular adaptations leading to peripheral insulin resistance in type 2 diabetes.

#### 4. Calcium signaling and diabetic muscle

The reduction of the dystrophin-dystroglycan complex in diabetic fibres described above appears to be restricted to skeletal muscle. Dystrophin-related structural weakening of the muscle fibre periphery does not seem to occur in the diabetic heart. However, abnormal  $\text{Ca}^{2+}$  handling has been implicated in the pathophysiological mechanisms leading to contractile weakness of cardiac fibres in diabetes (67,68). Though the dystrophin-glycoprotein complex is of central importance to the stabilization and maintenance of the  $\text{Ca}^{2+}$ -regulatory apparatus involved in excitation-contraction coupling, the cellular dystrophin network might nevertheless be indirectly involved in certain aspects of cardiac abnormalities in type 2 diabetes. Calcium cycling represents a central physiological mechanism that regulates the excitation-contraction-relaxation cycle in cardiac and skeletal muscle fibres (69). The highly coordinated temporal and spatial movement of  $\text{Ca}^{2+}$  ions within muscle cells is mediated by a large array of ion-regulatory proteins, including voltage sensors, surface  $\text{Ca}^{2+}$  channels, triadic  $\text{Ca}^{2+}$ -release channels,  $\text{Ca}^{2+}$ -binding complexes,  $\text{Ca}^{2+}$ -shuttle proteins, sarcolemmal  $\text{Ca}^{2+}$  pumps, sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases and  $\text{Ca}^{2+}$  exchangers (70). Disturbances in the abundance of these  $\text{Ca}^{2+}$ -handling proteins and/or altered protein-protein interactions within ion-regulatory protein complexes have been shown to be associated with various cardiac muscle diseases (71-73).

The diagram in Fig. 3 outlines the potential connection between the dystrophin complex, elements of the insulin signaling pathway and the  $\text{Ca}^{2+}$ -handling apparatus. In skeletal muscle, excitation-contraction coupling is mediated by direct physical interaction between the II-III loop domain of the voltage-sensing  $\alpha_{1S}$ -dihydropyridine receptor and a cytoplasmic region of the RyR1 isoform of the ryanodine receptor  $\text{Ca}^{2+}$ -release channel at the triad junction (74). In the heart, voltage sensing and sarcolemmal  $\text{Ca}^{2+}$  entry is mediated

by the  $\alpha_{1C}$ -subunit of the dihydropyridine receptor (75), which translates into a  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release mechanism that is provided by the RyR2 isoform of the junctional ryanodine receptor complex (76). The triadic linker molecule between the  $\text{Ca}^{2+}$ -release channel and the  $\text{Ca}^{2+}$ -reservoir complex of the terminal cisternae is represented by triadin (77). An elevated cytosolic  $\text{Ca}^{2+}$  level induces muscle contraction by its modulation of the troponin-tropomyosin system. Subsequent fibre relaxation is triggered by the efficient removal of  $\text{Ca}^{2+}$  ions via the sarcolemma and the sarcoplasmic reticulum. In skeletal muscle, the SERCA1 isoform of the  $\text{Ca}^{2+}$ -ATPase and its regulatory subunit sarcolipin mediate efficient  $\text{Ca}^{2+}$  re-uptake (78). In heart muscle fibres, key players in this process are the SERCA2 isoform of the  $\text{Ca}^{2+}$ -ATPase and its regulatory subunit phospholamban, the calmodulin-dependent surface PMCA  $\text{Ca}^{2+}$ -ATPase, and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger in physiological combination with the  $\text{Na}^+/\text{K}^+$ -ATPase (79). Thus, directly or indirectly,  $\text{Ca}^{2+}$  removal from the cytosol is a highly energy-dependent process. To keep the bioenergetic cost of ATP hydrolysis to a minimum, sophisticated  $\text{Ca}^{2+}$ -shuttle and luminal  $\text{Ca}^{2+}$ -binding systems provide an optimum ion reservoir environment, so that the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase has to pump against a less steep gradient of free  $\text{Ca}^{2+}$ -concentration. This gives the  $\text{Ca}^{2+}$ -shuttle element sarcalumenin of 160 kDa and  $\text{Ca}^{2+}$ -binding proteins, such as calsequestrin of 63 kDa, the calsequestrin-like proteins of higher molecular mass and the histidine-rich  $\text{Ca}^{2+}$ -binding protein HRCBP, a central role as mediators of the excitation-contraction-relaxation cycle (80).

