Glucagon-like Peptide 1 Can Directly Protect the Heart Against Ischemia/Reperfusion Injury

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Glucagon-like peptide 1 (GLP-1), a gut incretin hormone that stimulates insulin secretion, also activates antiapoptotic signaling pathways such as phosphoinositide 3-kinase and mitogen-activated protein kinase in pancreatic and insulinoma cells. Since these kinases have been shown to protect against myocardial injury, we hypothesized that GLP-1 could directly protect the heart against such injury via these prosurvival signaling pathways. Both isolated perfused rat heart and whole animal models of ischemia/reperfusion were used, with infarct size measured as the end point of injury. In both studies, GLP-1 added before ischemia demonstrated a significant reduction in infarction compared with the valine pyrrolidide (an inhibitor of its breakdown) or saline groups. This protection was abolished in the in vitro hearts by the GLP-1 receptor antagonist exendin (9-39), the cAMP inhibitor Rp-cAMP, the PI3kinase inhibitor LY294002, and the p42/44 mitogen-activated protein kinase inhibitor UO126. Western blot analysis demonstrated the phosphorylation of the proapoptotic peptide BAD in the GLP-1–treated groups. We show for the first time that GLP-1 protects against myocardial infarction in the isolated and intact rat heart. This protection appears to involve activating multiple prosurvival kinases. This finding may represent a new therapeutic potential for this class of drug currently undergoing clinical trials in the treatment of type 2 diabetes. *Diabetes* 54:146–151, 2005

Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted mainly by the enteroendocrine cells of the intestine in response to the presence of nutrients. It facilitates glucose-induced insulin release, and therefore, analogs of GLP-1 are of potential interest as a possible approach for the treatment of diabetes. Native GLP-1 has a short half-life of minutes, being rapidly degraded by dipeptidyl peptidase-IV (DPPIV), to generate an NH_2-terminally truncated metabolite in addition to undergoing renal excretion. Therefore, to assess the roles of intact GLP-1, it is necessary to use a DPPIV inhibitor such as valine pyrrolidide (VP) as a means of preventing its degradation (1). The GLP-1 receptor is widely expressed in islet cells, kidney, lung, brain, the gastrointestinal tract, and, interestingly, also in the heart (2). The GLP-1 receptor is a G protein–coupled receptor and is a distinct member of the glucagon-secretin receptor super family that has been shown to function by causing intracellular calcium influx in addition to upregulating cAMP (3). Interestingly, cAMP has been demonstrated to protect against apoptosis in several cell types other than myocardial (4–6). In isolated cardiac myocytes, GLP-1 has also been shown to elevate cAMP in addition to demonstrating chronotropic effects (7). Furthermore, in vivo experiments (8,9) have also shown elevated blood pressure and heart rate as a result of GLP-1 infusion. Studies using GLP-1 receptor knockout mice have also suggested a role for the GLP-1 receptor in the control of cardiac structure and function (10). More importantly, a recent clinical study (11) demonstrated that administration of GLP-1 improved left ventricular function in patients with acute myocardial infarction and left ventricular dysfunction. However, of specific interest is the fact that GLP-1 has also been shown to promote the activity of phosphoinositide 3-kinase (PI3K) in β-cells (12). This kinase has been clearly associated with myocardial protection in the setting of ischemic/reperfusion injury (13) as well as myocardial preconditioning (14,15).

