CaMKII Hyperactivity: Cytoprotective in the Human Diabetic Heart?

Danny Nam

Supervisor: Dr Jeffrey Erickson

Department of Physiology

University of Otago Summer Studentship 2013/2014

Introduction

The most common cause of death in diabetic patients is a myocardial infarction (MI), and these patients are twice as likely to die compared to non-diabetics (Luo *et al.*, 2013). This phenomenon appears to be independent of commonly associated clinical hallmarks, and the underlying factors that make diabetic patients more prone to increased mortality remains poorly understood, warranting further investigation into potential molecular mechanisms.

Recent evidence in the scientific literature has implicated the role of Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) hyperactivity in a plethora of cardiovascular pathologies, ranging from apoptotic cardiomyopathy to arrhythmia (Erickson *et al.*, 2011). In the resting state, CaMKII is inactive but upon Ca^{2+}/Calmodulin binding, it undergoes a conformation change which removes the autoinhibition of the kinase domain by the regulatory domain, leading to its activation. CaMKII serves multiple functions, coupling increases in intracellular calcium to activation of ion channels, gene transcription, calcium homeostasis, inflammation, and apoptosis. As opposed to this calcium-dependent activation, calcium-independent activation can also occur, and it is hypothesized that the interconversion between the different forms of activated CaMKII is the difference between physiologically normal calcium transients and pathological activity (Erickson *et al.*, 2008).

Recent findings in the scientific literature regarding CaMKII's role in the context of diabetes suggest increased oxidation-induced CaMKII activation (ox-CaMKII) in diabetic hearts post-MI as shown in streptozotocin (STZ)-treated diabetic mice (type 1 diabetic model), which showed increased sinoatrial node pacemaker cell apoptosis and 2-fold increased mortality from severe bradycardia compared to control mice (Luo *et al.*, 2013). Diabetes is well known to increase oxidative stress, resulting in the autonomous activation of CaMKII via oxidation of M281/282 residues of the regulatory subunit (ox-CaMKII) (Erickson *et al.*, 2008). ox-CaMKII mediates $p38^{MAPK}$ and JNK activation, along with activation of other pro-apoptotic proteins such as bcl-2 and caspase-3, resulting in apoptosis (Lim *et al.*, 2013). It also increases I_{MCU} via phosphorylation of specific serine residues of the mitochondrial calcium uniporter (MCU) and this increase in mitochondrial calcium can lead to opening of the

mitochondrial permeability transition pore (mPTP) and disruption of the mitochondrial membrane potential (Joiner *et al.*, 2012). Therefore it is not surprising past research has demonstrated increased apoptosis in the diabetic group compared to the non-diabetic group, obtained from caspase-3 activity assays and TUNEL staining for DNA fragmentation due to apoptosis, in the rat diabetic model (Luo *et al.*, 2013; Marsh *et al.*, 2013). All effects were significantly attenuated in STZ-treated oxidation resistant CaMKII mice models. This suggests ox-CaMKII contributes towards increased mortality seen in diabetic patients post-MI.

Recently, another mechanism of Ca^{2+}-independent CaMKII activation was identified by Erickson *et al* (2013) indicating that diabetic hyperglycemia is able to autonomously activate CaMKII via O-linked N-acetylglucosamine (O-GlcNAc) at S279, leading to arrhythmogenic calcium waves via increased RyR2 phosphorylation in cardiomyocytes, while further exacerbating CaMKII hyperactivation. O-GlcNAcylation has also been implicated in blunted autophagic signalling, an endogenous cell-survival process normally involving recycling and degrading cytoplasmic components and proteins (Marsh *et al.*, 2013). Immunoprecipitation has shown Bcl-2 and Beclin-1 are targets of O-GlcNAcylation, and has been suggested to result in decreased capability of autophagy, and increased apoptosis.

However with most studies using rat diabetic models, a gap in the knowledge exists in regards to the role of CaMKII in the human diabetic heart. Evidence outlined above from ox-CaMKII or O-GlcNAcylation induced CaMKII activation suggests there should be increased apoptosis in the human diabetic heart. Thus, we hypothesized increased apoptotic activity of cardiomyocytes in the human diabetic heart compared to the human non-diabetic heart, which could be shown as increased activity of downstream targets of the CaMKII apoptotic signalling pathway in the heart, such as caspase-3.

Methods

Cardiomyocyte Isolation

With collaboration from the cardiothoracic surgeons at Dunedin Hospital, samples fresh right atrial appendages were obtained from consenting diabetic and non-diabetic patients during cardiac surgery with no excluding factors such as age, gender, treatment, and lifestyle. A minimum of 50mg of tissue

was obtained, and tissue was used immediately from being transported from the operating theatre in buffer. Cardiomyocytes were isolated via Bullet Blender homogenization (Next Advance, Inc.) and centrifuged at 15000 rcf for 15 minutes.

