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Potential Target Molecules in Diabetic Cardiomyopathy: Hepatocyte Growth Factor (HGF) and Ryanodine Receptor 2 (RyR2)

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Additional information is available at the end of the chapter

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1. Introduction

Patients with diabetes mellitus have an increased cardiovascular mortality rate and, in particular, cardiovascular complications are the leading cause of diabetes-related morbidity and mortality. Diabetes mellitus is a well-recognized risk factor for developing heart failure and it also can affect cardiac structure and function even in the absence of traditional cardiovascular risk factors. Four decades ago Rubler and colleagues introduced the term 'diabetic cardiomyopathy' describing diabetic patients with congestive heart failure and normal coronary arteries [1]. Since then, many epidemiological and clinical studies have documented the existence of this entity in humans [2].

Individuals with diabetes mellitus (DM), both type 1 DM as well as type 2 DM, have an increased risk of developing end-organ damage. Clinically, the concept of diabetic cardiomyopathy is defined as ventricular dysfunction that occurs independently of coronary artery disease and hypertension, *i.e.* as a distinct primary disease process which develops secondary to a metabolic insult and results in structural and functional abnormalities of the myocardium leading to heart failure. Diabetic cardiomyopathy in humans is predominantly manifested by diastolic dysfunction, which may precede the development of systolic dysfunction [3]. Interestingly, only approximately 30% of type 2 DM and type 1 DM patients develop diabetic nephropathy, in contrast to diabetic cardiomyopathy that is present in 50% type 2 DM patients and diabetic retinopathy diagnosed in more than 90% of type 1 DM patients [4; 5]. This indicates a different time-course of end-organ damage in DM. Consequently, individual cell types are differentially sensitive to high blood glucose-induced damage likely because of different expression or activity of molecular factors responsible for damage activation and progression.

The prevalence of heart failure (HF) in the general population ranges from 1 to 4%, but in diabetic patients it is 12%, rising to 22% in those over the age of 64 years [6; 7]. The Framingham Heart Study reported a 2.4-fold increase in the incidence of HF in diabetic men and a 5.1-fold increase in diabetic women, when compared with age-matched controls [8; 9]. Diabetic patients are also more likely than non-diabetic patients to develop HF following myocardial infarction, despite comparable infarct sizes [10].

Based on the assumption of detrimental effects of hyperglycaemia on various tissues, reduction of blood glucose would reverse (or at least reduce) the development of end-organ damage. However, recent data showed unexpected findings. Castagno and colleagues conducted a meta-analysis of randomized controlled trials comparing strategies of more versus less intensive glucose-lowering that reported HF events. Interestingly, it became evident that tight glycemic control in patients with type 2 DM did not reduce the risk of HF and, additionally, when glucose lowering was achieved with thiazolidinediones, it increased that risk [11]. Additionally, evidence for a direct, causal link between insulin resistance, a hallmark of diabetes, and ventricular dysfunction has not been established [12]. The reason why intensive glucose control does not lead to the reduction in risk of HF predicted by epidemiological studies is uncertain but it may reflect an insufficient duration of treatment or follow-up, a treatment intervention too late in the course of the disease, off-target toxicity of the treatments used, or the possibility that hyperglycemia *per se* does not directly govern the development of HF in diabetic patients. In other words, hyperglycaemia could be a trigger of molecular changes causing end-organ damage, which are later regulated at least partially independently from the systemic glucose changes. These assumptions foster the search for local signalling molecules involved in end-organ damage in DM.

It is widely accepted that the pathogenesis of diabetic cardiomyopathy is multifactorial. Beyond the stereotypical function of metabolism as a provider of ATP, alterations in metabolic flux within the cell create essential signals for the adaptation of the heart to disturbed blood sugar regulation and insulin abnormalities in DM. In general, the prevailing concept of the heart's response to changes in its environment is a complex network of interconnecting signal transduction cascades where the focus is on communication of various cell surface receptors, heterotrimeric G-proteins, protein kinases, and transcription factors [13]. Several hypotheses have been proposed, including autonomic dysfunction, metabolic derangements, abnormalities in ion homeostasis, alteration in structural proteins, and interstitial fibrosis, and increased glycation of interstitial proteins such as collagen, which results in myocardial stiffness and impaired contractility [2]. Collectively, metabolic imbalance induces alterations in downstream transcription factors which result in changes in gene expression, myocardial substrate utilization, myocyte growth, endothelial function and myocardial compliance [14]. Indeed, alterations in gene expression have been observed for a number of key inducer or transducer molecules in diabetic cardiomyopathy. In particular, oxidative stress due to increased production of reactive oxygen species (ROS) by multiple sources, such as the NADPH oxidase or dysfunctional nitric oxide (NO)-signaling cascade, is considered to be a principal mechanism involved in the development of diabetic cardiomyopathy [15; 16; 17]. However, this review focuses on two of them which are currently not in the centre of interest but which might play

a role not only in beginning but also in development of diabetic cardiomyopathy. These are (1) hepatocyte growth factor/c-Met signalling cascade, and (2) calcium release system.

2. Hepatocyte growth factor/c-Met signalling

2.1. Structure and function of HGF and c-Met

The hepatocyte growth factor (HGF) is known to be involved in a huge variety of cellular processes playing a major role in the repair and regeneration of various tissues, including the liver, kidney, lung, and stomach. In addition, it is involved in embryogenesis, organ development and also carcinogenesis, and its role in autoimmune diseases has been suggested as well [18; 19].