Recent data have suggested that GLP-1 can exert a direct cytoprotective effect via inhibition of apoptosis either directly in target cells expressing the GLP-1 receptor or possibly via the activation of survival factors (16). GLP-1 has also been shown to protect against apoptosis in insulinoma cell lines through both cAMP and PI3K (6). In this context, it is important to bear in mind that insulin has been shown to activate prosurvival kinases such as PI3K (17), which have been proposed as integral components of antiapoptotic cascades involved in myocardial protection. Therefore, the aim of this study was to examine the effect of GLP-1 on ischemic injury in both the in vivo (in presence of endogenous insulin) and in vitro (in absence of insulin) rat myocardium, assessing myocardial infarct size as an end point of injury in both models. Furthermore, an additional aim was to elucidate the mechanism underlying the GLP-1 effect using specific inhibitors of prosurvival signaling pathways.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (300–450 g; Charles River, Bicester, U.K.) were used in all of these studies. They were fed a standard diet, housed under the

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DPPIV, dipeptidyl peptidase-IV; GLP-1, glucagon-like peptide 1; PI3K, phosphoinositide 3-kinase; VP, valine pyrrolidide.

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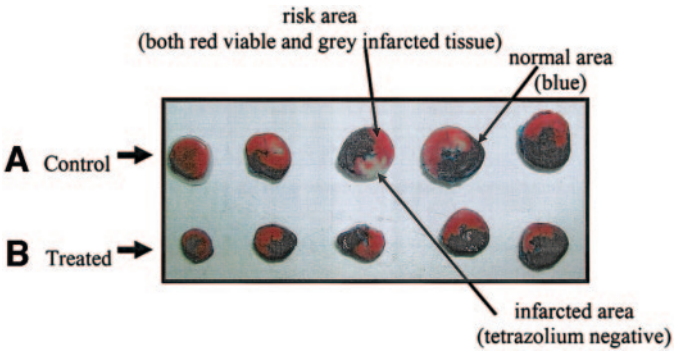

FIG. 1. Representative figure of myocardial infarction. Tetrazolium-stained heart slices taken from control hearts (*A*) and treated hearts (*B*) that have been subjected to 35 min of left main coronary artery occlusion followed by 120 min of reperfusion. The slices show areas of infarction (gray), ischemic area or area at risk (red and gray), and nonrisk normal area (blue).

same standardized conditions, and treated in accordance with the U.K. Animals (Scientific Procedures) Act of 1986. GLP-1 and VP were supplied by NovoNordisk (Bagsværd, Denmark). U0126 (a p44/42 inhibitor) and LY24002 (a PI3K inhibitor) were obtained from Tocris (Bristol, U.K.), exendin (9-39) from Bachem (Merseyside, U.K.), and Rp-cAMP from Calbiochem (Nottingham, U.K.). All other reagents were of analytic standard.

In vivo procedure. Rats were anesthetized with midazolam (5 mg/ml), fentanyl (0.315 mg/ml), and fluanisone (10 mg/ml) together with heparin (1 IU/kg) given by intraperitoneal injection. Further doses of anesthetic were given intravenously as needed. A tracheosotomy was performed, and an endotracheal tube was positioned to allow ventilation. A catheter was placed in the right jugular vein for drug infusion. The left carotid artery was cannulated to measure mean arterial pressure and heart rate. Intermittent arterial blood gas analysis was performed, and ventilation was adjusted to maintain a physiological pH (7.35–7.45), pCO2 (4.7–6.4 kPa), and pO2 (11.1–14.4 kPa). A left thoracotomy was performed and the heart exposed. A 6.0 suture was placed around the left anterior coronary artery, midway between the apex of the heart and the left atrial appendage, and threaded through a plastic snare to permit reversible occlusion of the coronary artery. To induce ischemia, the snare was tightened around the artery and confirmed by regional ischemic pallor, hypokinesia, a fall in blood pressure, and electrocardiogram recordings. The snare was released at the end of the 30-min ischemia to allow reperfusion for 120 min.

Isolated heart preparation (in vitro). Rats were anesthetized with sodium phenobarbitol (50 mg/kg i.p.). Heparin (1 IU/g) was administered concomitantly. Hearts were excised and retrogradely perfused via the aorta at a constant pressure of 75 mmHg, with oxygenated Krebs-Henseleit buffer (15), pH 7.3–7.5 at 37°C. A saline-filled latex balloon connected to a pressure transducer was inserted into the left ventricle and baseline end-diastolic pressure set at 5–10 mmHg. Heart rate, left ventricle end-diastolic pressure, and left ventricle developed pressure were recorded continuously. A 3.0 suture was placed around the left main coronary artery, halfway between the apex of the heart and the root of the pulmonary artery, and threaded through a plastic snare to permit reversible occlusion of the coronary artery. Coronary occlusion was induced for 35 min by clamping the snare onto the heart. Reperfusion was achieved by releasing the snare.

