Exendin-4 induces myocardial protection through MKK3 and Akt-1 in infarcted hearts

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Du J, Zhang L, Wang Z, Yano N, Zhao YT, Wei L, Dubielecka-Szczerba P, Liu PY, Zhuang S, Qin G, Zhao TC. Exendin-4 induces myocardial protection through MKK3 and Akt-1 in infarcted hearts. Am J Physiol Cell Physiol 310:C270–C283, 2016. First published January 6, 2016; doi:10.1152/ajpcell.00194.2015.—We have demonstrated that glucagon like peptide-1 (GLP-1) protects the heart against ischemic injury. However, the physiological mechanism by which GLP-1 receptor (GLP-1R) initiates cardioprotection remains to be determined. The objective of this study is to elucidate the functional roles of MAPK kinase 3 (MKK3) and Akt-1 in mediating exendin-4-elicited protection in the injured hearts. Adult male myocardial infarction (MI) was created by ligation of the left descending artery. Wild-type, MKK3−/−, Akt-1−/−, and Akt-1−/−; MKK3−/− mice were divided into one of several groups: 1) sham: animals underwent thoracotomy without ligation; 2) MI: animals underwent MI and received a daily dose of intraperitoneal injection of vehicle (saline); 3) MI + exendin-4: infarcted mice received daily injections of exendin-4, a GLP-1R agonist (0.1 mg/kg, ip). Echocardiographic measurements indicate that exendin-4 treatment resulted in the preservation of ventricular function and increases in the survival rate, but these effects were diminished in MKK3−/−, Akt-1−/−, and Akt-1−/−; MKK3−/− mice. Exendin-4 treatments suppressed cardiac hypotrophy and reduced scar size and cardiac interstitial fibrosis, respectively, but these beneficial effects were lost in genetic elimination of MKK3, Akt-1, or Akt-1−/−; MKK3−/− mice. GLP-1R stimulation stimulated angiogenic responses, which were also mitigated by deletion of MKK3 and Akt-1. Exendin-4 treatment increased phosphorylation of MKK3, p38, and Akt-1 at Ser129 but decreased levels of active caspase-3 and cleaved poly (ADP-ribose) polymerase; these proteins were diminished in MKK3−/−, Akt-1−/−, and Akt-1−/−; MKK3−/− mice. These results reveal that exendin-4 treatment improves cardiac function, attenuates cardiac remodeling, and promotes angiogenesis in the infarcted myocardium through MKK3 and Akt-1 pathway.

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GLUCAGON-LIKE PEPTIDE-1 (GLP-1) is a naturally occurring incretin that is implicated in the control of appetite and satiety (23). GLP-1 has been studied extensively in type 2 diabetes as a novel insulinotropic peptide whose actions are dependent on the ambient glucose concentration. GLP-1 acts through the GLP-1 receptor (GLP-1R), a 463-amino acid member of the G protein-coupled receptor superfamily (23). GLP-1 is rapidly cleaved by dipeptidyl-peptidase-4 (DPP4), which results in the generation of largely inactive molecular GLP-19–36 amide and GLP-19–37 forms (14). The majority of GLP-1 leaving the intestinal venous circulation has already been cleaved by DPP4 expressed in capillary surrounding gut L cells, which provides an estimated half-life of 1–2 min for intact GLP-1 in vivo (12). The GLP-1 receptor is widely distributed in tissues, including brain, pancreas, intestine, lung, stomach, and kidney. Recently, multiple GLP-1 receptor agonists with longer duration of effect in vivo have been explored, among which is exendin-4, a 39-amino acid peptide that shares 53% sequence homology with GLP-1, making it a more favorable target for GLP-1 for a therapeutic approach in treating diabetes (16, 22, 26, 40). More importantly, patients with myocardial infarction (MI), metabolic syndrome, and diabetes exhibited a higher prevalence in association with higher risks of deaths and major cardiovascular events. Emerging evidence indicates that the use of GLP-1-based therapies as metabolic modulators in heart disease represents a new therapeutic approach in this important cardiovascular domain (1).