Liver regeneration has long been a subject of active research, because it has impressive regenerative capacities. Humoral factors that trigger liver cell growth, and so hepatic regeneration, have been detected in the blood circulation of liver-injured animals, and many researchers have tried to isolate these factors ensuring this liver characteristic. One of them, HGF was first recognised as a molecule that stimulates hepatocyte proliferation and so to be a key player in the regulation of liver regeneration. It was originally identified in the plasma of partially hepatectomised rats and initially thought to be a liver-specific mitogen [20; 21; 22]. Actually, HGF is known to be a multifunctional cytokine and such as it has been a subject of immense research efforts during the past decade [23].

Physiologically, HGF is a mesenchyme-derived pleiotropic growth factor which consists of two polypeptide chains, heavy 69-kDa alpha-chain and a light 34-kDa beta-chain which are held together by a disulfide bond. Like plasminogen, HGF is synthesised as pro-HGF, an 82-kDa single-chain inactive precursor, and subsequently transformed in the active heterodimer [24]. HGF is latent in normal states, and is activated specifically at the site of tissue injury, predominantly by HGF activator - HGFA [19; 25; 26]. The domains of HGF are very similar to those of proteases in the blood coagulation and fibrinolytic system, HGF shows the highest similarity to plasminogen (about 40% amino acid similarity). Thus, the HGF system is functionally linked to the blood coagulation and fibrinolytic system.

The HGF receptor c-Met is a transmembrane tyrosine kinase that mediates several biological responses after stimulation by its cognate ligand. c-Met is synthesised as a precursor (170 kDa) and then, it is converted into the active disulphide-linked heterodimer composed of a 50 kDa extracellular alpha-chain and a longer 145 kDa beta-chain with a transmembrane helix and a cytoplasmic portion. The alpha-chain is exposed extracellularly, while the beta-chain is a transmembrane subunit containing an intracellular tyrosine kinase domain [27; 28; 29; 30].

Multiple biological effects of HGF/c-Met system on a wide variety of cells have been documented. HGF is a cytokine regulating cell growth, cell motility and morphogenesis of various types of cells. Additionally, mitogenic, motogenic, chemotactic and anti-apoptotic activities of HGF have been documented on multiple cell type. This cytokine stimulates endothelial proliferation and, consequently, angiogenesis, as well as stimulates growth of other target cells

including melanocytes, epithelial cells, and haemopoietic cells. Thus, HGF is considered a humoral mediator of the epithelial-mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis [23; 31]. According to current hypothesis, HGF is an activating ligand of c-Met receptor, whose activity is essential for normal tissue development and organ regeneration but abnormal activation of c-Met has been implicated in growth, invasion, and metastasis of many types of solid tumors. While HGF is produced in various mesenchymal cells mostly in response to tissue injury, its receptor c-Met is expressed on epithelial cells, irrespective of the organ. This ligand-receptor pair (HGF/c-Met) has a role during embryogenesis, organogenesis and carcinogenesis [28; 32; 33; 34; 35] as well as in the homeostasis of adult tissues [27; 28]. The paracrine signalling between HGF and c-Met plays an important role in regulating epithelial-mesenchymal cell interactions.

2.2. HGF/c-Met in cardiovascular pathology

HGF and its receptor c-Met are expressed in several tissues, including the heart although at low levels. Several reports have focused on the role of HGF/c-Met in cardiovascular pathophysiology and, comparable to pleiotropic effects in other organs, a huge variety of actions have been described also in cardiovascular system. Animal studies demonstrated that treatment with HGF gene or protein may reduce acute myocardial ischemia and reperfusion injury, decrease infarct size, and improve cardiac perfusion and function in acute myocardial infarction. These beneficial effects are associated with angiogenesis and reduced apoptosis. HGF protects cardiomyocytes against oxidative stress and can counteract the loss of cardiomyocytes usually observed in cardiac diseases [36; 37]. In addition to its beneficial effects on cardiomyocytes under acute stress, recent research has demonstrated that HGF also exerts beneficial effects on cardiac function in animal models of chronic heart diseases, including ischemic cardiomyopathy following old myocardial infarction and hereditary cardiomyopathy. In those cases, the main mechanisms appeared to be a hypertrophic effect on cardiomyocytes as well as angiogenic and antifibrotic actions.

Interestingly, several injuries alter the expression of HGF in cardiac tissue. In the heart, acute myocardial infarction, ischemia reperfusion injury, and congestive heart failure induce expression of HGF [37; 38; 39]. In myocardial ischemia, HGF has been suggested to counteract damage and to mediate a regenerative response (Nakamura et al. 2000). Apparently, there is a time dynamics in HGF production and/or secretion in the diseased myocardium. Although there is an upregulation of cardiac HGF/cMet in the acute phase of myocardial infarction, the cardiac HGF production appears to be downregulated in the late phase of myocardial infarction (MI). HGF quantity measured 24 hours after infarction was confirmed to be higher in the border than in the remote myocardium [40]. Aoki and colleagues observed that the cardiac HGF level was significantly decreased at 14 days after MI [41]. Others supported this time dynamics. In rats, myocardial expression of HGF mRNA and c-Met receptor mRNA are significantly elevated following myocardial ischemia/reperfusion induced by transient coronary ligation or MI induced by permanent coronary ligation. Both levels rapidly increase, peak between 24-72 hours, and remain significantly elevated for at least 5-7 days. Myocardial gene expression of HGF and c-Met remained activated for one month after cardiac ischemia/

