Liraglutide Preserves Intracellular Calcium Handling in Isolated Murine Myocytes Exposed to Oxidative Stress

Short tittle: Liraglutide preserves cardiac intracellular calcium handling

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SUMMARY

In ischemic/reperfusion (I/R) injured hearts, severe oxidative stress occurs and is associated with intracellular calcium (Ca^{2+}) overload. Glucagon-Like Peptide 1 (GLP-1) analogues have been shown to exert cardioprotection in I/R heart. However, there is little information regarding the effects of GLP-1 analogue on the intracellular Ca^{2+} regulation in the presence of oxidative stress. Therefore, we investigated the effects of GLP-1 analogue, (liraglutide, 10μM) applied before or after hydrogen peroxide (H_2O_2, 50μM) treatment on intracellular Ca^{2+} regulation in isolated cardiomyocytes. We hypothesized that liraglutide can attenuate intracellular Ca^{2+} overload in cardiomyocytes under H_2O_2-induced cardiomyocyte injury. Cardiomyocytes were isolated from the hearts of male Wistar rats. Isolated cardiomyocytes were loaded with Fura-2/AM and fluorescence intensity was recorded. Intracellular Ca^{2+} transient decay rate, intracellular Ca^{2+} transient amplitude and intracellular diastolic Ca^{2+} levels were recorded before and after treatment with liraglutide. In H_2O_2 induced severe oxidative stressed cardiomyocytes (which mimic cardiac I/R) injury, liraglutide given prior to or after H_2O_2 administration effectively increased both intracellular Ca^{2+} transient amplitude and intracellular Ca^{2+} transient decay rate, without altering the intracellular diastolic Ca^{2+} level. Liraglutide attenuated intracellular Ca^{2+} overload in H_2O_2-induced cardiomyocyte injury and may be responsible for cardioprotection during cardiac I/R injury by preserving physiological levels of calcium handling during the systolic and diastolic phases of myocyte activation.

Keywords: Liraglutide; Calcium regulation; Cardiomyocyte; Ischemic/Reperfusion; Cardioprotective

List of abbreviations

Ca^{2+}	=	Calcium
GLP-1	=	Glucagon-Like Peptide 1
H_2O_2	=	Hydrogen peroxide
I/R	=	Ischemic/reperfusion
NCX	=	Sodium-calcium exchanger
NSS	=	Normal saline solution
PKC	=	Protein kinase C
SERCA	=	Sarco/endoplasmic reticulum Ca^{2+}-ATPase

MAIN BODY OF THE TEXT

Since the risk of coronary heart disease is increased 2 to 4 times in type-2 diabetic patients (Beckman *et al.* 2002), anti-diabetic drugs that are associated with the reduction of cardiovascular events may have beneficial effects for this group of patients. Glucagon-Like Peptide 1 (GLP-1) is an incretin peptide secreting from intestinal L-cells, which has a potent effect on glycemic control (Amori *et al.* 2007). The GLP-1 receptors were expressed in ventricular myocytes (Ban *et al.* 2008, Richards *et al.* 2014). Liraglutide is one of a long-acting GLP-1 analogue which has potent glucose lowering effects for treatment of hyperglycemia in type 2 diabetes patients (Amori *et al.* 2007). Recent studies demonstrated that GLP-1 analogues exert potent cardioprotective effects in both clinical trials and animal models (Amori *et al.* 2007, Arturi *et al.* 2016, Chen *et al.* 2016, Kumarathurai *et al.* 2016, Nikolaidis *et al.* 2005, Sonne *et al.* 2008). In animal models, growing evidence demonstrates the cardioprotective effects of GLP-1 in addition to its glycemic control properties (Nikolaidis *et al.* 2005). GLP-1 analogues have been shown to improve cardiac function in ischemic/reperfusion (I/R) injury of porcine model via reduced oxidative stress and increased phosphorylated Akt and Bcl-2 expression (Timmers *et al.* 2009) and activate cytoprotective pathways after I/R injury by modulating the expression and activity of cardioprotective genes including Akt, GSK3beta, PPARbeta-delta, Nrf-2, and HO-1(Noyan-Ashraf *et al.* 2009). Recent reports also support these basic studies by demonstrating that GLP-1 analogues have exerted potent cardioprotective effects in clinical trials by improved left ventricular ejection fraction, cardiac output, and left ventricular end-diastolic diameter in patients with myocardial infarction and chronic heart failure (Arturi *et al.* 2016, Chen *et al.* 2016, Chen *et al.* 2015, Kumarathurai *et al.* 2016). During I/R period, severe oxidative stress occurs and has been shown to be associated with intracellular Ca^{2+} overload, thus facilitating both electrical and mechanical dysfunction in the heart (Shintani-Ishida *et al.* 2012). Therefore,