Caspase-3 Activity Assays

Isolated cardiomyocytes were lysed in assay lysis buffer (200 mM Tris, pH 7.5, 2 mM NaCl, 20 mM EDTA, 0.2% TRITON X-100) and total protein concentration was determined by Nanodrop 2000 (Thermo Fisher Scientific Inc.). Caspase-3 activity was determined by EnzChek Caspase-3 Assay Kit #2 (Thermo Fisher Scientific Inc.) using a fluorescence microplate reader.

Statistical Analysis

Data was analysed using Prism 6 (Graphpad Software.). Data was expressed as mean ± SEM. Student's (unpaired, two-tailed) t-test was used to detect any significant differences between mean standardized caspase-3 activity between the diabetic and non-diabetic human right atrial appendage cardiomyocytes. Statistical significance for differences between means was set at $p < 0.05$.

Results

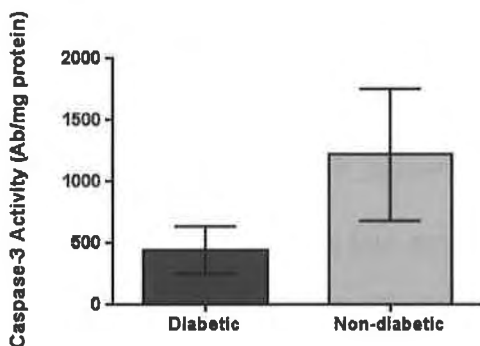

Figure 1. Comparison in caspase-3 activity in diabetic and non-diabetic human right atrial appendage. Results are expressed as mean ± SEM; n = 3 (Diabetic), n = 5 (Non-Diabetic).

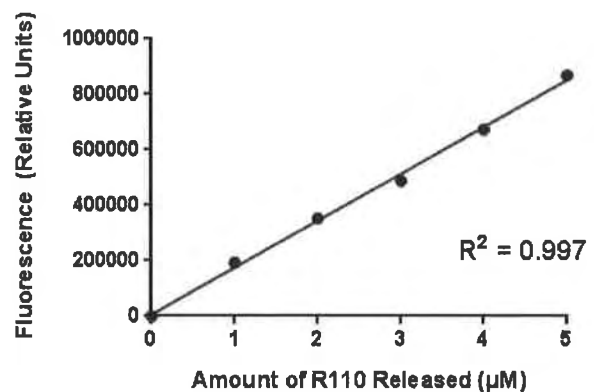

Figure 2. Rhodamine 110 (R110) reference standard curve for quantification of the amount of R110 released in the reaction.

We found no significant difference (P = 0.23) in caspase-3 activity (measure of cell death), between the human non-diabetic right atrial appendage (1220 ± 536, n = 5), compared to the human diabetic right atrial appendage (446 ± 192, n = 3) (Figure 1). Our standard curve was plotted to quantify the amount of R110 released which is a proxy of how much caspase-3 was present (Figure 2). This highlights the unusually weak signal from our assays, and despite efforts to optimize output signal,

which included using fresh samples as opposed to frozen ones after testing in rat tissue, we still continued to observe a weak signal.

Discussion

We hypothesized to observe higher levels of apoptosis shown by higher caspase-3 activity in the diabetic group compared to the non-diabetic group. Unexpectedly, less apoptosis was observed in the diabetic group compared to the non-diabetic group. Despite statistical insignificance most likely due to a very small sample sizes, and a lot of noise from human samples, there appears to be a trend towards significance. Power analysis using G*Power 3.1.9 (Heinrich-Heine-Universität Düsseldorf) indicates the sample size required is 17 and 29 for diabetic and non-diabetic groups respectively, to yield significant results. The current p-value indicates if the null hypothesis was true, our observations would not be surprising. Even though we may not be able to reject the null hypothesis, our initial hypothesis has still come into question.

Could glycophagy play a cytoprotective role in the human diabetic heart?

A glycogen-specific autophagy signalling pathway (also known as "glycophagy") regulated by insulin has been demonstrated in cardiac tissue, and may be involved in glycogen handling in the diabetic heart (Mellor *et al.*, 2013). This could help explain the reduced apoptosis observed in the human diabetic heart compared to the non-diabetic heart. The diabetic heart would be expected to be in a state of intracellular 'glucose deprivation', thus activating autophagy. However, diabetes is associated with an accumulation of cardiac glycogen, paradoxically. The mechanisms and physiological role behind this phenomenon are yet to be fully elucidated. Glycophagy is exhibited by an accumulation of glycogen-filled autophagosomes in the myocardium, with glycogen degradation for the release of free glucose, closely associating glycogen and autophagy (Reichelt *et al.*, 2013). Triggered by low ATP, AMP-activated kinase (AMPK) increases glucose uptake in the heart by increasing glucose-6-phosphate, an activator of glycogen synthase. Autophagy via AMPK occurs via phosphorylation of mTOR to remove its inhibitory effect, resulting in simultaneous regulation of glycogen storage and autophagy. Autophagy is involved in the clearance of harmful organelles such as depolarizing