reperfusion in rats. The peak in c-Met expression occurs 24 hours after ischemia/reperfusion, whereas HGF gene expression peaks at 72 hours. The peak mRNA levels increase by 4-fold for HGF and 8.3-fold for c-Met. The c-Met mRNA returned to near normal levels by one week, and HGF gene expression is substantially reduced from the peak level by one week and then gradually returns to baseline levels over 15-30 days [42]. Thus increased blood HGF may reflect a defensive reaction and possibly participate in cardioprotection during myocardial infarction [36]. From a mechanistic point of view, HGF exerts anti-apoptotic and angiogenic properties by activating its c-Met receptor and a downstream ERK1/2-mediated signalling pathway in cardiomyocytes and endothelial cells both on the myocardial infarction border zone and in regions of the heart remote from the infarct.

Several experimental studies have shown that HGF can stimulate myocardial regeneration by inducing endogenous cardiac stem cells to migrate, differentiate, and proliferate in situ to replace lost cardiomyocytes. c-Met is expressed on different populations of putative cardiac stem cells and cardiac progenitor cells migration, proliferation and homing are predominantly modulated by the HGF/c-Met receptor system [43; 44]. Thus, HGF/c-Met signalling might play a key role in self-renewal of myocardial tissue.

2.3. HGF as a biomarker of cardiac damage

Currently, the use of HGF as a clinical marker of cardiovascular injury is under intensive debate as several papers reported increased serum HGF in patients with heart failure. Lamblin and colleagues investigated the prognostic value of 2 cytokines, vascular endothelial growth factor (VEGF) and HGF, in patients evaluated for a reduced left ventricular ejection fraction [45]. Vascular endothelial growth factor was shown to have limited prognostic utility. However, increased levels of HGF were strongly associated with markers of congestive heart failure severity such as higher NYHA class and lower left ventricular ejection fraction, as well as clinical outcomes including both cardiac and overall mortality (*see figure 1*). The association of HGF with adverse outcomes persisted in multivariable analysis that incorporated state-of-the-art risk factors such as BNP and peak oxygen consumption, an important step when assessing a new biologic marker. In detail, HGF levels were higher in patients with a cardiovascular event (1001 [741-1327] pg/mL) than in the patients without it (773 [610-1045] pg/mL, $P < 0.0001$). Similar results were found when overall mortality was considered. HGF levels were higher in the patients who died of any cause (940 [748-1306] pg/mL) than in patients who did not. Importantly, HGF concentrations were strongly associated with age, diabetes mellitus, and all markers of congestive heart failure severity. Consequently, the survival curves indicated a worse outcome for patients with high HGF levels. Similarly, in a small clinical study, Ueno and colleagues found 5.3 times higher serum HGF levels as compared to healthy volunteers in patients with acute exacerbation of congestive heart failure [39]. In another study of Lamblin and colleagues studied patients with a first anterior Q-wave myocardial infarction [46]. They observed that plasma HGF levels were positively associated with left ventricular volumes, wall motion systolic index, early transmitral velocity to mitral annular early diastolic velocity ratio, and BNP levels. High HGF levels were associated with higher C-reactive protein levels. On the other hand, HGF levels were negatively associated with left ventricular ejection

fraction. Multivariate analysis showed that both BNP and C-reactive protein were independently associated with HGF levels at 3 and 12 months. Patients who died or were re-hospitalized for HF during follow-up had higher HGF levels at 1 month, 3 months, and 1 year after myocardial infarction. Thus, circulating HGF levels correlate with all markers of LV remodelling after MI and are associated with re-hospitalization for heart failure.

