Glucagon-like Peptide-1 Modulates Calcium Homeostasis in Human Coronary Microvascular Endothelial Cells after Ischemia and Reperfusion

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Abstract

Diabetic patients are at extreme risk for cardiovascular disease. Endothelial dysfunction plays a key role in the development of cardiovascular complications in diabetes. The effects of diabetes on the endothelium are multi-factorial, and include impaired intracellular calcium [Ca²⁺]i handling, particularly during reperfusion after ischemia. Glucagon-like peptide-1 (GLP-1) forms the basis of a group of agents currently used for glycemic control in type 2 diabetic patients. GLP-1 also improves endothelial function and is cardioprotective after ischemia and reperfusion. The mechanisms behind these effects are unknown. We determined the effects of GLP-1 on cytosolic calcium [Ca²⁺]i in human coronary microvascular endothelial cells (HCMVECs), under physiologic conditions and also after simulated ischemia and reperfusion. In HCMVECs, [Ca²⁺]i was measured with and without treatment with GLP-1 (10 nM) and with and without simulated ischemia and reperfusion. We found that GLP-1 significantly modulates calcium handling in HCMVECs. The rise of ATP-induced [Ca²⁺]i was attenuated after treatment with GLP-1, both under physiologic conditions and after ischemia and reperfusion. These data reveal a role for GLP-1 in the modulation of [Ca²⁺]i in HCMVECs, which may be one mechanism by which it improves endothelial function. Given the increased risk of coronary microvascular dysfunction in type 2 diabetes, these findings suggest another potential benefit to the use of GLP-1 based therapies in diabetic patients.

ABBREVIATIONS

[Ca²⁺]i: Cytosolic (intracellular) Calcium; GLP-1: Glucagon-Like Peptide-1; HCMVECs: Human Coronary Microvascular Endothelial Cells; ATP: Adenosine Triphosphate; cAMP: Cyclic AMP; GPCR: G-Protein Coupled Receptor

INTRODUCTION

Diabetic patients are at extreme risk for cardiovascular disease [1]. Endothelial dysfunction plays a key role in the development of cardiovascular complications in diabetes [2]. The effects of diabetes on the endothelium are multi-factorial, and include an impairment of intracellular calcium [Ca²⁺]i handling [3], which is also altered during reperfusion after ischemia [4,5].

Cardioprotection after an ischemic event is clearly dependent on preservation of a functional coronary microvascular endothelium, without which the heart is unable to deliver adequate substrate to meet the metabolic demands of the myocardium [6]. The risk of reperfusion injury is greater in coronary endothelial cells than in cardiomyocytes [7], and consequently microvascular endothelial dysfunction typically precedes cardiomyocyte...
death after ischemia and reperfusion of the heart [8]. This dysfunction of the endothelium leads to inadequate perfusion of the myocardium and poor clinical outcomes after myocardial infarction, despite aggressive treatment [9-14].

Calcium (Ca\(^{2+}\)) overload appears to be an important contributor to cellular injury during reperfusion. Persistently high levels of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) during reperfusion lead to irreversible cell damage which to a large degree is dependent on excess Ca\(^{2+}\) uptake by mitochondria, dissipation of the mitochondrial membrane potential, and ultimately cell death via signaling processes leading to either necrosis or apoptosis [4,5]. Moreover, the endothelium appears to be particularly susceptible to intracellular Ca\(^{2+}\) overload and subsequent dysfunction during reperfusion [15-18].