In diabetic muscle fibres, impaired insulin signaling and insufficient recruitment of glucose transporters are believed to play an important role in insulin resistance. Disturbed levels of the insulin receptor or its major docking partner, the insulin receptor substrate IRS1, as well as alterations in factors downstream of the insulin receptor pathway such as the PI3-kinase, the growth factor receptor-bound element Grb2 or the glucose transporter GLUT4, may be involved in the molecular pathogenesis of the diabetic heart (81). While skeletal muscles from the GK rat do not seem to exhibit changes in the central elements of insulin signaling (20), the insulin receptor IRS1 and GLUT4 are all affected in the heart of this animal model of type 2 diabetes (82). Building on these observations and the fact that altered  $\text{Ca}^{2+}$  homeostasis has previously been reported to play a role in cardiac weakness in diabetes (67), the analyses shown in Figs. 4 and 5 investigated the abundance of the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase in GK heart muscle. Although these findings are preliminary results that require further validation, they provide a good indication of the pathobiochemical fate of the major  $\text{Ca}^{2+}$ -ATPase in non-obese type 2 diabetes. In contrast to comparable levels of the surface  $\text{Na}^+/\text{K}^+$ -ATPase in normal versus GK specimens (Fig. 4B), the SERCA2 isoform of the  $\text{Ca}^{2+}$  pump was drastically reduced in the diabetic heart (Fig. 4C). The silver-stained protein band pattern of electrophoretically separated heart membranes showed no major differences between control and diabetic preparations (Fig. 4A), and expression of the regulatory protein phospholamban was unaltered in cardiac GK fibres (Fig. 4D). In comparison, the skeletal muscles SERCA1  $\text{Ca}^{2+}$ -ATPase and  $\text{Na}^+/\text{K}^+$ -ATPase exhibited no major changes in their relative density in control versus diabetic preparations (Fig. 4E and F).