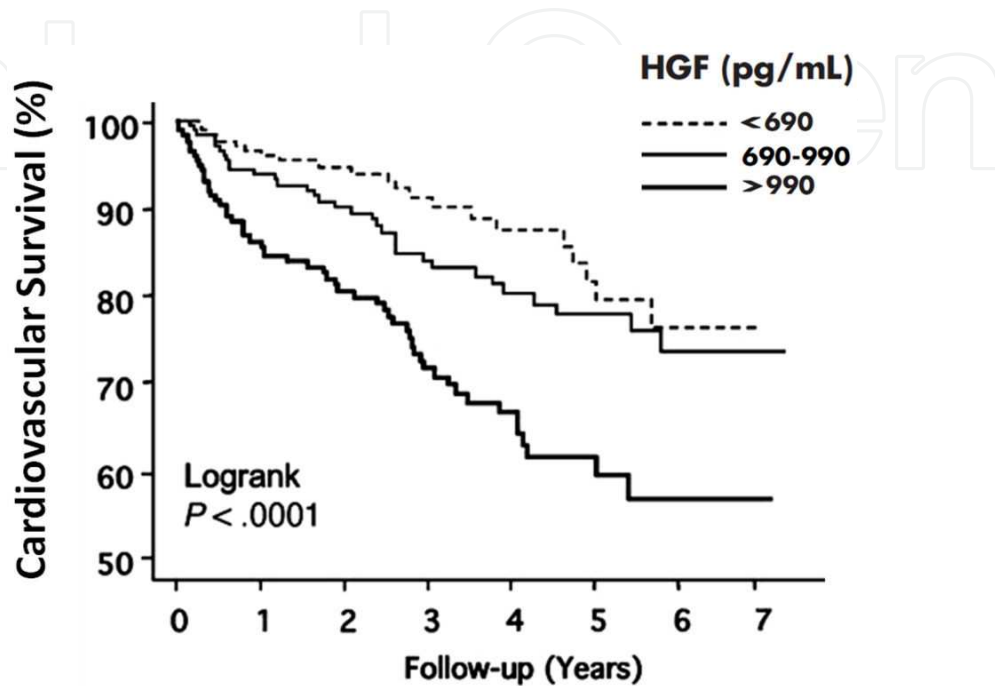


Figure 1. Kaplan-Meier survival curves according to the tertiles of HGF. These survival curves indicate a worse outcome for patients with high HGF levels [45].

Rychli and colleagues assessed the prognostic value of HGF in heart failure in a prospective cohort study [47]. They demonstrated that the risk of all-cause mortality increases with endogenous HGF concentrations in patients with advanced HF with a 3.1-fold higher risk in the third tertile compared with the first tertile. Interestingly, additional subgroup analysis stratifying by the aetiology of HF showed that the prognostic value of HGF was only present in patients with ischaemic HF and not in those with HF of other aetiology. In patients with ischaemic HF they observed a 4.4-fold higher risk in the third tertile compared with the first tertile. The main increase of risk was between the first and the second tertile of HGF. Therefore, it might be speculated that a certain threshold of HGF has to be exceeded to initiate mechanisms linked with a poor survival. Additional analysis evaluating the predictive potential of HGF for the secondary end point cardiovascular mortality yielded similar results as reported for all-cause mortality. In patients with ischaemic HF the adjusted hazard for a cardiovascular death was 6.2-fold higher in the third tertile of HGF compared with the first tertile. The predictive value of HGF was independent of BNP and other potential predictors of outcome in patients with HF. Stratified analyses evaluating the combined risk prediction by HGF and BNP levels showed that high HGF indicates a poor prognosis even in patients with low BNP. This subgroup of patients had a comparable risk to those with elevated BNP, but a low HGF.

As expected, the greatest risk was found when both factors were raised. This additive prognostic value of HGF might help to identify patients at high risk who would benefit from intensive treatment.

Although several figures have been suggested such as 0.26, 0.39, 0.69, and 0.99 ng/ml [45; 48; 49], no practical cutoff values discriminating between normal and abnormal HGF concentrations linked to human cardiovascular pathology have been defined yet. Moreover, although HGF was associated with adverse outcomes in heart failure patients, Wang and colleagues show that its prognostic value for mortality and heart transplant necessity in various forms of cardiomyopathy is poor [50]. In their prospective cohort study, HGF concentrations were measured in patients with Chagas' disease related dilated cardiomyopathy or idiopathic dilated cardiomyopathy. When compared to healthy individuals, no difference was detected for patients with NYHA class I–II but HGF was significantly increased in advanced HF patients (NYHA III–IV) in both groups of patients. In addition, there was a strong correlation between HGF and left ventricular ejection fraction in patients suffering from Chagas' disease but HGF failed to predict mortality and necessity for heart transplant in both groups of patients.

In spite of still controversial findings, HGF might be an attractive biomarker in patients with congestive heart failure because it is increased in the setting of cardiomyocyte apoptosis and active remodeling, thereby identifying individuals who are at increased risk of adverse clinical outcomes. However, based on available evidence, the etiology of cardiac abnormality has to be considered before applying HGF as a biomarker [51].

2.4. HGF as a therapeutic factor

It remains unclear what mechanism is responsible for the cardioprotective effects of HGF but its therapeutic potential is undisputable. In current literature, the focus is stressed on influence of HGF in stem cell mobilization in damaged myocardium. HGF belongs to factors that increase recruitment of progenitor cells to damaged myocardium by its chemotactic effects on cardiac progenitor cells [52]. In other words, HGF attracts cardiac stem cells to start transport and differentiate in infarcted area.

Several methods were developed to affect injured hearts in a variety of animal models. Most of investigators use gene transfections. HGF gene therapy decreases adverse ventricular remodeling and improves cardiac function in various species. In a hamster model of dilated cardiomyopathy, transfection with the HGF gene attenuates the progression of cardiac impairment, including the reduction of myocardial fibrosis and reorganization of the cytoskeletal proteins and these changes lead to an improvement in life expectancy [53; 54; 55]. Li and colleagues studied its chronic effects on post-infarction left ventricular remodeling and heart failure in mice. They applied adenovirus encoding human HGF and observed improved left ventricular remodeling and function and hypertrophied cardiomyocytes near infarcted area at 4 weeks after induction of myocardial infarction. Postinfarction HGF gene therapy improved LV remodeling and dysfunction through hypertrophy of cardiomyocytes, infarct wall thickening, preservation of vessels, and antifibrosis [56]. In rats, HGF gene transfer following a large myocardial infarction results in preservation of ventricular geometry and function, and is associated with enhanced angiogenesis and a reduction in apoptosis (Jaya-