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that exhibits cardioprotective effects [19-24]. GLP-1 improves coronary microvascular endothelial function after ischemia and reperfusion [22,23], and prevents hypoxia-induced endothelial cell dysfunction [25] and death [26]. The mechanisms by which GLP-1 promotes these effects have not been fully elucidated. Moreover, the effects of GLP-1 on Ca\(^{2+}\) handling in the endothelium have not been studied. GLP-1-based therapies are currently being widely used to treat patients with type 2 diabetes; arguably, this population is at higher risk for heart disease than any other. In addition, several agents that were previously used to treat type 2 diabetes have been found later to contribute to heart disease in patients with type 2 diabetes (the thiazolidinedione rosiglitazone being the most recent example [27-29]). Since coronary microvascular dysfunction is common among diabetic patients [30-34], who also are at extreme risk of ischemic heart disease [35-37], it is critical to understand the mechanisms by which GLP-1 protects the coronary microcirculation, and therefore the heart.

In the present study, we used an in vitro model of endothelial ischemia and reperfusion to test the hypothesis that GLP-1 modulates Ca\(^{2+}\) handling in the coronary microvascular endothelium under these conditions. The underlying hypothesis is that a modulatory effect of GLP-1 on Ca\(^{2+}\) homeostasis mitigates reperfusion injury. In human coronary microvascular endothelial cells (HCMVECs), we observed that GLP-1 attenuated the purinergic agonist-induced rise in [Ca\(^{2+}\)]\(_i\), both under physiologic conditions, and also after ischemia and reperfusion. These findings demonstrate, for the first time, a direct role for GLP-1 on Ca\(^{2+}\) handling in endothelial cells, specifically from the human coronary microcirculation. We propose that the ability of GLP-1 to attenuate Ca\(^{2+}\) overload in the coronary endothelium may be an important mechanism of its cardioprotective effect.

**MATERIALS AND METHODS**

**Cell culture**

Human coronary microvascular endothelial cells (HCMVECs) were obtained from Lonza, Inc. (Walkersville, MD) at passage 3 and were grown in culture per manufacturer’s instructions. Cells were used between passage 5 and 6. Quality assurance from the manufacturer verified pure endothelial cells using immunohistologic staining for the presence of acetylated LDL and von Willebrand’s antigen as well as the absence of smooth muscle α-actin and mycoplasma contamination. Cells were used per manufacturer’s protocols.

**Single-cell [Ca\(^{2+}\)]\(_i\) measurements**

Cells were grown on coverslips in 6-well plates and loaded with the fluorescent Ca\(^{2+}\) sensitive dye fura-2(AM) as previously described [38]. After rinsing for 20 min to allow for hydrolysis of the acetoxymethylester, a coverslip with dye-loaded cells was placed in a chamber held at 37°C while mounted on the stage of an inverted Olympus IX-70 microscope equipped with a 40x 1.4 numerical aperture (NA) ultrafluor objective and a 300-W Xe lamp as the excitation source. Fura-2, loaded within the cells, was alternately excited at 340 and 380 nm using a filter wheel. The emitted light was filtered at 510 nm (10 nm band pass) before focusing the cell image onto a charge-coupled device (CCD) camera. After dye loading and rinsing, cells were incubated in the presence of 10 nM GLP-1 (American Peptide Co., Sunnyvale, CA) or vehicle (saline) for 5 min, then 10 µM ATP was added to stimulate a rise in [Ca\(^{2+}\)]\(_i\). Prior to termination of each experiment, the Ca\(^{2+}\) ionophore ionomycin (2.5 µM) was used as a positive control to verify probe sensitivity to Ca\(^{2+}\). In a separate series of experiments, HCMVECs were grown to confluence on coverslips, and a subset was subjected to simulated ischemia and reperfusion (I/R) as described below. Immediately prior to the hypoxia period, GLP-1 (10 nM, American Peptide Co., Sunnyvale, CA) was added to 50% of wells containing cells to be exposed to I/R and also to 50% of wells not exposed to I/R. After 30 min of simulated ischemia, cells were returned to physiologic conditions (reperfusion), immediately loaded with fura-2, and [Ca\(^{2+}\)]\(_i\) determined as described above.