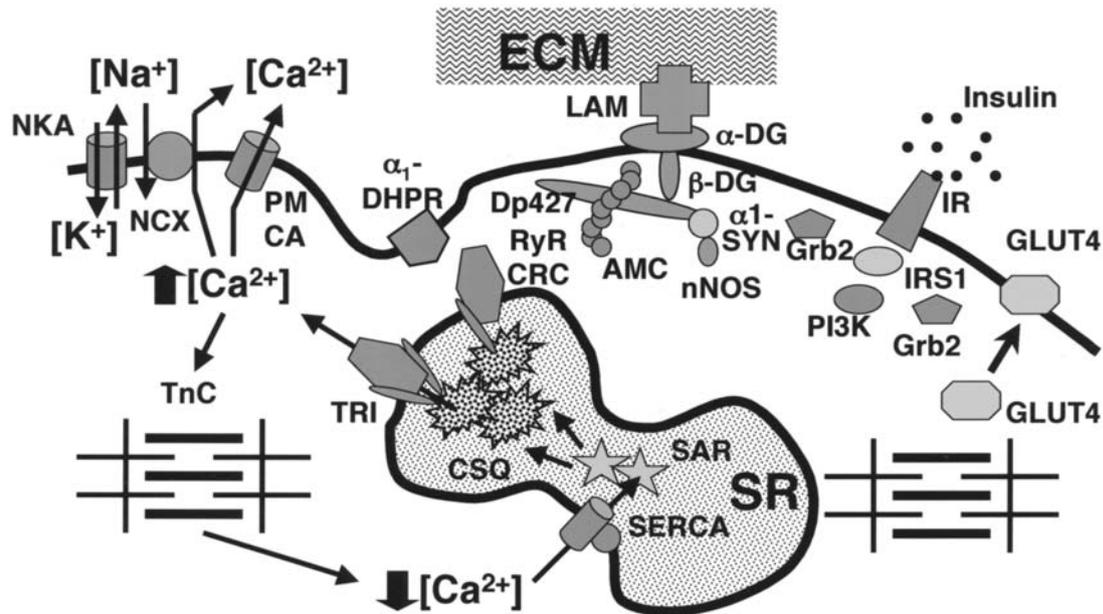


Figure 3. Overview of the functional connection between the insulin signaling pathway, the dystrophin-glycoprotein complex and the Ca<sup>2+</sup>-handling apparatus of muscle fibres. The diagram provides an overview of key proteins that have recently been implicated in the pathophysiological mechanisms that lead to peripheral insulin resistance in diabetic muscle fibres. Some of the listed proteins differ in their isoform between skeletal muscle and the heart, such as the ryanodine receptor Ca<sup>2+</sup>-release channel (RyR1 versus RyR2), the voltage-sensing subunit of the dihydropyridine receptor (α<sub>1S</sub>-DHPR versus α<sub>1C</sub>-DHPR) and the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA1 versus SERCA2). Immunoblot analysis has clearly shown that expression levels of the dystrophin-dystroglycan and syntrophin-nNOS complexes are reduced in diabetic skeletal muscle, and that an essential Ca<sup>2+</sup>-removal protein, the sarcoplasmic reticulum SERCA2 Ca<sup>2+</sup>-ATPase, is significantly decreased in the diabetic heart. This pathobiochemical status may trigger abnormal signaling downstream of the insulin receptor (IR) complex. Besides the activation of the insulin receptor substrate 1 (IRS1), IRS-1-associated phosphoinositide-3-kinase (PI3K) and growth factor receptor-bound protein Grb2, the recruitment of the glucose transporter GLUT4 is a crucial step in glucose uptake. Possibly, reduced dystrophin expression results in impaired anchoring of the actin membrane cytoskeleton, which in turn may interfere with the proper recruitment of GLUT4 units to the surface membrane. The decrease in the neuronal isoform of the nitric oxide synthase (nNOS) and syntrophin (α1-SYN) could trigger abnormal physiological signaling and reduced cytoprotection in diabetic skeletal muscle. On the other hand, impaired SERCA2 functioning in the diabetic heart may be the underlying reason for delayed relaxation kinetics in diabetic cardiomyopathy. Since Ca<sup>2+</sup>-handling involves many different cardiac proteins, including the luminal Ca<sup>2+</sup>-binding proteins calsequestrin (CSQ) and sarcalumenin (SAR), and the surface elements Na<sup>+</sup>-Ca<sup>2+</sup>-exchanger (NCX), Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) and calmodulin-dependent Ca<sup>2+</sup>-ATPase (PMCA), altered SERCA2 activity may trigger overall disturbed ion handling, and thereby contractile weakness.