sankar et al. 2003, Jayasankar et al. 2005). Iwasaki and colleagues reported that recombinant human HGF delivered by ultrasound-mediated destruction of microbubbles into the cardiomyopathic hearts prevents cardiac dysfunction in an animal model of doxorubicin-induced cardiomyopathy [57]. In this form of anthracycline induced cardiomyopathy in mice, findings of Esaki and colleagues suggest that HGF gene delivery by adenoviral vector exerts therapeutic antiatrophic/degenerative and antifibrotic effects on myocardium and mitigation of cardiac dysfunction. These beneficial effects appear to be related to HGF-induced MAPK/ERK activation and upregulation of c-Met, GATA-4, and sarcomeric proteins [58]. Okayama et al demonstrated in transgenic mice that HGF reduced cardiac fibrosis by inhibiting endothelial mesenchymal transition and the transformation of fibroblasts into myofibroblasts. The amount of cardiac fibrosis significantly decreased in pressure-overloaded HGF-transgenic mice compared with pressure-overloaded nontransgenic controls, particularly in the perivascular region. This pattern was accompanied by a reduction in the expression levels of fibrosis-related genes and by significant preservation of echocardiographic measurements of cardiac function in the HGF-transgenic mice [59]. In dogs with intracoronary microembolization-induced heart failure, intramyocardial injections of HGF naked DNA plasmid attenuated the expression abnormalities of the SR Ca^{2+} -cycling proteins, improved regional and global left ventricular function and prevented progressive LV remodeling [60]. In a ventricular rapid pacing heart failure canine model, gene transfection of HGF promoted angiogenesis, improved perfusion, decreased fibrosis and apoptosis, promoted recovery from myocyte atrophy, and thereby attenuated cardiac remodeling and improved myocardial function in the failing canine hearts [61]. The gene therapy with hepatocyte growth factor-complementary DNA plasmids reduced coronary artery ligation-induced cardiac impairment in goats (Shirakawa et al. 2005). Taken together, a number of experimental data support the potential therapeutic value of HGF.

Importantly, the concept of gene therapy using HGF has been used in human as well. The intracoronary administration of adenovirus vector encoding the human HGF gene in patients with coronary heart disease resulted in high levels of gene expression of HGF and its receptor c-Met, as well as increased serum concentrations of HGF. Adenovirus vector encoding the human HGF gene effectively induced temporarily high expression of the HGF gene in peripheral blood mononuclear cells and consequently increased serum HGF levels [62]. Nevertheless, the clinical utility of HGF therapy in the myocardium still remains enigmatic. It is still unclear which mechanisms are the most important for the cardioprotective effect of HGF. For a successful translation to clinical application of a protein, a clearly defined primary mode of action and knowledge on pharmacokinetic properties are necessary for the rational development of the protein as a therapeutic [52].

2.5. HGF/c-Met in diabetic cardiomyopathy

The role of HGF/c-Met signalling in cardiac tissue is predominantly linked to ischemic damage and little is known about its role in diabetic cardiomyopathy. Since HGF contributes to the protection or repair of vascular endothelial cells and decreased serum and tissue HGF levels are related to the progression of endothelial cell damage induced by diabetes [63], the same might be true for cardiac tissue. In general, increased HGF is believed to be a marker of

complications. However, local HGF production in vascular cells was shown to be markedly suppressed by high D-glucose [64] what suggests that decreased local HGF production may accelerate the progression of atherosclerotic vascular changes as well as cardiomyocytes injury in DM. In turn, an adaptive increase of HGF in advanced DM might support the hypothesis that serum HGF concentrations are elevated in response to various organ injuries.

The results of a clinical study [65] showed for the first time that serum HGF concentrations are increased in type 1 diabetic patients. Interestingly, these results were similar at the diagnosis time point and after more than 10 years duration with or without pathologic albumin excretion. This finding suggests a stable increase of HGF during development and progression of DM 1. In contrary, Nakamura and colleagues [63] found a decrease of serum HGF concentration in DM patients without hypertension but an increase in patients suffering from both DM as well as arterial hypertension. In the latter group, HGF concentration progressively increased with the stage of hypertension and it positively correlated with systolic blood pressure in DM patients. In addition, both animal and clinical data showed that serum HGF concentration were negatively correlated with HbA1c in subjects without any complications, suggesting a loss of this endothelial protection in accordance with the severity of diabetes. In fact, the serum HGF concentration in DM patients may be determined by a balance of stimulating factors (hypertension, atherosclerosis, *etc.*) and suppressing factors (high glucose, TGF-beta, Ang II, *etc.*). Consequently, the elevation of the serum HGF concentration may be considered as an index of the severity of complications in DM. Systemic HGF may work in tissue regeneration as a humoral mediator, although it might be insufficient to promote tissue regeneration, owing to a decrease in local HGF production. In conclusion, the HGF/c-Met signalling might play a crucial role in cardiac damage such as diabetic cardiomyopathy (*see figure 2*) and exact identification of this role may pave new ways towards drug development and to contribute to better management of DM in future.