treatment options which prevent intracellular Ca^{2+} overload could potentially be beneficial for I/R hearts. Although currently there is only one study reporting the benefit of GLP-1 on improving intracellular Ca^{2+} homeostasis in Hl-1 cells (Huang *et al.* 2016) and one study reporting the neutral effects of liraglutide in cardiac I/R model (Kristensen *et al.* 2009), there is no available information regarding the effects of liraglutide on intracellular Ca^{2+} regulation in the ventricular cardiomyocyte. Therefore, we investigated the effect of liraglutide on the intracellular Ca^{2+} transient in isolated rat cardiomyocytes in this study. Hydrogen peroxide (H_2O_2) was used to induce severe oxidative stress similar to that observed during I/R injury. We hypothesized that liraglutide can attenuate intracellular Ca^{2+} overload in cardiomyocytes under H_2O_2-induced cardiomyocyte injury.

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine, Chiang Mai University. All the animals were fed with normal rat chow and water ad libitum for two weeks prior to experimentation. Male Wistar rats (8-10-week old, 250-300 g) were used. The rats were deeply anesthesized with thiopental (0.5 mg/kg; Research institute of antibiotics and biotransformations, Roztoky, Czech Republic) after which the hearts were removed for single ventricular myocyte isolation (Palee *et al.* 2016, Palee *et al.* 2013).

The isolated cardiomyocytes were used in each study protocol for the measurement of intracellular Ca^{2+} transient. In the first protocol, cardiomyocytes were divided into 3 groups (n = 8 cells/rat and 8 rats/group) as shown in Figure 1A. The real-time Ca^{2+} measurements were performed at the beginning of the study (baseline). Then, cardiomyocytes in Group I were treated with normal saline solution (NSS) for 5.0 minutes as a control group. Group II's cells were treated with NSS for 2.0 minutes and then H_2O_2 for 3.0 minutes to simulate I/R injury. Group III's cells were treated with liraglutide (10 μM) (Novo Nordisk A/S, Denmark) for 5.0 minutes. We used liraglutide at a clinically relevant dose; patients receive the

maximum clinical dose of 1.8 mg once a day (Margulies *et al.* 2016). The concentration we used for an in vitro study in this study was 10 μM of liraglutide which was approximately similar to the dose used in human (Langlois *et al.* 2016).

In the second protocol, cardiomyocytes were divided into 4 groups (n = 8 cells/rat and 8 rats/group) as shown in Figure 2A. The real-time Ca^{2+} measurements were performed at the beginning of the study (baseline). Then, cardiomyocytes in Group I were treated with NSS for 10.0 minutes followed by H_2O_2 for 3.0 minutes as a control group. Group II's cells were treated with NSS for 5.0 minutes followed by liraglutide (10 μM) for 5.0 minutes and then H_2O_2 for 3 minutes. Group III's cardiomyocytes were treated with NSS for 5.0 minute followed by H_2O_2 for 3.0 minutes and then NSS for 5.0 minute as another control group. Group IV were treated with NSS for 5.0 minutes followed by H_2O_2 for 3.0 minutes and then liraglutide (10 μM) for 5.0 minutes. The real-time Ca^{2+} measurements were performed after drug treatment in all groups (Palee *et al.* 2016).

In this study, we used H_2O_2 (50 μM) to induce oxidative stress, to simulate the oxidative stress that is generated by ischemia/reperfusion injury. H_2O_2 concentration at 50 μM has been widely used to trigger oxidative stress-induced intracellular Ca^{2+} dyshomeostasis in cardiomyocytes. H_2O_2 has been shown to decrease sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and sodium-calcium exchanger (NCX) activities (Huang *et al.* 2014) by inhibiting protein kinase C (PKC) activities, leading to the alteration of the intracellular Ca^{2+} homeostasis (Goldhaber 1996, Reeves *et al.* 1986).