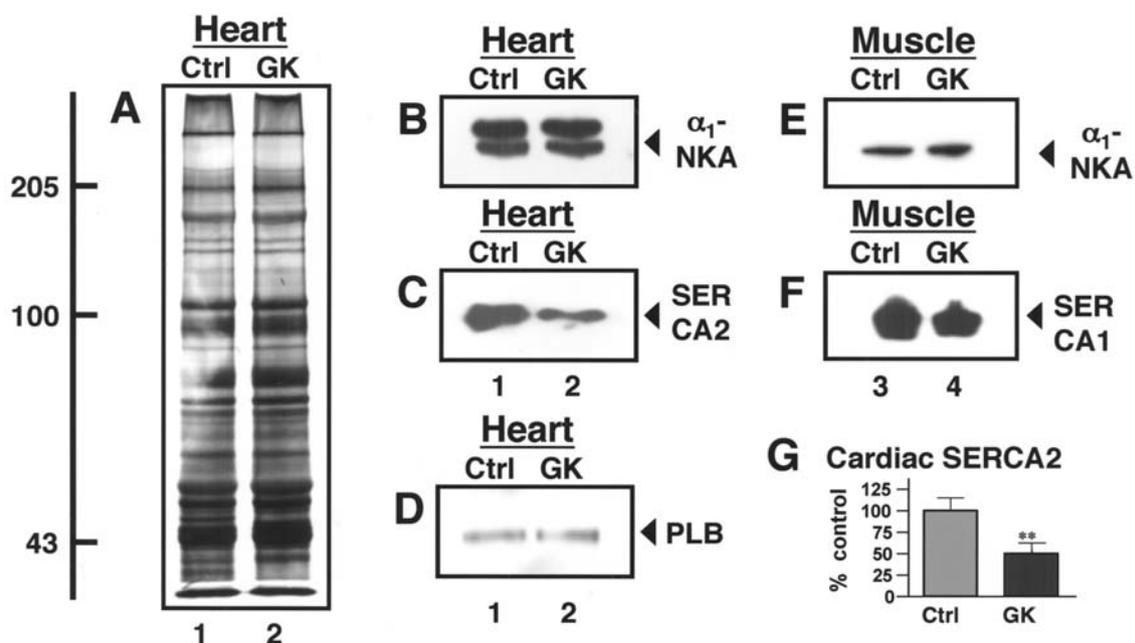


Figure 4. Immunoblot analysis of the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase in the diabetic GK heart. (B-F) Immunoblots labeled with antibodies to the α<sub>1</sub>-subunit of the Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA; B and E), the slow cardiac SERCA2 (C) and fast skeletal muscle SERCA1 (F) isoforms of the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase, and the regulatory subunit phospholamban (PLB; D). (G) Graphical presentation of the immunoblot analysis of SERCA2 (n=10; \*\*p<0.01; unpaired t-test). (A) A representative silver-stained gel of cardiac preparations. Molecular mass standards (in kDa) are indicated on the left. Lanes 1 and 2, membranes from normal control and diabetic GK heart, respectively; lanes 3 and 4, membranes from normal control and diabetic GK skeletal muscle, respectively. Arrowheads mark the positions of immuno-decorated proteins.