3. Calcium release and Ryanodine receptor 2

3.1. Regulation of calcium cycle in cardiac cells

One of the long reported general hypotheses of cardiac impairment is based on the calcium overload. Fleckenstein's calcium theory of myocardial cell necrosis from 1970' is widely quoted in literature as a general mechanism of myocardial cell damage [66]. It must be noted that intracellular calcium dysregulation is present in all types of advanced cardiomyopathy and apparently is a late stage event that represents a final common pathway for myocardial cell damage and death. There is now increasing evidence that depression of contractility in heart failure is linked to a malfunction of calcium regulation in cardiomyocytes, in particular to sarcoplasmic reticulum (SR) Ca²⁺ uptake and/or release [67; 68].

Sarcoplasmic reticulum (SR) Ca²⁺ release is maintained by a macromolecular protein complex consisting of the ryanodine receptor (RyR) – a Ca²⁺ release channel, calsequestrin (CSQ), triadin, and junctin that is activated by L-type Ca²⁺ current [69; 70]. Aside from cytosolic Ca²⁺, RyR activity is also regulated by SR luminal Ca²⁺ [71; 72]. Its storage and release are under the

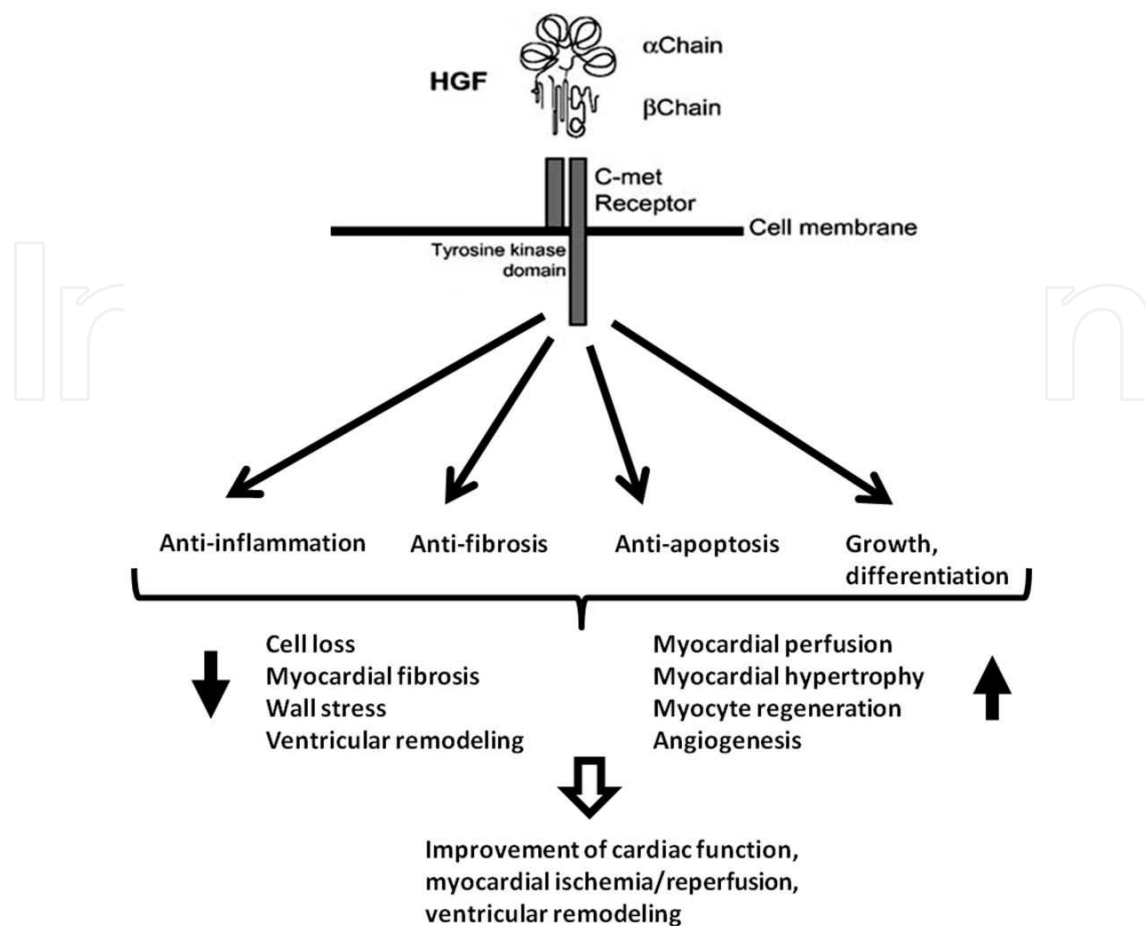


Figure 2. A schematic illustration of potential effects of HGF/c-Met in diabetic cardiomyopathy [24; 42].

control of CSQ [71], whereas triadin and junctin may serve as linker proteins between CSQ and the RyR [70; 73]. The tethering of CSQ to the inner surface of the SR allows it to sequester Ca^{2+} in the vicinity of the RyR during SR Ca^{2+} cycling [74]. CSQ may act as a Ca^{2+} sensor that inhibits the RyR at low SR luminal Ca^{2+} via interaction with triadin/junctin [75]. An increase of SR luminal Ca^{2+} disrupts the inhibition of the RyR because the CSQ Ca^{2+} binding sites become more occupied with Ca^{2+} , resulting in a weakened interaction between CSQ and triadin/junctin and an increased open probability of the channel [76]. Sorcin, a 22-kDa Ca^{2+} -binding protein, also binds to cardiac RyR with high affinity, and its interaction with RyR is facilitated by annexin A7 in a Ca^{2+} -dependent manner. Thus the interaction between these proteins appears to be critical for the regulation of SR Ca^{2+} release. For relaxation to occur, calcium ions must be removed from the cytosol, the majority of which is pumped back into the SR by cardiac specific SERCA2a (sarcolemmal/endoplasmic reticulum Ca^{2+} -ATPase 2a), while the remainder is ejected out of the cell through the sarcolemmal NCX ($\text{Na}^{2+}/\text{Ca}^{2+}$ exchange), PMCA (plasma-membrane Ca^{2+} -ATPase) or mitochondrial calcium uniport.