Cardiomyocytes were isolated from the hearts of male Wistar rats using a method described previously (Palee *et al.* 2016). In brief, under deep anesthesia, the heart was immediately removed and placed into a modified Langendroff apparatus. The hearts were perfused with modified Krebs solution as previously described (Palee *et al.* 2016) for 5 minutes, followed by calcium-free solution (100 μM EGTA) for 4 minutes, Tyrode's

solution with collagenase (0.1 mg/ml) for 10 minutes, and modified Krebs solution containing 100 μM $CaCl_2$ and 1 mg/ml type II collagenase for another 8 minutes. The ventricles were removed from the cannula, cut into small pieces and incubated in 10 ml of collagenase solution gassed with 100% O_2 for 7 minutes at 37ºC. A pipette was used to pipette the cell suspension up and down in order to dissociate cardiac tissue into single cells. The cardiomyocytes were separated from undigested ventricular tissues by filtering through cell strainer, and were settling into a loose pellet. Then, the supernatant was removed and replaced with modified Krebs solution containing 1% BSA and 500 μM $CaCl_2$. This process was repeated with modified Krebs solution containing 1 mM $CaCl_2$. After this procedure, the cardiomyocytes were ready for recording.(Palee *et al.* 2013) The isolated cardiomyocytes were placed in a modified Krebs solution containing 1 mM $CaCl_2$. Intracellular Ca^{2+} transient were measured using the CELL^R imaging software (Olympus Soft Imaging Solutions GmbH, Germany). The isolated cardiomyocytes were loaded with Fura-2/AM at a final concentration of 5 μM and fluorescent intensity (excitation wavelengths are 340 nm and 380 nm, and emission wavelength is 510 nm) was recorded during electrical pacing (1 Hz, 10 ms duration, 15 V) (Palee *et al.* 2016). The ratio of the emissions wavelengths (510 nm) is directly related to the amount of intracellular Ca^{2+}. Data are shown as mean ± SD. Comparisons of variables were performed using the one-way ANOVA followed by LSD post-hoc test. $P<0.05$ was considered statistically significant.

We investigated the effects of liraglutide on intracellular Ca^{2+} handling in isolated rat cardiac myocytes exposed to hydrogen peroxide solution to provoke oxidative stress. H_2O_2 significantly decreased both intracellular Ca^{2+} transient amplitude (Figure 1B) and intracellular Ca^{2+} transient decay rate (Figure 1C). However, intracellular diastolic Ca^{2+} levels were not altered (Figure 1D), when compared to the control group (i.e. cardiomyocytes treated with NSS). Moreover, liraglutide (10 μM) significantly increased the intracellular

158 Ca^{2+} transient amplitude (Figure 1B) and Ca^{2+} transient decay rate (Figure 1C), but did not
159 alter intracellular diastolic Ca^{2+} levels (Figure 1D), when compared to the control group. The
160 representative Ca^{2+} transient tracings are shown in Figure 1E.

161 In the simulated I/R injury protocol, our results demonstrated that cardiomyocytes
162 pretreated with liraglutide significantly increased the intracellular Ca^{2+} transient amplitude
163 (Figure 2B) and the intracellular Ca^{2+} transient decay rate (Figure 2C), when compared to the
164 H_2O_2 treated group. However, in all experimental groups, the levels of intracellular diastolic
165 Ca^{2+} levels did not differ (Figure 2D. The representative Ca^{2+} transient tracings are shown in
166 Figure 2E. Interestingly, we found that when liraglutide was given after H_2O_2 application to
167 cardiomyocytes, it still significantly increased the intracellular Ca^{2+} transient amplitude and
168 intracellular Ca^{2+} transient decay rate, when compared to the H_2O_2 treated group (Figure 2B,
169 2C). Similar to the results of pretreatment, liraglutide given after H_2O_2 application did not
170 alter the intracellular diastolic Ca^{2+} levels.

171 Since patients with type-2 diabetes mellitus have a higher risk (2 to 4 fold) for
172 developing coronary heart disease including myocardial infarction (Beckman *et al.* 2002),
173 anti-diabetic drugs with cardioprotection will be beneficial to these patients. It is known that
174 fatal arrhythmias and LV dysfunction are often observed following acute myocardial
175 infarction (Takamatsu 2008). Importantly, impaired intracellular Ca^{2+} regulation has been
176 shown to be an important factor responsible for these pathological effects (Takamatsu 2008).
177 Therefore, treatment options which can attenuate the impairment of intracellular Ca^{2+}
178 homeostasis could provide cardioprotection for the ischemic heart. In the present
179 study, our results clearly demonstrated that liraglutide exerted cardioprotective effects against
180 H_2O_2-induced cardiomyocyte injury by attenuating intracellular Ca^{2+} overload.