Experimental studies and clinical data demonstrate that infusion of GLP-1 promotes regional and global left ventricular (LV) function recovery in patients with acute MI (23, 25). Recent observation suggests a possible link between the GLP-1R agonist and mitogen-activated protein kinase pathway in initiating cellular signalings (13). Our previous studies showed that activation of p38 protected the heart against ischemia/reperfusion injury (43). We have demonstrated that GLP-1 protected the heart against ischemia/reperfusion injury, which is related to p38 phosphorylation. Noticeably, activation of p38 has been demonstrated to play an essential role in GLP-1-induced protective effects in heart failure (8). Therefore, direct inhibition of p38 by genetic ablation of MAPK kinase 3 (MKK3) will provide...
evidence for the role of p38 in GLP-1R-mediated cardiac protection. Akt-1 pathway is essential in protecting against myocardial loss in ischemic hearts (20). Inhibitors of phosphoinositide-3-kinase (PI3K) abolished the GLP-1-induced infarct size limitation in rat hearts (9, 10). Exendin-4 treatment was found to induce phosphorylation of Akt-1 in mice with dilated cardiomyopathy (36). However, the functional role of Akt-1 and MKK3 in direct linkage with GLP-1R in the protection of ischemic heart remains to be determined.

In the present study, we employed MKK3−/−, Akt−/−, and Akt−/−;MKK3−/− mice to define the effect of exendin-4 treatment on MKK3 and Akt-1 and to further elucidate the functional roles of MKK3 and Akt-1 in GLP-1R stimulation by restoring cardiac function, attenuating interstitial fibrosis, sup-

Fig. 1. Exendin-4 (Ex-4) treatment resulted in increases in phosphorylated Akt-1 (A and C) and phosphorylated MAPK kinase 3 (MKK3) (B and D) in the myocardial infarction (MI). C and D show densitometric analyses of protein levels in each group, and values represent mean ± SE (n = 3/per group). *P < 0.05 vs. sham; #P < 0.05 vs. MI. WT, wild-type.

Fig. 2. Exendin-4 treatment increases Kaplan-Meier survival rate after MI. Percentage survival of sham-operated mice and post-MI mice injected with saline or Exendin-4. A: WT (n = 10–12/per group). B: Akt−/− mice (n = 10–11/per group). C: MKK3−/− (n = 10–11/per group). D: Akt−/−;MKK3−/− mice that underwent sham, vehicle-treated MI, and exendin-4 treatment (n = 9–11/per group). Values represent means ± SE, *P < 0.05 vs. vehicle-treated MI.
pressing cardiac hypertrophy, and promoting angiogenesis in the infarcted heart. Our results reveal that exendin-4 treatment elicits a protective effect through MKK3 and Akt-1 in the infarcted myocardium.

MATERIALS AND METHODS

Animals. Adult C57BL/6 mice were purchased from Charles River Laboratory (Bar Harbor, ME); Akt-1<sup>−/−</sup>, MKK3<sup>−/−</sup>, and Akt-1<sup>−/−</sup>; MKK3<sup>−/−</sup> mice were bred and maintained. Akt-1<sup>−/−</sup>;MKK3<sup>−/−</sup> mice double-transgenic were obtained from breeding between Akt-1<sup>−/−</sup> and MKK3<sup>−/−</sup> mice. All animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

MI and study protocol. The mouse MI model was created as described previously (44). MI was created by ligation of the left anterior descending coronary artery. Mice in the sham group were anesthetized and underwent thoracotomy without coronary ligation. Following MI, exendin-4 at a dose of (0.1 mg/kg) or vehicle (saline, 0.1 ml) was immediately intraperitoneally injected to infarcted mice anesthetized and underwent thoracotomy without coronary ligation. Mice in the sham group were anesthetized with 1.5% isoflurane, and temperature was main-