Cardiac specific ryanodine receptor 2 (RyR2), a Ca^{2+} -activated Ca^{2+} channel situated in the SR membrane, plays the dominant role in Ca^{2+} release from the SR in cardiac myocytes. In general, the RyR is a huge tetrameric protein with each monomer constituted of around 5000 amino

acids (Mw: 565 kDa). Three isoforms of RyR have been described in mammalian tissues (RyR1, RyR2 and RyR3) of which RyR2 is predominant in cardiac muscle. The RyR is a tetramer consisting of four subunits and forms a complex with other proteins of which the FK506-binding protein (FKBP), calsequestrin, triadin 1 and junctin were identified in cardiac muscle. FKBP is known for immunosuppressive properties; however, members of this protein family, FKBP12 and FKBP12.6, also bind to the cytoplasmic part of the RyR in skeletal and cardiac muscle and seem to modulate the gating properties of the RyR. Calsequestrin is a 55-kDa high-capacity calcium binding protein located in the lumen of the cardiac or skeletal junctional SR storing the calcium to be released by the RyR. Both triadin and junctin are transmembrane proteins in the junctional SR which bind directly to the RyR and to calsequestrin suggesting that these proteins attach calsequestrin to the RyR. Initially, two functional cardiac isoforms of triadin with apparent molecular weights of 35 kDa (triadin 1) and 40 kDa (triadin 2) were cloned of which triadin 1 is predominant and representing more than 95% of cardiac triadin. Junctin was first identified as a 26-kDa calsequestrin-binding protein in cardiac and skeletal muscle. Triadin and junctin are encoded on different genes but exhibit structural and amino acid similarities with single membrane spanning domains (62% identity within this domain), short cytoplasmic N-terminal segments and long highly-charged basic C-terminal domains situated in the lumen of the SR [67; 68; 75; 77].

Indeed, several disorders of the SR Ca^{2+} release complex have been identified as causes of heart disease. Hyperphosphorylation of the RyR by PKA and Ca/Calmodulin-dependent protein kinase II (CaMKII) induces a Ca^{2+} leak during diastole, which can cause heart failure and lead to fatal arrhythmias [78; 79; 80; 81]. The forced expression of triadin or junctin in rat myocytes resulted in an increase of the RyR open probability or a depressed contractility, respectively [77; 82]. Consistently, the ablation of junctin was associated with enhanced cardiac function and increased Ca^{2+} cycling parameters in mice [83]. Similarly, overexpression of CSQ induces rapid development of heart failure in transgenic mice [84].

3.2. Calcium regulation abnormalities in diabetic cardiomyopathy

Predominantly, diabetic cardiomyopathy is related to diastolic abnormalities. In both Type 1 and Type 2 rodent models of diabetes, altered expression, activity and function of all transporters involved in excitation–contraction coupling, SERCA2a, NCX, and PMCA, leading to dysfunctional intracellular calcium signalling. In particular, abnormalities of SERCA2a, the major splice variant in the heart have been documented in diabetic cardiomyopathy. Protein, mRNA, and also activity of this protein decreases in response to diabetes [10].

Depressed SERCA activity causes inefficient sequestration of calcium in the SR, resulting in cytosolic calcium overload, impaired relaxation and hence diastolic dysfunction. On the other hand, cardiac overexpression of SERCA improves Ca^{2+} homeostasis and contraction in diabetic models [12; 85; 86; 87; 88; 89]. Because heart muscle from diabetic animals exhibits a diastolic dysfunction, SERCA2a has been considered a major site for contractile dysfunction. Indeed, perfusion of hearts with glucose can lead to lowered SERCA2a mRNA levels [2; 13]. Several factors may alter proteins regulating cardiomyocytes calcium homeostasis. The process of advanced glycation has been related directly to alterations in myocardial calcium handling

and hence contractility. The advanced glycation of SERCA2a has been shown to lead to a decrease in its activity and a prolongation of cardiac relaxation [14].