Infarct assessment in both in vivo and in vitro models. At the end of the 120-min reperfusion, the left main coronary artery was religated and the risk zone delineated with Evans blue dye infused via the aortic root. Hearts were frozen, sectioned transversely from the apex to the base in five slices of 2-mm thickness, and incubated in 1% triphenyl-tetrazolium chloride in phosphate buffer (pH 7.4, 37°C) for 12 min to stain viable tissue red and necrotic tissue white (Fig. 1). The sections were fixed in 4% formalin for 24 h and then drawn on acetate by an independent operator. The hearts in which the risk zone was lower than 0.4 cm^3 or higher than 0.7 cm^3 were excluded. The infarct-to-risk volume ratios were determined by computerized planimetry. (Planimetry + version 1.0 for Windows; Boreal Software, St. Catharines, Canada).

Study protocols

In vivo. Animals were randomly assigned to one of three study groups: the *1*) control group, where they were subjected to 30 min of regional ischemia followed by 120 min of reperfusion; the *2*) VP control group, in which they received, in addition to the control protocol, a subcutaneous injection of VP (20 mg/kg) 30 min before anesthesia in order to inhibit DPPIV activity; and *3*) the GLP-1 + VP group, in which the animals were given, in addition to the subcutaneous dose of VP, an intravenous infusion of GLP-1 (4.8 pmol · kg^{-1} · min^{-1}) commencing during stabilization and continuing throughout the procedure.

In vitro. Animals were randomly assigned to one of the following seven groups: the *1*) control group, where they were subjected to 35 min of regional ischemia followed by 120 min of reperfusion, and the treated hearts groups, which received the following drugs added to buffer during stabilization and continued throughout the experiment: the *2*) VP (20 mg/l) group; the *3*) GLP-1 (0.3 nmol) + VP (20 mg/l) group; the *4*) GLP-1 (0.3 nmol) + VP (20 mg/l) + PI3K inhibitor LY24002 (15 μmol/l) group; the *5*) GLP-1 (0.3 nmol) + VP (20 mg/l) + p44/42 inhibitor UO126 (10 μmol/l) group; the *6*) GLP-1 (0.3 nmol) + VP (20 mg/l) + cAMP inhibitor Rp-cAMP (1.5 μmol/l) group; and *7*) GLP-1 (0.3 nmol) + VP (20 mg/l) + the GLP-1 R antagonist exendin (9-39) (3 nmol/l) group. In addition, some hearts only received the inhibitors or the antagonists (LY294002, UO126, Rp-cAMP, and exendin (9-39)) to rule out any influence they may have directly upon infarction.

Western blot analysis and protein extraction. In vitro experiments were performed to examine the phosphorylation of the proapoptotic peptide BAD. GLP-1 (0.3 nmol)-treated hearts (10-min stabilization followed by 5 min of drug administration) were compared with control hearts. The hearts were frozen in liquid nitrogen and then extracted in a lysis buffer as previously described (15). The tissue was homogenized and centrifuged at 11,000 rpm for 10 min at 4°C. Protein samples and a prestained protein marker (New England Biolabs, Hitchin, U.K.) were separated by SDS-PAGE and transferred onto Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Biosciences, Amersham, U.K.) overnight. Equal loading and transfer of proteins were confirmed by Poinceau's red staining. Membranes were incubated with phospho-specific polyclonal antibodies (1:1,000) against total BAD and phospho-BAD (Ser 136) (New England Biolabs, U.K.) and, subsequently, with an anti-rabbit secondary antibody (1:2,500). Proteins were detected using enhanced chemiluminescence Western blotting reagent (Amersham Biosciences, U.K.), and bands were visualized by autoradiography (15).