Histological analysis. Sections (10 μm) were prepared from par-

Table 1. Echocardiographic parameters at baseline between wild-type and transgenic mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>Akt-1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>MKK3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Akt-1&lt;sup&gt;−/−&lt;/sup&gt;;MKK3&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVID&lt;sub&gt;d&lt;/sub&gt;, mm</td>
<td>2.8 ± 0.3</td>
<td>2.9 ± 0.2</td>
<td>2.9 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>LVID&lt;sub&gt;s&lt;/sub&gt;, mm</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>EF, %</td>
<td>73.1 ± 4.6</td>
<td>67.1 ± 6.3</td>
<td>62.4 ± 4.2</td>
<td>69.9 ± 6.2</td>
</tr>
<tr>
<td>FS, %</td>
<td>39.3 ± 3.5</td>
<td>34.5 ± 3.3</td>
<td>36.2 ± 4.1</td>
<td>36.7 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 12 for WT, n = 11 for Akt-1<sup>−/−</sup>, n = 11 for MKK3<sup>−/−</sup>, n = 9 for Akt-1<sup>−/−</sup>;MKK3<sup>−/−</sup>), WT, wild-type; LVID<sub>d</sub> left ventricular internal dimension in diastole; LVID<sub>s</sub>, left ventricular dimension in systole; EF, ejection fraction; FS, fractional shortening.
endothelial cell adhesion molecule-1) (Millipore, Billerica, MA). The total number of vessels from each group was calculated and normalized to the tissue area. The stained numbers of each section were counted in ~10 randomized fields of the tissue sections, which were taken in the middle plane of each heart and contained infarct and border regions.

**Western blot analysis for molecular signaling.** We prepared samples from the myocardium and performed immunoblotting analysis. Proteins (50 μg/lane) were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The blots were incubated with their respective antibodies: phosphorylated and non-phosphorylated rabbit-MKK3 polyclonal, phosphorylated and nonphosphorylated rabbit-Akt-1 polyclonal, phosphorylated and non-phosphorylated p38 polyclonal, p85 subunit of PI3K (Cell Signaling, Danvers, MA), rabbit monoclonal phosphorylated Akt-1, active caspase-3, cleaved poly (ADP-ribose) polymerase (PARP) monoclonal antibodies, and p85 subunit of PI3K (Cell Signaling, Danvers, MA). Treatment groups included: Akt1−/−, M KK3−/−, WT, and MI+Saline. The blots were developed with an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ) and exposed to X-ray film.

**Fig. 4. Echocardiographic measurements of ventricular function.** Echographic measurements of ventricular functional parameter: ejection fraction (EF) (A–D). Representative images are shown as the M-mode short-axis ultrasound among the groups (E). Values represent means ± SE (n = 4–7/per group). **P < 0.01 vs. saline-treated MI in WT mice.
Table 2. FS, LVID;d, and LVID;s in sham and treated mice

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>Sham</th>
<th>MI + Saline</th>
<th>MI + Ex-4</th>
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<tbody>
<tr>
<td>Fractional Shortening, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>39.0 ± 3.0</td>
<td>12.0 ± 2.1</td>
<td>27.0 ± 1.5*</td>
</tr>
<tr>
<td>Akt-1⁻⁻⁻</td>
<td>25.0 ± 2.0</td>
<td>11.4 ± 0.8</td>
<td>15.1 ± 3.3</td>
</tr>
<tr>
<td>MKK3⁻⁻⁻⁻</td>
<td>30.0 ± 1.8</td>
<td>9.0 ± 4.0</td>
<td>14.0 ± 3.0</td>
</tr>
<tr>
<td>Akt-1⁻⁻⁻MKK3⁻⁻⁻⁻</td>
<td>28.5 ± 3.9</td>
<td>9.0 ± 5.5</td>
<td>9.8 ± 4.2</td>
</tr>
</tbody>
</table>