**Ischemia-reperfusion model**

HCMVECs were grown to confluence on coverslips in 6-well plates and a sub-set (n=6 wells) was subjected to hypoxic conditions (5% O2 at 37°C) and acidic media (pH 6.5) for 30 minutes, to simulate ischemia. After 30 minutes, cells were returned to physiologic conditions (simulated reperfusion), immediately loaded with fura-2, and rinsed as above. An additional 6-well plate, maintained under physiologic conditions, was also studied in a paired fashion.

Data are expressed as means ± S.E., and were analyzed using unpaired Students t-tests. P values of <0.05 were taken to indicate statistical significance.

**RESULTS**

**Time course of response to ATP**

In order to evaluate the effect of GLP-1 on Ca\(^{2+}\) regulation, we required a modulator of [Ca\(^{2+}\)]\(_i\) in endothelial cells. Purinergic agents such as ATP are known to activate Ca\(^{2+}\) flux, therefore we evaluated [Ca\(^{2+}\)]\(_i\) after exposure to ATP in the absence and presence of GLP-1. Shown in Figure 1 is the time course of [Ca\(^{2+}\)]\(_i\) in response to 10 µM ATP. In the absence of pretreatment with GLP-1, a significant elevation of [Ca\(^{2+}\)]\(_i\) was observed in response to ATP, followed by recovery to baseline within 2.5 min. Treatment with GLP-1 (for 5 min prior to taking resting measurements) was found to depress the ATP induced [Ca\(^{2+}\)]\(_i\) elevation (Figure 1).
Simulated ischemia-reperfusion (I/R) causes resting Ca\(^{2+}\) overload in HCMVEC-Cs which is prevented by pretreatment with GLP-1.

GLP-1 treatment by itself slightly, but not significantly, increased resting [Ca\(^{2+}\)]\(i\). Forskolin, which acts to elevate cAMP by direct activation of adenylate cyclase, caused a modest increase in resting [Ca\(^{2+}\)]\(i\) (Figure 2). After cells were subjected to simulated I/R, resting [Ca\(^{2+}\)]\(i\) was substantially increased, consistent with previous reports of coronary endothelial Ca\(^{2+}\) overload associated with I/R [39-41]. Impressively, treatment with GLP-1 prior to ischemia completely prevented the elevation of resting [Ca\(^{2+}\)]\(i\) during simulated reperfusion to levels seen with GLP-1 or forskolin under normoxic conditions (Figure 2).

GLP-1 attenuates the rise in ATP-induced [Ca\(^{2+}\)]\(i\) both under physiologic conditions and during reperfusion after ischemia.

There was no difference in the effect of GLP-1 on the ATP-induced [Ca\(^{2+}\)]\(i\) between cells under physiologic conditions and cells subjected to I/R (Figure 3). These findings suggest a potential protective effect of GLP-1 in coronary microvascular endothelial cells subjected to I/R.

**DISCUSSION**

In this manuscript, we report that GLP-1 has a significant effect on Ca\(^{2+}\) handling in human coronary microvascular endothelial cells (HCMVECs). Exposure of the cells to GLP-1 blunts the agonist-induced rise in [Ca\(^{2+}\)]\(i\). In addition, the attenuation of agonist-induced [Ca\(^{2+}\)]\(i\) by GLP-1 persists in the cells after they are subjected to simulated ischemia and reperfusion. These findings may help to explain the ability of GLP-1 to improve endothelial function in diabetes, [42] as well as to provide cardioprotection after ischemia and reperfusion [21,22,24,43-45].

A growing body of evidence supports beneficial vaso- and cardio-protective effects of GLP-1, independent of its ability to promote glycemic control. GLP-1 improves peripheral endothelial function [42,46,47] and coronary microvascular function *in vivo*

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**Figure 1** Measurement of [Ca\(^{2+}\)]\(i\) under resting conditions, and after addition of the purinergic agent ATP (10 µM). Cells were maintained in either standard incubation media (control) or media containing 10 nM GLP-1 for 5 minutes prior to initiating the measurement period. Exposure to GLP-1 blunts the ATP-induced rise in [Ca\(^{2+}\)]\(i\). Squares or diamonds indicate means ± SE. Control, n=37 cells; GLP-1, n=37 cells. *P<0.05, GLP-1 vs. control.