Statistical analysis. Data are expressed as means ± SE. Infarcts-to-risk ratios and risk volumes were analyzed using the one-way ANOVA factorial test. Statistical significance between group means was defined as $P < 0.05$.

RESULTS

Exclusions. A total of 116 rats were used, 91 for the in vitro and 25 for the in vivo study. In the in vivo study, three animals were lost during the surgical procedure due to complications such as hemorrhage. In the in vitro study, one experiment was excluded, as the risk zone was <0.4 cm^3, while five others were excluded due to technical reasons.

Hemodynamic effects. In the in vivo preparation, mean arterial pressure and heart rate were recorded throughout the experimental protocol. No statistically significant differences between these values during stabilization, ischemia, or reperfusion were observed (Table 1). As expected, ischemia produced bradycardia and hypotension when compared with stabilization. These data were reproduced in the in vitro model, with heart rate and rate pressure product being comparable among all the groups throughout the experimental protocols. (Table 2 shows data for the three main groups. The rest of the data are not shown.)

Infarction in vivo and in vitro models. GLP-1 + VP was seen to protect the myocardium (Fig. 2) from ischemia/reperfusion injury in the in vivo heart model, demonstrating a significant reduction in infarction compared with the VP or saline groups (20.0 ± 2.8% vs. 47.3 ± 4.3% and 44.3 ± 2.4%, $P < 0.001$, $n = 8$ per group). In the in vitro study (Fig. 3), those hearts treated with GLP-1 + VP again demonstrated a significantly reduced infarct size compared with control and VP groups (26.7 ± 2.7% vs. 58.7 ± 4.1% in control and 52.6 ± 4.7% in the VP group, $P < 0.0001$).

In vitro infarct size and GLP-1 receptor inhibition. Exendin (9-39), a specific inhibitor of the GLP-1 receptor (18,19) when given concomitantly with GLP-1 + VP

MPI EXHIBIT 1231 PAGE 2

TABLE 1
In vivo mean arterial blood pressure and heart rate

Groups	Stabilization	5-min ischemia	20-min ischemia	15-min reperfusion
Control group (n = 6)				
Mean arterial blood pressure (mmHg)	120 ± 4	80 ± 7	73 ± 4	76 ± 3
Heart rate (bpm)	400 ± 8	368 ± 4	364 ± 10	364 ± 16
VP group (n = 8)				
Mean arterial blood pressure (mmHg)	127 ± 4	79 ± 6	77 ± 5	78 ± 7
Heart rate (bpm)	404 ± 8	360 ± 5	354 ± 9	358 ± 12
GLP-1 group (n = 8)				
Mean arterial blood pressure (mmHg)	125 ± 3	74 ± 7	78 ± 4	75 ± 4
Heart rate (bpm)	406 ± 5	356 ± 9	362 ± 12	354 ± 14

Data are means ± SE.

throughout the experiments (Fig. 4), completely abolished the GLP-1–mediated myocardial protection (57.3 ± 3.8% vs. 26.7 ± 2.7%, $P < 0.001$).

In vitro infarct size and inhibitors of the prosurvival pathways. The cAMP inhibitor Rp-cAMP, administered with GLP-1 + VP throughout the experiments (Fig. 4), abolished the protection (57.5 ± 5.0% vs. 26.7 ± 2.7%, $P < 0.001$). In addition, the protection we observed was also abolished by the PI3K inhibitor LY294002 (43.4 ± 3.9% vs. 26.7 ± 2.7%, $P < 0.05$) and by the p44/42 mitogen-activated protein kinase inhibitor UO126 (48.3 ± 8.6%, $P < 0.01$). None of these antagonists or inhibitors had any effect on infarct size when given alone (data not shown).