181 GLP-1 receptor is expressed in the heart and ventricular myocyte and has a high
182 affinity with a specific GLP-1 receptor agonist liraglutide (Pyke *et al.* 2014, Saraiva *et al.*

183 2014). Therefore, in this study the cardioprotective effect of liraglutide is mediated by the
184 GLP-1 receptor dependent pathway via increased phosphorylation of Akt and GSK3β which
185 are involved in the reperfusion injury survival kinase (RISK) pathway (Hausenloy *et al.*
186 2005). This finding was supported by previous studies reported the cardioprotective effects
187 of GLP-1 in animal models (Bose *et al.* 2005, Bose *et al.* 2007, Kavianipour *et al.* 2003,
188 Nikolaidis *et al.* 2005). Liraglutide pre- and post-treatment in cardiac I/R injury has been
189 shown to provide cardioprotective effects in both animals and clinical studies (Chen *et al.*
190 2016, McCormick *et al.* 2015, Noyan-Ashraf *et al.* 2009, Salling *et al.* 2012).

191 In the present study using H_2O_2-induced cardiomyocyte injury, our results
192 demonstrated that intracellular Ca^{2+} transient amplitude was impaired by H_2O_2 and both of
193 liraglutide pre- and post-treatment significantly increased intracellular Ca^{2+} transient
194 amplitude. Our finding consistent with previous studies reported that liraglutide exerts
195 cardioprotective effects by activating GLP-1 receptors in cardiomyocytes by coupled with the
196 G-protein/adenyl cyclase complex to increase cyclic adenosine monophosphate (cAMP)
197 production. Then, activates protein kinase A (PKA) and Ca^{2+}channel phosphorylation,
198 respectively. Finally, increase Ca^{2+}influx and increasing cardiomyocyte contractility
199 (Kristensen *et al.* 2009). Moreover, cAMP activate sarco/endoplasmic reticulum Ca2+-
200 ATPase (SERCA2a) activity and then increases Ca^{2+} reuptake into the endoplasmic reticulum
201 (Younce *et al.* 2013), leading to cardiomyocyte relaxation. Moreover, we found that
202 liraglutide increased intracellular Ca^{2+} transient decay rates. This finding is consistent with
203 previous findings which reported that liraglutide increased intracellular cAMP and activated
204 SERCA2a activity and then increased Ca^{2+} reuptake into the endoplasmic reticulum (Younce
205 *et al.* 2013). This finding also helped to explain the results in a previous report which
206 showed that a GLP-1 analogue improved diastolic functions in liraglutide-treated mice
207 (Noyan-Ashraf *et al.* 2009) and liraglutide also reduced the severity of left ventricular

dilation in that study (Noyan-Ashraf *et al.* 2009). Therefore, the ability of liraglutide to attenuate the impairment of physiological Ca^{2+} handling in a H_2O_2-induced cardiomyocyte injury model by increasing intracellular Ca^{2+} amplitude and decay rates, is a cardioprotective effect, in addition to its glycemic control, which is responsible for the improvement of cardiac function observed in previous reports. In addition, our results showed that liraglutide did not alter the intracellular diastolic Ca^{2+} level. Even though there is a high level of intracellular Ca^{2+} transient amplitude which reflect an increased intracellular Ca^{2+} during systolic period, there was a high rate of Ca^{2+} elimination which represented by intracellular Ca^{2+} transient decay rate. The balance on this intracellular calcium regulation could be contributed to the unaltered intracellular diastolic calcium level as seen in this study. Although we did not assess the oxidative stress parameters, previous studies demonstrated that liraglutide activated of PI3K-Akt-eNOS-NO signaling pathway and inhibited of oxidative stress (Inoue *et al.* 2015, Liu *et al.* 2016, Noyan-Ashraf *et al.* 2009).

Conflict of Interest

The authors declare that they have no conflict of interest.

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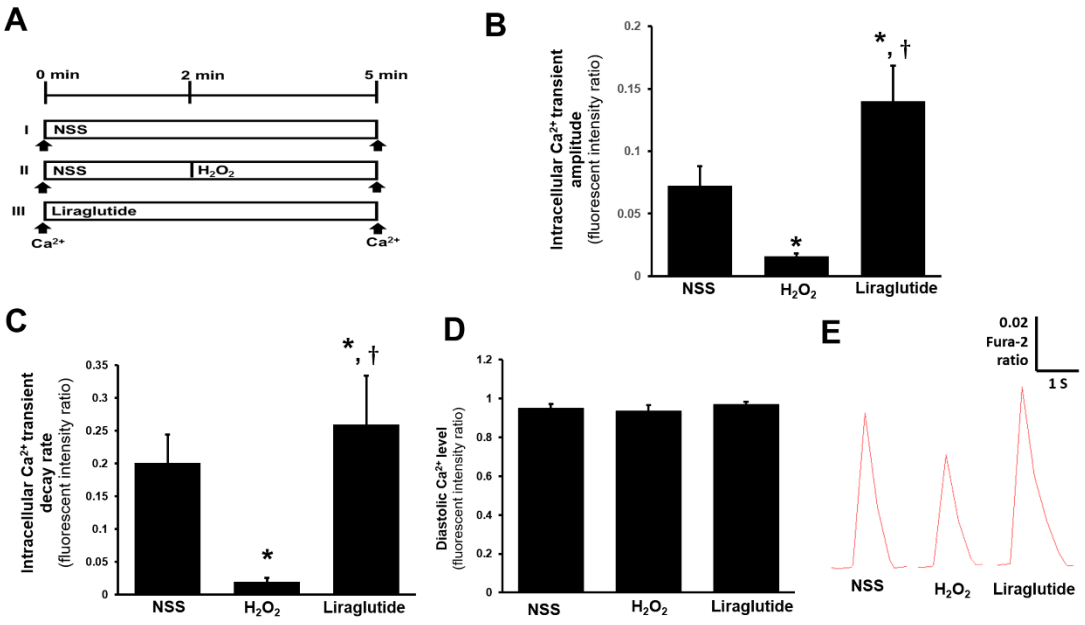