**Figure 2** Effects of ischemia/reperfusion (I/R) and GLP-1 treatment on resting [Ca\(^{2+}\)]\(i\) in HCMVECs. To simulate I/R, a subset of HCMVECs (where indicated) was subjected to hypoxic conditions (5% O\(_2\) at 37°C) and acidic media (pH 6.5) for 30 minutes, followed by a return to physiologic conditions. Where indicated, GLP-1 (10 nM) was added to the cell culture media immediately prior to the 30 minute period of hypoxia or normoxia. Measurements of [Ca\(^{2+}\)]\(i\) were taken in the resting state, immediately after returning cells to physiologic conditions. Bars are means ± SE. Normoxia, n=37 cells; Hypoxia, n=20 cells; Normoxia + GLP-1, n=37 cells; Hypoxia + GLP-1, n=16 cells; forskolin, n=23 cells.

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**Figure 3** Peak change in [Ca\(^{2+}\)]\(i\) measured following addition of 10 µM ATP to induce an increase. Where indicated, cells were incubated with GLP-1 for 5 minutes prior to initiation of the experimental period. **Left panel:** GLP-1 pretreatment attenuates ATP induced [Ca\(^{2+}\)]\(i\) transients under normoxic conditions. **Right panel:** Cells were pre-treated with GLP-1 and subjected to simulated ischemia (%5 O\(_2\) and 37°C, in acidic media (pH 6.5) for 30 minutes, then returned to physiologic conditions (simulated reperfusion), loaded with fura-2, and stimulated with ATP, immediately followed by measurement of [Ca\(^{2+}\)]\(i\). GLP-1 attenuates the ATP-induced rise in [Ca\(^{2+}\)]\(i\), both under physiologic conditions and in cells subjected to I/R. Bars are means ± SE. Normoxia, n=37 cells; Normoxia + GLP-1, n=37 cells; I/R, n=20 cells; I/R + GLP-1, n=16 cells. *P<0.05, GLP-1 vs. placebo.
Although the clinical relevance of these findings is not clear, Ca\textsuperscript{2+} vasodilators [64,65] and vasoconstrictors [57,60,66,67].

The underlying mechanisms for these phenomena are not completely understood, however, [Ca\textsuperscript{2+}]\textsubscript{i} is an important and complex regulator of endothelial function (recently reviewed by Moccia et al.) [55] and thus may play a significant role. Modulation of endothelial [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis can have profound effects on the balance of vasoactive factors synthesized by the endothelium, and thus underlying vascular tone. A rise in endothelial [Ca\textsuperscript{2+}]\textsubscript{i} activates nitric oxide synthase and enhances production of the vasodilator nitric oxide (NO) [56]. On the other hand, a rise in [Ca\textsuperscript{2+}]\textsubscript{i} can also enhance production of endothelium-dependent contracting factors, particularly under conditions of oxidative stress [22,57-60]. In addition, endothelial Ca\textsuperscript{2+} overload is a key mechanism of reperfusion injury in the coronary microcirculation [41], and exaggerated agonist-induced elevations in [Ca\textsuperscript{2+}]\textsubscript{i} are present in diabetic coronary endothelial cells [3], conditions both relevant to our findings.