Western blot analysis. We found no significant difference in the content of total BAD between control and GLP-1–treated hearts, whereas chemiluminescence demonstrated the presence of phospho-BAD (Ser 136) in GLP-1–treated hearts compared with control hearts (Fig. 5).

DISCUSSION

GLP-1 possesses a number of properties, which makes it a potentially ideal antidiabetic agent (20,21). It also possesses other properties which have the potential for it to exert a direct cardioprotective effect. In this regard and in addition to its incretin actions, GLP-1 has also been shown to reduce pancreatic β-cell apoptosis (22,23). The localization of the GLP-1 receptor in the heart and the demonstration that GLP-1 promotes the activity of PI3K in β-cells (6), a kinase that has been clearly associated with myocardial protection in the setting of ischemic/reperfusion injury (13) as well as preconditioning (14,15), allows one to hypothesize a novel and independent action of GLP-1 in the setting of ischemia/reperfusion.

GLP-1 induces an increased level of cAMP in cardiomyocytes (7), which, in turn, activates protein kinase A. GLP-1 has an antiapoptotic action on insulin-secreting cells mediated by cAMP and PI3K (6). Activation of PI3K leads to the phosphorylation and inactivation of the proapoptotic peptide BAD by causing it to bind to 14-3-3 proteins (24). BAD is a proapoptotic member of the Bcl-2 family that can displace Bax from binding to Bcl-2 and Bcl-xl, resulting in cell death. Our Western blot results confirmed phosphorylation of BAD at serine 136 by GLP-1. Therefore, it is possible that GLP-1 has a direct antiapoptotic effect on cardiac muscle in our model; however, this needs to be explored in greater detail.

Elevated levels of cAMP have previously been thought to be detrimental in ischemic cardiomyocytes. The amount of cAMP produced may play a role in determining divergent signaling pathways that lead to antiapoptotic pathways. The cAMP produced may also be located in particular microdomains, described as compartmentalization, that restrict its actions (25). GLP-1–mediated increases in cAMP (comparable to isoproterenol) failed to cause any inotropic or lusitropic effect (7), supporting the suggestion for such compartmentalization. Compartmentalization of G protein–coupled signaling has been the subject of numerous reports, and it is increasingly recognized that spatiotemporal regulation of protein kinase A activity involves regulation of discrete cAMP pools (26).

GLP-1 has been shown to increase blood pressure and heart rate in rats (9), although we, and others using pigs (27), failed to demonstrate any hemodynamic changes. This may be due to the differences in dose, method of delivery, or species. GLP-1 has been shown to have effects on the central control of blood pressure and pulse (28);

TABLE 2
Rate pressure product analysis of major groups throughout the ischemia/reperfusion protocol

Groups	n	Stabilization	5-min ischemia	30-min ischemia	15-min reperfusion
Control group	9	24,533 ± 3,356	12,340 ± 1,763	13,137 ± 1,549	11,528 ± 2,129
VP group	10	31,450 ± 3,674	19,967 ± 2,278	16,655 ± 1,233	16,365 ± 1,945
GLP-1 group	13	25,469 ± 3,225	16,424 ± 2,362	16,905 ± 2,046	13,246 ± 1,667

Data are means ± SE. The rate pressure product analysis for the in vitro groups is mmHg/bpm.

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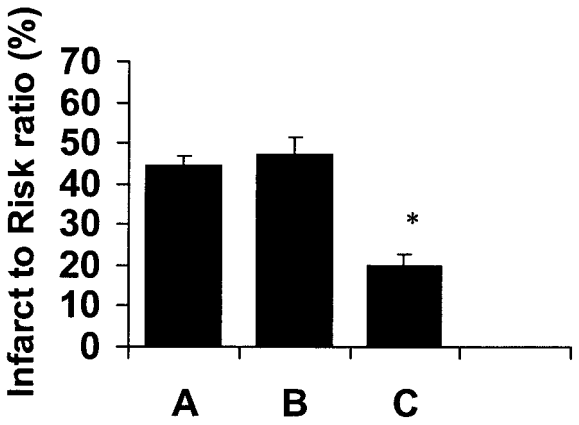

FIG. 2. In vivo myocardial infarct size expressed as a percentage of the risk zone. *A*: Control (n = 6); *B*: VP (n = 8); and *C*: GLP-1 (n = 8). Results are presented as means ± SE (*P < 0.001 vs. control and VP).

however, this central mechanism is excluded in the in vitro setting.

