SHORT COMMUNICATION

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

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Summary
In ischemic/reperfusion (I/R) injured hearts, severe oxidative stress occurs and is associated with intracellular calcium (Ca²⁺) 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 Ca²⁺ regulation in the presence of oxidative stress. Therefore, we investigated the effects of GLP-1 analogue, (liraglutide, 10 µM) applied before or after hydrogen peroxide (H₂O₂, 50 µM) treatment on intracellular Ca²⁺ regulation in isolated cardiomyocytes. We hypothesized that liraglutide can attenuate intracellular Ca²⁺ overload in cardiomyocytes under H₂O₂-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 Ca²⁺ transient decay rate, intracellular Ca²⁺ transient amplitude and intracellular diastolic Ca²⁺ levels were recorded before and after treatment with liraglutide. In H₂O₂ induced severe oxidative stressed cardiomyocytes (which mimic cardiac I/R injury), liraglutide given prior to or after H₂O₂ administration effectively increased both intracellular Ca²⁺ transient amplitude and intracellular Ca²⁺ transient decay rate, without altering the intracellular diastolic Ca²⁺ level. Liraglutide attenuated intracellular Ca²⁺ overload in H₂O₂-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.

Key words
Liraglutide • Calcium regulation • Cardiomyocyte • Ischemic/Reperfusion • Cardioprotective

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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). Using I/R period, severe oxidative stress was shown to improve cardiac function in ischemic/reperfusion (I/R) model (Kristensen et al. 2016). The concentration we used for an in vitro study in this study was 10 µM of liraglutide which is 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 Ca2+ measurements were performed at the beginning of the study (baseline). Then, cardiomyocytes in Group I were treated with normal saline solution (NSS) for 5 min as a control group. Group II's cells were treated with NSS for 2 min and then H2O2 for 3 min to simulate I/R injury. Group III's cells were treated with liraglutide (10 µM) (Novo Nordisk A/S, Denmark) for 5 min. 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).

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 anesthetized 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 Ca2+ 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 Ca2+ measurements were performed at the beginning of the study (baseline). Then, cardiomyocytes in Group I were treated with normal saline solution (NSS) for 5 min as a control group. Group II's cells were treated with NSS for 2 min and then H2O2 for 3 min to simulate I/R injury. Group III's cells were treated with liraglutide (10 µM) (Novo Nordisk A/S, Denmark) for 5 min. 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 is approximately similar to the dose used in human (Langlois et al. 2016).

In this study, we used H2O2 (50 µM) to induce oxidative stress, to simulate the oxidative stress that is generated by ischemia/reperfusion injury. H2O2 concentration at 50 µM has been widely used to trigger oxidative stress-induced intracellular Ca2+ dyshomeostasis in cardiomyocytes. H2O2 has been shown to decrease sarco/endoplasmic reticulum Ca2+-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 Ca2+ homeostasis (Goldhaber 1996, Reeves et al. 1986).
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Fig. 1. A schematic of study protocol I (A) and the effects of liraglutide on intracellular Ca\textsuperscript{2+} transient amplitude (B), intracellular Ca\textsuperscript{2+} transient decay rate (C), intracellular diastolic Ca\textsuperscript{2+} levels (D) and the representative images of Ca\textsuperscript{2+} transient tracing (E). * P<0.05 vs. NSS, † P<0.05 vs. H\textsubscript{2}O\textsubscript{2} + NSS. NSS = normal saline solution, H\textsubscript{2}O\textsubscript{2} = hydrogen peroxide, Ca\textsuperscript{2+} = intracellular calcium measurement.

Fig. 2. A schematic of study protocol II (A) and the effects of liraglutide administration before and after H\textsubscript{2}O\textsubscript{2} application on intracellular Ca\textsuperscript{2+} transient in cardiomyocytes. Liraglutide significantly increased intracellular Ca\textsuperscript{2+} transient amplitude (B) and increased intracellular Ca\textsuperscript{2+} transient decay rate (C), but did not alter intracellular diastolic Ca\textsuperscript{2+} levels (D), when compared with the H\textsubscript{2}O\textsubscript{2} group and the representative images of Ca\textsuperscript{2+} transient tracing (E). * P<0.05 vs. NSS + H\textsubscript{2}O\textsubscript{2}, † P<0.05 vs. H\textsubscript{2}O\textsubscript{2} + NSS. NSS = normal saline solution, H\textsubscript{2}O\textsubscript{2} = hydrogen peroxide, Ca\textsuperscript{2+} = intracellular calcium measurement.
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 Langendorff apparatus. The hearts were perfused with modified Krebs solution as previously described (Palee et al. 2016) for 5 min, followed by calcium-free solution (100 µM EGTA) for 4 min, Tyrode’s solution with collagenase (0.1 mg/ml) for 10 min, and modified Krebs solution containing 100 µM CaCl₂ and 1 mg/ml type II collagenase for another 8 min. The ventricles were removed from the cannula, cut into small pieces and incubated in 10 ml of collagenase solution gassed with 100 % O₂ for 7 min at 37 °C. 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 CaCl₂. This process was repeated with modified Krebs solution containing 1 mM CaCl₂. 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 CaCl₂. Intracellular Ca²⁺ transient were measured using the CELL® 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 Ca²⁺. 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 Ca²⁺ handling in isolated rat cardiac myocytes exposed to hydrogen peroxide solution to provoke oxidative stress. H₂O₂ significantly decreased both intracellular Ca²⁺ transient amplitude (Fig. 1B) and intracellular Ca²⁺ transient decay rate (Fig. 1C). However, intracellular diastolic Ca²⁺ levels were not altered (Fig. 1D), when compared to the control group (i.e. cardiomyocytes treated with NSS). Moreover, liraglutide (10 µM) significantly increased the intracellular Ca²⁺ transient amplitude (Fig. 1B) and Ca²⁺ transient decay rate (Fig. 1C), but did not alter intracellular diastolic Ca²⁺ levels (Fig. 1D), when compared to the control group. The representative Ca²⁺ transient tracings are shown in Figure 1E.