Recently, attention has been focused on abnormalities of calcium release in diabetic conditions. In diabetic subjects, oxidative stress arises from an imbalance between production of reactive oxygen and nitrogen species and capability of the system to readily detoxify reactive intermediates. Importantly, it is now well established that RyR channels are highly susceptible to modification by various endogenous redox agents. Furthermore, RyR channels serve a role as intracellular redox sensors, via redox induced Ca^{2+} release and they are likely to connect cellular redox state with Ca^{2+} signaling cascades. Indeed, endogenous redox active molecules enhance RyR2 channel activity and RyR2 is one of the well-characterized redox-sensitive ion channels in heart. In general, oxidizing conditions increase RyR2 activity and so stimulate SR Ca^{2+} and causing Ca^{2+} leak (see figure 3). In addition, RyR2 is activated also by reactive nitrogen species and S-nitrosylation increases RyR open probability in cardiac muscle and leads to increased Ca^{2+} leak [14]. Redox reactions by biological oxidants and antioxidants have been shown to alter the kinetics of Ca^{2+} -induced Ca^{2+} release in the heart tissue. Besides several potential phosphorylation sites, the tetrameric RyR2 channel contains ~84 free thiols and is S-nitrosylated in vivo. S-Nitrosylation of up to 12 sites (3 per subunit) led to progressive channel activation that was reversed by denitrosylation. RyR2 is activated also by reactive nitrogen species. For example, nNOS is expressed in SR and can supply NO to RyR2 in the immediate vicinity for S-nitrosylation, which increases RyR2 open probability in cardiac muscle and leads to increased Ca^{2+} release. Thus, sulfhydryl-oxidizing agents, hydrogen peroxide and diamide, diminished RyR2-FKBP12.6 binding [90].

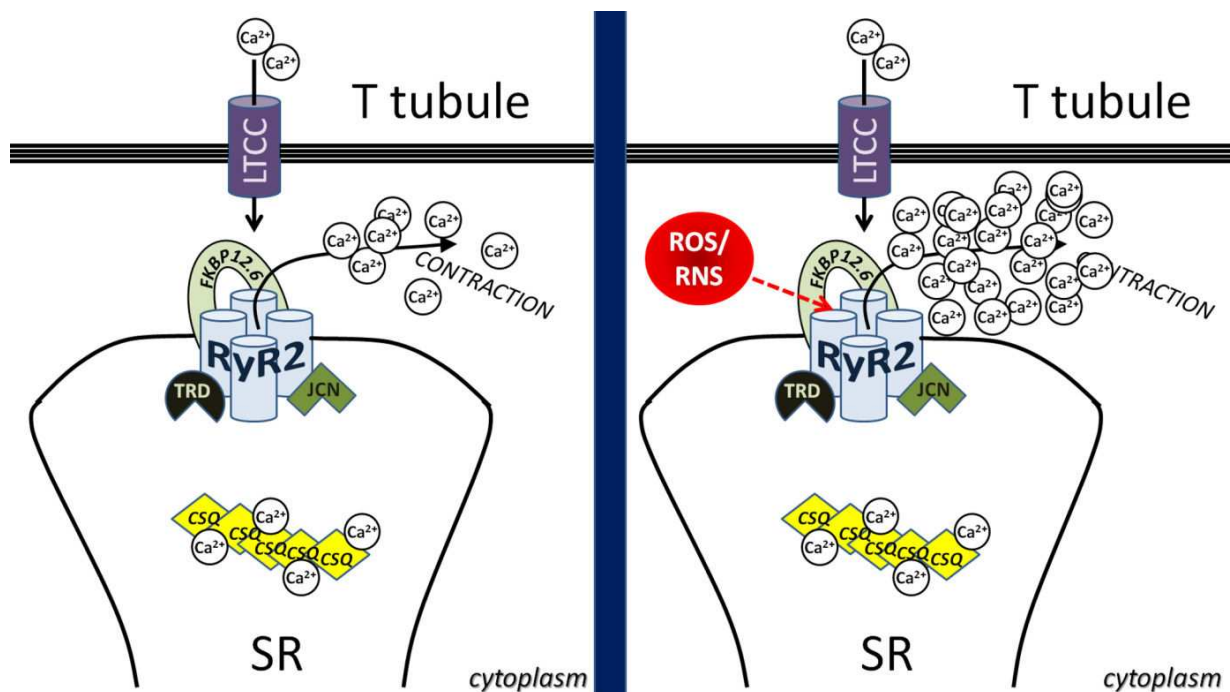


Figure 3. Intracellular calcium regulation and influence of oxidizing molecules (ROS, reactive oxygen species; RNS, reactive nitrogen species) on RyR2 function.

Modulation of cardiomyocyte Ca^{2+} handling by RyR2 is long known to occur by caffeine and tetracaine, which increase RyR2 open probability. More recently, flecainide was reported to prevent catecholamine polymorphic ventricular tachycardia as a result of decreasing RyR2 conductance and RyR2 open time. RyR2s from these hearts were S-nitrosylated and depleted of FKBP12.6, resulting in leaky RyR2 channels and a diastolic SR- Ca^{2+} leak. Inhibiting the depletion of calstabin2 from the RyR2 complex with the Ca^{2+} channel stabilizer S107, a novel RyR2-specific benzothiazepine derivative compound, inhibited the SR- Ca^{2+} leak and prevented arrhythmias in vivo. Similarly in skeletal muscle, S107 which binds to RyR1 and recovers the binding of FKBP12.6 to the nitrosylated channel inhibits SR Ca^{2+} leak, improves muscle function, and increases exercise performance in muscular dystrophic-deficient mouse model [90; 91]. Taken together, these data opens new era of new drugs – stabilizers of RyR complex (rycals), in regulation of calcium in various cells what could have an impact also in treatment of diabetic cardiomyopathy in the future.

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