LVID;d, mm

| WT         | 3.1 ± 0.3 | 5.5 ± 0.2 | 4.1 ± 0.2* |
| Akt-1⁻⁻⁻     | 3.1 ± 0.1 | 4.9 ± 0.1 | 5.2 ± 0.3 |
| MKK3⁻⁻⁻⁻     | 3.5 ± 0.3 | 5.5 ± 0.2 | 4.9 ± 0.3 |
| Akt-1⁻⁻⁻MKK3⁻⁻⁻⁻ | 3.4 ± 0.3 | 5.7 ± 0.4 | 5.5 ± 0.3 |

LVID;s, mm

| WT         | 1.8 ± 0.3 | 4.8 ± 0.2 | 2.3 ± 0.6* |
| Akt-1⁻⁻⁻     | 2.1 ± 0.2 | 4.3 ± 0.3 | 3.8 ± 0.1 |
| MKK3⁻⁻⁻⁻     | 2.3 ± 0.3 | 5.0 ± 0.2 | 4.0 ± 0.4 |
| Akt-1⁻⁻⁻MKK3⁻⁻⁻⁻ | 2.8 ± 0.1 | 4.6 ± 0.1 | 4.2 ± 0.6 |

Values are means ± SE (n = 5–8/group). LVID;d and LVID;s were taken at 9 week of post-myocardial infarction (MI) from all groups. *P < 0.01 vs. MI + saline. Ex-4; exendin-4.

Table 3. Heart weight, tibia length, and body weight in WT and treated mice

<table>
<thead>
<tr>
<th>Heart Weight/Tibia Length, mg/mm</th>
<th>Heart Weight/Body Weight, mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
</tr>
<tr>
<td>WT</td>
<td>9.58 ± 0.24</td>
</tr>
<tr>
<td>Akt-1⁻⁻⁻</td>
<td>8.50 ± 0.60</td>
</tr>
<tr>
<td>MKK3⁻⁻⁻⁻</td>
<td>9.70 ± 0.30</td>
</tr>
<tr>
<td>Akt-1⁻⁻⁻MKK3⁻⁻⁻⁻</td>
<td>10.46 ± 2.20</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 3–7/group). *P < 0.05 vs. sham; †P < 0.05 vs. MI + saline.
came indistinguishable between vehicle- and exendin-4-treated groups (Fig. 4, Table 2). There is no difference in heart rates among groups (data not included). Furthermore, treatment of Akt-1\(^{-/-}\);MKK3\(^{-/-}\) mice with exendin-4 did not result in functional improvements, as shown in wild-type mice (Fig. 4, Table 2). Genetic deletion of both MKK3 and Akt-1 did not cause additional ventricular function depression, suggesting that the synergistic effects of MKK3 and Akt-1 may not play an important role in exendin-4 treatment-induced myocardial protection in MI hearts.