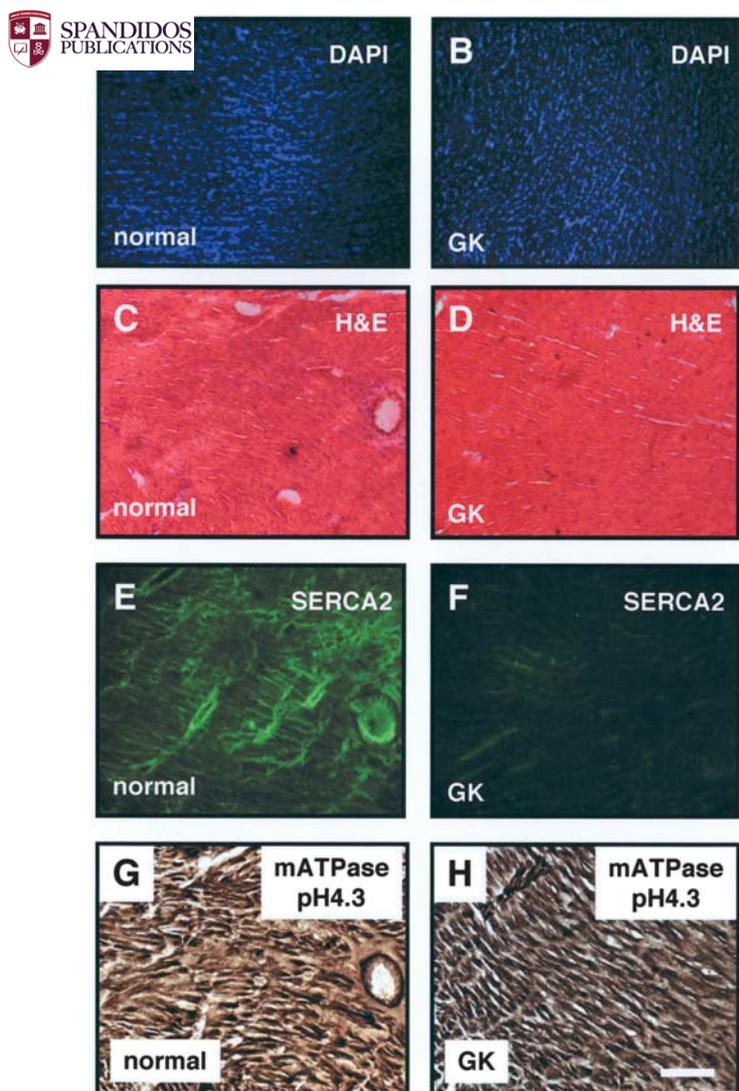


Figure 5. Immunofluorescence localization of sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase in the diabetic GK heart. (A-H) Cardiac cryosections stained with the DNA-binding dye diamidinophenylindole (A and B), haematoxylin and eosin (H&E; C and D), antibodies to the slow/cardiac SERCA2 isoform of the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (E and F) and labeled histochemically for myosin ATPase activity at pH 4.3 (G and H). Cryosections of  $12\ \mu\text{m}$  thickness were prepared from whole hearts with the help of a cryostat at  $-20^\circ\text{C}$ , and subsequently mounted on positively-charged microscope slides. Fixation, incubation with primary and fluorescein-conjugated secondary antibodies and washing steps were performed as previously described (20). (A, C, E and G) Normal heart; (B, D, F and H) the diabetic GK heart. Bar,  $20\ \mu\text{m}$ .

Analogous to the lower density of  $\text{Ca}^{2+}$ -ATPase in diabetic microsomes (Fig. 4G), comparative testing of enzyme activity of normal versus diabetic preparations revealed an  $\sim 50\%$  reduced  $\text{Ca}^{2+}$ -dependent ATPase activity in GK membranes. Since the reduced levels and activity of SERCA2 represented a notable initial finding that might explain the impaired relaxation kinetics of the diabetic heart, we wished to confirm this result at the cellular level. Standard immunofluorescence microscopy was employed to compare the localization and density of the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase in normal versus GK heart cryosections. Representative cell biological labeling patterns are shown, outlining nuclei staining with the DNA-binding dye diamidinophenylindole (Fig. 5A and B), and

general cellular structures using haematoxylin/eosin (Fig. 5C and D) and histochemical staining of the myosin ATPase at pH 4.3 (Fig. 5G and H). Fluorescein-conjugated antibody labeling of SERCA2 confirmed its reduced expression in the diabetic GK rat heart (Fig. 5E and F). Studies by Algenstaedt *et al* (83) suggest a tight interaction between SERCA2  $\text{Ca}^{2+}$ -ATPase units and the insulin receptor substrate IRS1. This interaction between two key regulators of the insulin signaling pathway and muscle relaxation may represent an important intersection in the regulation of normal muscle physiology. Thus, reduced expression of the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase might represent the molecular defect that underlies prolonged relaxation kinetics in the diabetic heart and may be involved in peripheral insulin resistance.