In the simulated I/R injury protocol, our results demonstrated that cardiomyocytes pretreated with liraglutide significantly increased the intracellular Ca²⁺ transient amplitude (Fig. 2B) and the intracellular Ca²⁺ transient decay rate (Fig. 2C), when compared to the H₂O₂ treated group. However, in all experimental groups, the levels of intracellular diastolic Ca²⁺ levels did not differ (Fig. 2D). The representative Ca²⁺ transient tracings are shown in Figure 2E. Interestingly, we found that when liraglutide was given after H₂O₂ application to cardiomyocytes, it still significantly increased the intracellular Ca²⁺ transient amplitude and intracellular Ca²⁺ transient decay rate, when compared to the H₂O₂ treated group (Fig. 2B, 2C). Similar to the results of pretreatment, liraglutide given after H₂O₂ application did not alter the intracellular diastolic Ca²⁺ levels.

Since patients with type-2 diabetes mellitus have a higher risk (2 to 4 fold) for developing coronary heart disease including myocardial infarction (Beckman et al. 2002), anti-diabetic drugs with cardioprotection will be beneficial to these patients. It is known that fatal arrhythmias and LV dysfunction are often observed following acute myocardial infarction (Takamatsu 2008). Importantly, impaired intracellular Ca²⁺ regulation has been shown to be an important factor responsible for these pathological effects (Takamatsu 2008). Therefore, treatment options which can attenuate the impairment of intracellular Ca²⁺ homeostasis could provide cardioprotection for the ischemic heart. In the present study, our results clearly demonstrated that liraglutide exerted cardioprotective effects against H₂O₂-induced cardiomyocyte injury by attenuating intracellular Ca²⁺ overload.

GLP-1 receptor is expressed in the heart and ventricular myocyte and has a high affinity with a specific GLP-1 receptor agonist liraglutide (Pyke et al. 2014, Saraiva et al. 2014). Therefore, in this study the cardioprotective effect of liraglutide is mediated by the GLP-1 receptor dependent pathway via increased phosphorylation of Akt and GSK3β which are involved in the reperfusion injury survival kinase (RISK) pathway (Hausenloy et al. 2005). This finding was supported by previous studies reported the cardioprotective effects of...
GLP-1 in animal models (Bose et al. 2005, Bose et al. 2007, Kavianipour et al. 2003, Nikolaidis et al. 2005). Liraglutide pre- and post-treatment in cardiac I/R injury has been shown to provide cardioprotective effects in both animals and clinical studies (Chen et al. 2016, McCormick et al. 2015, Noyan-Ashraf et al. 2009, Salling et al. 2012).

In the present study using H2O2-induced cardiomyocyte injury, our results demonstrated that intracellular Ca2+ transient amplitude was impaired by H2O2 and both of liraglutide pre- and post-treatment significantly increased intracellular Ca2+ transient amplitude. Our finding consistent with previous studies reported that liraglutide exerts cardioprotective effects by activating GLP-1 receptors in cardiomyocytes by coupled with the G-protein/adenylyl cyclase complex to increase cyclic adenosine monophosphate (cAMP) production. Then, activates protein kinase A (PKA) and Ca2+ channel phosphorylation, respectively. Finally, increase Ca2+ influx and increasing cardiomyocyte contractility (Kristensen et al. 2009). Moreover, cAMP activate sarco/endoplasmic reticulum Ca2+-ATPase (SERCA2a) activity and then increases Ca2+ reuptake into the endoplasmic reticulum (Younce et al. 2013), leading to cardiomyocyte relaxation. Moreover, we found that liraglutide increased intracellular Ca2+ transient decay rates. This finding is consistent with previous findings, which reported that liraglutide increased intracellular cAMP and activated SERCA2a activity and then increased Ca2+ reuptake into the endoplasmic reticulum (Younce et al. 2013). This finding also helped to explain the results in a previous report which showed that a GLP-1 analogue improved diastolic functions in liraglutide-treated mice (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 Ca2+ handling in a H2O2-induced cardiomyocyte injury model by increasing intracellular Ca2+ 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 Ca2+ level. Even though there is a high level of intracellular Ca2+ transient amplitude which reflect an increased intracellular Ca2+ during systolic period, there was a high rate of Ca2+ elimination which represented by intracellular Ca2+ 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
There is no conflict of interest.

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Abbreviations

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