Figure 1. A schematic of study protocol I (A) and the effects of liraglutide on intracellular Ca^{2+} transient amplitude (B), intracellular Ca^{2+} transient decay rate (C), intracellular diastolic Ca^{2+} levels (D) and the representative images of Ca^{2+} transient tracing (E). *$p<0.05$ vs. NSS, †$p<0.05$ vs. H_2O_2 + NSS. NSS = normal saline solution, H_2O_2 = hydrogen peroxide, Ca^{2+} = intracellular calcium measurement

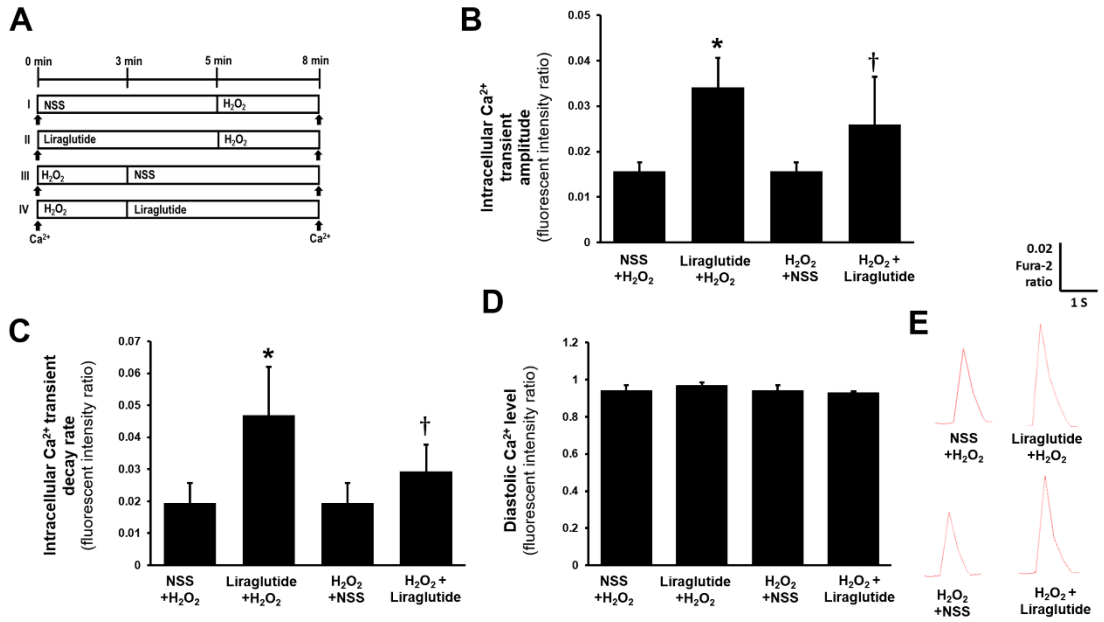

Figure 2. A schematic of study protocol II (A) and the effects of liraglutide administration before and after H_2O_2 application on intracellular Ca^{2+} transient in cardiomyocytes. Liraglutide significantly increased intracellular Ca^{2+} transient amplitude (B) and increased intracellular Ca^{2+} transient decay rate (C), but did not alter intracellular diastolic Ca^{2+} levels (D), when compared with the H_2O_2 group and the representative images of Ca^{2+} transient tracing (E). *$p<0.05$ vs. NSS + H_2O_2, †$p<0.05$ vs. H_2O_2 + NSS. NSS=normal saline solution, H_2O_2 = hydrogen peroxide, Ca^{2+} = intracellular calcium measurement