In the present study, we found that GLP-1 attenuated the ATP-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i} both under physiologic conditions and also during simulated reperfusion after ischemia. The physiologic relevance of these findings is unknown. Moreover, the mechanism by which GLP-1 elicits this effect needs to be determined. Though ATP is found in very low concentrations in the extracellular space under physiologic conditions [61], the presence of biologically active concentrations of ATP in plasma following vessel wall damage suggests that extracellular ATP may have a role in the control of vascular tone in pathologic states [62], and has been found 	extit{in vitro} to be deleterious to endothelial cells [63]. Signals from extracellular ATP are transduced via P2y receptors expressed in endothelial cells [62]. These G-protein-coupled receptors activate IP\textsubscript{3}-mediated mobilization of [Ca\textsuperscript{2+}]\textsubscript{i} from internal stores, and subsequently promote either relaxation or contraction of vascular smooth muscle, depending on the concentration, the vascular bed, and other factors [62]. The secretion of a variety of endothelium-derived vasoactive products are dependent on an agonist-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}, including vasodilators [64,65] and vasoconstrictors [57,60,66,67]. Although the clinical relevance of these findings is not clear, Ca\textsuperscript{2+} overload is a key mechanism of endothelial dysfunction during reperfusion following ischemia [15-18], and the attenuation of [Ca\textsuperscript{2+}]\textsubscript{i} has been shown to protect coronary endothelial cells against reperfusion injury [41].

Activation of the GLP-1 receptor causes an elevation of intracellular cAMP [68]. In the present study, the adenylate cyclase activator forskolin attenuated the rise in ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} to the same degree as GLP-1, suggesting that the GLP-1 effect may be mediated via cAMP. Consistent with our findings, Campos-Toimil et al. reported that two different type 4 phosphodiesterase (PDE) inhibitors, known to improve endothelial function [69], significantly attenuated the agonist-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i} in HUVECs [69]. While many PDE inhibitors work by inhibiting the enzyme that rapidly degrades cGMP, and thus promote sustained cGMP signaling [70], type 4-PDE inhibitors prevent degradation of cAMP [71]. In addition, forskolin attenuates agonist-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} in human umbilical vein endothelial cells (HUVECs) [72], and cAMP blunts ATP-induced, but not resting [Ca\textsuperscript{2+}]\textsubscript{i} in bovine aortic endothelial cells [73]. The mechanisms of these effects are poorly understood. Crosstalk between cAMP and [Ca\textsuperscript{2+}]\textsubscript{i} is complex, and differs between different regions of the vasculature [74]; thus, further studies are required to determine mechanisms behind the effects of GLP-1 and cAMP on endothelial Ca\textsuperscript{2+} handling in the heart.

There are several limitations of the present study that warrant discussion. We measured changes in global [Ca\textsuperscript{2+}]\textsubscript{i}, both in the resting state and after purinergic stimulation. Ca\textsuperscript{2+} signaling during GPCR activation is complex in endothelial cells, and varies according to the intensity of the stimulus and proximity and coupling to other actively signaling cells [75]. In addition, our model does not allow for the determination of the contribution of vascular smooth muscle. In the microcirculation, myoendothelial coupling is significant, and there is clear evidence that vascular smooth muscle may alter or initiate Ca\textsuperscript{2+} signaling in endothelium via direct communication across myoendothelial junctions [76,77]. Moreover, in the present study, HCMVECs were not exposed to physiologic shear stress which can alter agonist-induced signaling pathways and thus, the [Ca\textsuperscript{2+}]\textsubscript{i} response. Further studies, focused on spatially and temporally relevant aspects of real-time signaling, and the underlying mechanisms that provide for the changes in Ca\textsuperscript{2+} handling are necessary to fully elucidate the effects of GLP-1 on endothelial Ca\textsuperscript{2+} homeostasis.

CONCLUSION

In summary, we found that GLP-1 attenuates the agonist-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}. Under physiologic conditions, a rise in [Ca\textsuperscript{2+}]\textsubscript{i}, causes endothelium-dependent relaxation, and pathophysiology, our findings raise important questions that have implications for future basic research, as well as for translational science and clinical medicine. Ultimately, the role of GLP-1 in endothelial Ca\textsuperscript{2+} handling, particularly in the heart, may have broad implications for the cardiovascular complications of diabetes, and for the potential of GLP-1 based therapies to mitigate these complications in patients with type 2 diabetes.
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