mitochondria during the mitochondrial permeability transition, also known as mitophagy (Nishida *et al*., 2008). It is this mitophagy that has been implicated in inhibiting cell death. Our sample of human diabetic patients may only have been undergoing mild stress, and a corresponding low intensity insult during the mitochondrial permeability transition may have induced mitophagy, which would have conferred a cell survival role as opposed to cell death.

Could CaMKII play a cytoprotective role in the human diabetic heart?

Reduced apoptosis in the human diabetic heart may also be due to CaMKII's role in the NO signalling pathway. Recent evidence by Zhang *et al* (2014) is consistent with the notion that ROS is a critical step within the NO signalling-induced opening of myocardial K_{ATP} channels. Via an intracellular signalling cascade it is thought to activate CaMKII, functionally enhancing the opening of cytoprotective K_{ATP} channels. This would accelerate the phase 3 repolarization, shortening the duration of the cardiac action potential. This could inhibit calcium entry via L-type calcium channels into the heart, reducing calcium overload, something that CaMKII hyperactivation itself has been implicated in by RyR2 phosphorylation and also a factor which further exacerbates its own hyperactivation (Erickson *et al*., 2013).

CaMKII has also been implicated as a key mediator in the process of cellular O-GlcNAcylation. Inhibition via KN93 attenuated the increase in O-GlcNAc expected to be observed under stress (Zou *et al*., 2012). Increased O-GlcNAcylation has been implicated in cardioprotection, such as attenuating disruption of the mitochondrial membrane potential, as shown in pharmacological enhancement of O-GlcNAcylation by Jones *et al* (2008). This also follows previous studies in multiple cell lines showing increased O-GlcNAcylation increased cellular stress tolerance, whereas inhibition decreased cell survival (Boglarka *et al*., 2010). It is possible that hyperactivation of CaMKII via ROS/O-GlcNAcylation in diabetes may increase O-GlcNAcylation to confer a pro-survival response.

Why is there so much variation in human data compared to data generated from the rat?

Due to time constraints, our study was limited to having no discrimination when selecting human

diabetic and non-diabetic tissue sample. Past research has demonstrated the intrinsic increased oxidative stress in the aging heart, due to impaired transcription responses to oxidative stress, reduced endogenous antioxidant enzymes, and mitochondrial dysfunction in the heart (Van Empel *et al.*, Because we failed to adjust for age between the diabetic and non-diabetic group, confounding may have occurred, biasing the non-diabetic group to show increased apoptosis due to increased oxidative stress. Gender has also been implicated as a determinant of apoptosis. Guerra *et al* (1999) were the first to show increased apoptosis in the failing human male heart, compared to the female heart using Taq labelling to identify fragmented DNA resulting from apoptosis. TUNEL staining later by Mallat *et al* (2001) indicated that in the normal heart, apoptosis % was three-fold higher in the human male heart, compared to the female heart. It has been suggested the reduced apoptosis in the female heart may be due to estrogen-mediated phosphorylation of insulin-like growth factor-1 (IGF-1) receptors to have a pro-cell survival effect. For the same reason as above, this too may be a confounding factor in the present study.

In the future, a follow-up study would take into consideration certain factors not limited to age, gender, treatment, and cardiovascular history and profile, and with an increased sample size, a 2-way ANOVA would be possible to measure differences between these important sub-groups.

Concluding remarks

Our results indicate a novel phenomenon not yet demonstrated in the scientific literature. For the first time, we have shown reduced apoptosis in the human diabetic heart, compared to the human non-diabetic heart. Despite an overwhelming amount of data generated from the rat diabetic model which indicates there should be increased apoptosis in the diabetic heart, increased NO-signalling via diabetic ROS upregulation/CaMKII hyperactivity and CaMKII-mediated cardioprotective O-GlcNAcylation in addition to glycophagy could potentially provide enough cardioprotection to provide an explanation. However due to the lack of adjustment of other key factors such as age and gender, further research is warranted before a conclusion can be drawn.

Acknowledgements

Firstly, I would like to acknowledge the Otago Diabetes Research Trust (OMRF) for their financial support for this project to be undertaken. I would like to acknowledge my supervisor Dr Jeffrey Erickson for giving me this opportunity and for his helpful advice. I would also like to acknowledge Rachel Wallace for her generous technical support in the laboratory.

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