Fig. 5. Exendin-4 treatment suppresses cellular hypertrophic response in the infarcted myocardium. A: representative images of wheat germ agglutinin (WGA) staining showing sham, vehicle-treated MI, and exendin-4-treated MI in WT, Akt-1\(^{-/-}\), MKK3\(^{-/-}\), and Akt-1\(^{-/-}\);MKK3\(^{-/-}\) mice. Scale bar = 80 \(\mu\)m. B-D: Quantitative analyses of myocyte cross-sectional area; values represent means ± SE (n = 3–5/per group), *P < 0.05 vs. sham. #P < 0.05 vs. MI + saline.
Fig. 6. Interstitial fibrosis and hydroxyproline contents in MI hearts. A: representative images showing picrosirius red staining in sham, vehicle-treated MI, and exendin-4 treatments in WT, Akt-1−/−, MKK3−/−, and Akt-1−/−;MKK3−/− mice, respectively. B–E: quantitative analysis of myocyte interstitial collagen deposition. F–I: myocardial hydroxyproline from myocardium in sham, vehicle-treated MI, and exendin-4 treatment in WT, Akt-1−/−, MKK3−/−, and Akt-1−/−;MKK3−/− mice, respectively; values represent mean ± SE (n = 3/per group). *P < 0.05 vs. sham; #P < 0.05 vs. MI + saline. Scale bar = 50 μm.
Cardiac hypertrophy and interstitial fibrosis. We measured the heart weight/tibia length and heart weight/body weight ratio to evaluate the hypertrophic response at the organ level. As shown in Table 3, MI resulted in an increase in the heart weight/body weight ratio and heart/tibia ratio compared with sham control animals. Treatment of wild-type infarcted animals with exendin-4 dramatically reduced the heart/tibia ratio and heart weight/body weight ratio. However, deletion of Akt-1 and MKK3 abolished the effects of exendin-4 on the reduction of heart/tibia ratio and heart weight/body weight ratio (Table 3). As shown in Fig. 5A, WGA staining showed that MI led to an increase in the cross-sectional area of cardiomyocytes in all vehicle-treated mice compared with sham-operated groups. Compared with vehicle-treated wild-type MI mice, exendin-4-treated wild-type mice displayed a reduction in cross-sectional area of cardiomyocyte compared with wild-type mice treated with vehicle. However, the anti-hypertrophy effect of exendin-4 was totally lost in Akt-1−/−, MKK3−/−, and/or Akt-1−/−;MKK3−/− mice (Fig. 5, B–E).

The collagen content was significantly increased after MI in all MI mice receiving vehicle treatment compared with sham-operated group (Fig. 6). Compared with vehicle-treated wild-type mice, exendin-4 treatment resulted in a significant reduction of interstitial collagen following MI (Fig. 6, A and B). However, exendin-4 treatment did not show obvious antifibrotic effects in Akt-1−/−, MKK3−/−, and/or Akt-1−/−;MKK3−/− mice following MI (Fig. 6, C–E). As shown in Fig. 6, F–I, exendin-4 treatment resulted in the reduction of hydroxyproline in the
Fig. 8. A–D: phosphorylated MKK3 and Akt-1 following exendin-4 treatment in Akt-1<sup>−/−</sup> mice and MKK3<sup>−/−</sup> mice. A and B: phosphorylated MKK3 in Akt-1<sup>−/−</sup> mice. C and D: phosphorylated Akt-1 in MKK3<sup>−/−</sup> mice. B and D: densitometric analyses of protein levels in each group, and values represent means ± SE (n = 3/per group). *P < 0.05 vs. sham; #P < 0.05 vs. MI + exendin-4. E and F: deletion of MKK3 attenuated p38 phosphorylation in exendin-4-treated myocardium. E: representative blots showing decreased p38 phosphorylation in MKK3<sup>−/−</sup> myocardium that received exendin-4 treatment. F: densitometric analysis of phosphorylated p38, and values represent means ± SE (n = 3/per group); *P < 0.05 vs. sham and MI + exendin-4. The preparation of samples and protocol for detecting proteins is described in detail in MATERIALS AND METHODS. G–J: effect of exendin-4 treatment on phosphoinositide-3-kinase (PI3K)-p85 in Akt-1<sup>−/−</sup> mice. G and H: representative blots showing that exendin-4 treatment increased phosphorylated p85 and p85 and was not affected by ablation of Akt-1, downstream of PI3K. I and J: densitometric analyses of p-p85 and p85 in each group, and values represent means ± SE (n = 3/per group), *P < 0.05 vs. WT sham and MI + exendin-4; ¥P < 0.05 vs. Akt-1<sup>−/−</sup> sham and Akt-1<sup>−/−</sup> MI + exendin-4.
infarcted myocardium in wild-type mice, which was abolished in Akt-1−/−, MKK3−/−, and/or Akt-1−/−;MKK3−/− mice. In addition, exendin-4 treatment reduced the contents of collagen 1α1 (Fig. 7, A–F) and fibronectin (Fig. 7, A–C, and G–J) in the myocardium of wild-type mice (Fig. 7, A–I), but the deletion of Akt-1 and MKK3 abrogated the effect of exendin-4 on reducing collagen 1α1 and fibronectin (Fig. 7, A–I).