Insulin has been shown to be cardioprotective in animal models, activating PI3K and reducing postischemic myocardial apoptotic death (29,30) as well protecting against acute myocardial infarction in man (31,32). Importantly, the myocardial protection observed in our study is reproduced in both the in vivo and in vitro models; the latter being specifically relevant, as in this setting there is an absence of circulating insulin, implying that the protective effects that we observe may not be a direct consequence of insulin itself.

Initially it was surmised that GLP-1, acting as a potent incretin, could increase levels of insulin, thereby suggesting a possible mechanism to explain our findings in the in vivo study. However, although a rise in insulin and decrease in glucose is possible, it must be remembered that these studies were undertaken in nondiabetic animals that, although not fasted, were not postprandial, suggesting that any GLP-1–mediated stimulation of insulin release is likely to be small because insulinotropic effects of GLP-1 are glucose dependent. Furthermore, as discussed above in our studies using the in vitro–isolated perfused rat heart, we obtained a similar degree of myocardial protection. Any residual insulin in these hearts at the time of harvesting from the rats would be lost from the tissues

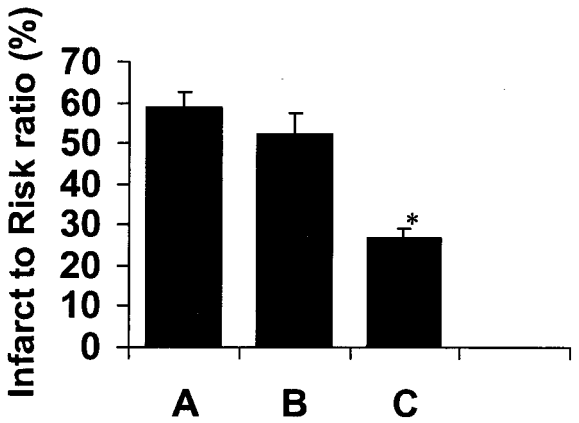

FIG. 3. In vitro myocardial infarct size expressed as a percentage of the risk zone. *A*: Control (n = 9); *B*: VP (n = 10); and *C*: GLP-1 (n = 13). Results are presented as means ± SE (*P < 0.001 vs. control and VP).

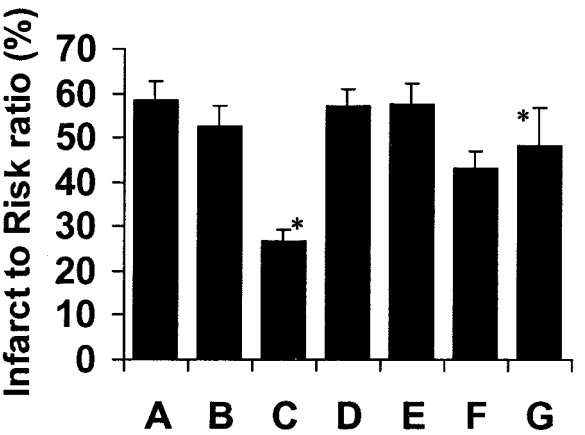

FIG. 4. In vitro myocardial infarct size expressed as a percentage of the risk zone. *A*: Control (n = 9); *B*: VP (n = 10); *C*: GLP-1 (n = 13); *D*: exendin (9-39) and GLP-1 (n = 6); *E*: Rp-cAMP and GLP-1 (n = 6); *F*: LY294002 and GLP-1 (n = 6); and *G*: UO126 and GLP-1 (n = 6). Results are presented as means ± SE (*P < 0.001 vs. all other groups).

during the stabilization period on the Langendorff apparatus.