It is possible that the inefficient removal of  $\text{Ca}^{2+}$  ions contributes to diastolic dysfunction (84). Thus, type 2 diabetic complications in the heart are most likely associated with abnormal  $\text{Ca}^{2+}$  removal and impaired muscle relaxation rather than modified voltage sensing, an altered  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release mechanism or a sub-optimum linkage between the  $\text{Ca}^{2+}$ -release channel and the luminal  $\text{Ca}^{2+}$ -reservoir region. Chronically reduced rates of  $\text{Ca}^{2+}$  uptake from the cytosol into the lumen of the sarcoplasmic reticulum could seriously impair cardiac muscle function in diabetes. This is evident from our knowledge of an established disease of muscle relaxation named Brody's disease, whose pathophysiology is due to mutations in the fast skeletal muscle SERCA1 gene (85). Although  $\text{Ca}^{2+}$  removal is regulated differently in cardiac versus skeletal muscles, the principle that the main protein components involved in relaxation are reduced could be used as an analogy between Brody's disease and the diabetic GK heart. In the long term, altered relaxation patterns may render the diabetic heart more susceptible to cardiomyopathy. Since the regulatory SERCA2 subunit phospholamban did not show a concomitant reduction in the GK heart as compared to the  $\text{Ca}^{2+}$ -ATPase, it has a potentially stronger repressor effect on the reduced number of SERCA2 units in the diabetic heart. Phospholamban functions by decreasing the affinity of the  $\text{Ca}^{2+}$ -ATPase enzyme for  $\text{Ca}^{2+}$  ions (86), therefore a drastically altered SERCA-to-phospholamban ratio may impair cytosolic  $\text{Ca}^{2+}$  uptake into the lumen of the sarcoplasmic reticulum. This idea is in agreement with the fact that  $\text{Ca}^{2+}$ -ATPase activity is reduced more than the density of SERCA2 protein in GK heart preparations.

## 5. Conclusions

Recent studies on type 2 diabetic muscle tissues have indicated that abnormal hormonal signaling, impaired surface membrane stabilization and/or altered ion handling may play a critical role in peripheral insulin resistance. The loss of the dystrophin-dystroglycan complex in selected diabetic fibres from skeletal muscle might impair the integrity of the sarcolemma, thereby causing structural weakening of the muscle periphery and abnormal signaling. The additional reduction in the syntrophin-nNOS complex probably results in impaired muscle activation and a decreased cytoprotective mechanism in diabetes. On the functional level of glucose removal, the impaired anchoring of cortical actin due to reduced dystrophin levels might interfere with the proper recruitment of GLUT4 units to the surface

membrane, which would interfere with the skeletal muscle metabolism following stimulation by insulin or muscle contraction. GLUT4 vesicles are probably abnormally accumulated in a dense membrane region, where they may be unable to be recruited by insulin signaling (87). In this respect, the analysis of the dystrophin-glycoprotein complex in the diabetic GK rat (20) has furthered our general understanding of how insulin resistance may be triggered in diabetic skeletal muscles. Reduced expression of Dp427,  $\alpha$ -dystroglycan,  $\beta$ -dystroglycan, syntrophin and nNOS may aggravate insulin resistance characteristic of non-obese type 2 diabetic skeletal muscle fibres. Therefore, the rescue of the dystrophin network through cell-based therapies suggests itself as a new therapeutic option in preventing the diabetic phenotype. Up-regulation of the dystrophin-dystroglycan and syntrophin-nNOS complexes might improve insulin-induced glucose uptake and reduce diabetes-related skeletal muscle weakness.

Since cardiovascular disease represents a leading cause of morbidity in diabetic patients, there is an urgent need to establish novel treatment targets to counteract diabetic complications. The preliminary findings presented in this review, which need further validation by in-depth analysis of the fate of all key ion-regulatory muscle proteins in diabetes, suggest that modulation of disturbed  $\text{Ca}^{2+}$  cycling might be useful to reduce the effect of an impaired excitation-contraction-relaxation cycle in the diabetic heart. The observed decreased expression and enzyme activity of the SERCA2  $\text{Ca}^{2+}$ -ATPase in diabetes suggests a central pathophysiological role of insufficient relaxation kinetics in diabetic cardiomyopathy, and clearly identifies this ion-handling protein as a potential pharmacological target for developing new drug-based strategies to counteract cardiac contractile weakness in type 2 diabetes (67). Since cardiovascular complications cause the majority of all diabetes-related deaths worldwide (8), novel pharmacological interventions at the level of  $\text{Ca}^{2+}$  homeostasis may represent a promising method of treating at least some of the most serious clinical side effects of diabetes mellitus.

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