Recombinant GLP-1 has been shown in a porcine model of myocardial ischemia to prevent the accumulation of pyruvate and lactate but failed to show any decrease in the infarction (33). However, it must be appreciated that in that particular study, no inhibitor of DDPIV was used, and it could be argued that the GLP-1 may have been partly degraded and as such been unable to have a direct effect on the myocardium (as seen by our study), even though insulin and glucose were shown to be modulated by the incretin. Furthermore, the severity of the experimental model, i.e., the use of very long periods of ischemia in a noncollaterised heart may have masked any protective effect that could have been induced by the GLP-1.

Our results demonstrate myocardial protection by GLP-1 when accompanied by an inhibitor of DPPIV VP, which appears to confer no benefit by itself. Antagonism of the GLP-1 receptor by exendin (9-39) appears to also completely inhibit the action of GLP-1 on myocardial preservation, affirming its role as signal transducer for GLP-1–induced cardioprotection. The cAMP inhibitor Rp-cAMP abolished protection, confirming this known GLP-1 pathway as a possible mechanism. The protection was also abolished by the PI3K inhibitor LY294002 and by the p44/42 mitogen-activated protein kinase inhibitor UO126, implicating both these well-demonstrated prosurvival pathways in the cardioprotection mediated by GLP-1. Each of these pathways appears to be essential for the protection afforded by GLP-1, as inhibiting them individually abrogates the protection, suggesting that they may act in parallel.

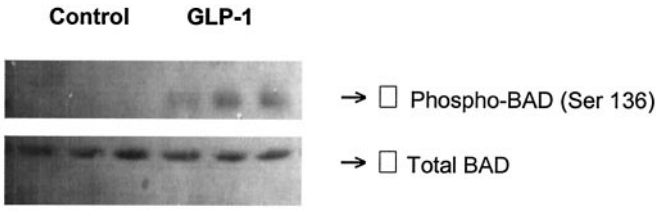

FIG. 5. Western blot showing total BAD and phospho-BAD (Ser 136) in control and GLP-1–treated isolated hearts (n = 3 each).

MPI EXHIBIT 1231 PAGE 4

VP is a prototype DPPIV inhibitor, and GLP-1 analogs are stabilized forms of GLP-1 (optimized for once-daily administration). Both DPPIV inhibitors and GLP-1 agonists are currently of considerable interest as potentially new therapeutic approaches for both the prevention and treatment of type 2 diabetes, a condition characterized by increased risk of acute myocardial infarction (34). DPPIV inhibitors are thought to act via inhibiting the breakdown of intact biologically active versions of both GLP-1 and another incretin hormone, glucose-dependent insulinotropic polypeptide (35,36), and it is therefore interesting to observe that the cardioprotective effect of exogenous GLP-1 was not observed in hearts pretreated with VP alone. It may be speculated that the magnitude of any rise in the intact version of GLP-1 as a result of DPPIV inhibition is insufficient to protect the heart and that pharmacological concentrations of GLP-1, such as those achieved by administration of exogenous GLP-1, are necessary to achieve cardioprotection. Our data suggest, therefore, that GLP-1 analogs may possess an additional benefit (i.e., cardioprotection) that is not shared by DPPIV inhibitors, a property that, if also observed in the human species, may help distinguish between these newly emerging therapeutic approaches. Furthermore, it may be of interest, therefore, to study the effects of stable GLP-1 analogs in future experiments.

In conclusion, we believe our data describe for the first time a cardioprotective effect of GLP-1 in rat heart and the cellular mechanisms of that effect, and, furthermore, provide a new insight into this possible therapeutic potential for GLP-1 agonists, a class of drugs currently undergoing trials in the treatment of type 2 diabetes.

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MPI EXHIBIT 1231 PAGE 5

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MPI EXHIBIT 1231 PAGE 6