**Signaling proteins.** As shown in Fig. 8, A and B, ablation of Akt-1 did not result in a noticeable change in phosphorylated MKK3 following exendin-4 treatment. However, exendin-4 treatment was unable to increase phosphorylated Akt-1 in MKK3−/− mice (Fig. 8, C and D). Additionally, exendin-4 treatment mitigated the magnitude of reduction of the phosphorylated p38, which was absent in MKK3 mice (Fig. 8, E and F). Exendin-4 treatment caused increases in the phosphorylation of p85, but the ablation of Akt-1 did not affect phosphorylated p-85 (Fig. 8, G–J).

**Apoptosis in the infarcted myocardium.** To estimate whether exendin-4 treatment would mitigate the occurrence of apoptosis and death in the infarcted mice, we detected the protein levels of cleaved PARP and active caspase-3 in both noninfarcted remote and border zones of an infarcted myocardium. As shown in Fig. 9, A–D, both cleaved PARP and active caspase-3 increased in all vehicle-treated MI mice compared with sham-operated animals in both noninfarcted remote and border zone infarcted myocardium. The densitometry analyses indicated that exendin-4 treatment dramatically reduced the levels of cleaved PARP and active caspase-3 in the infarcted wild-type mice compared with vehicle-treated mice (Fig. 9, E–L). However, deletion of Akt-1 (Fig. 9, A, B, E, F, I, and J), MKK3 (Fig. 9, C, D, G, H, K, and L), and/or both MKK3 and Akt-1 (Fig. 10, A–F) abrogated the effects of exendin-4 treatment on the suppression of cleaved PARP and active caspase-3. These results suggest that the antiapoptotic effect of exendin-4 treatment is dependent on Akt-1 and MKK3 in the MI heart.

**Angiogenesis in post-MI myocardium.** Vascular endothelial cell (CD31) and vascular α-SMA were utilized to examine microvessels in the infarcted myocardium. Exendin-4 treatment resulted in a robust increase in CD31 (Fig. 11A) and α-SMA positive capillary density (Fig. 11E) in the border area of infarct heart compared with vehicle-treated infarcted wild-type mice. However, the increase in CD31-stained angiogenesis following exendin-4 treatment was not observed in mice deficient of MKK3 and Akt-1 (Fig. 11, B–D). Likewise, there were no significant differences in α-SMA-positive microvessels in MKK3−/−, Akt-1−/−, and Akt-1−/−;MKK3−/− mice following the administration of exendin-4 (Fig. 11, F–H).

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**Fig. 9.** Exendin-4 treatment reduced active caspase-3 and cleaved poly(ADP-ribose)polymerase (PARP) in MI hearts. Representative blots of cleaved PARP and active caspase-3 in remote and border areas in sham, vehicle-treated MI, and exendin-4 treatment in WT, Akt-1−/− (A and B), and MKK3−/− mice (C and D), respectively. E–H: densitometric analysis of active caspase-3 in Akt-1−/− mice (E and F) and MKK3−/− mice (G and H). Cleaved PARP in sham, vehicle-treated MI, and exendin-4 treatment in WT, Akt-1−/− mice (I and J), and MKK3−/− mice (K and L), respectively. Values represent means ± SE (n = 3/per group), *P < 0.05 vs. WT sham; WT MI + exendin-4; #P < 0.05 vs. Akt-1−/− or MKK3−/− sham; &P < 0.05 vs. WT MI + exendin-4.
DISCUSSION

Salient findings. Our study is the first to use genetic and physiological approaches to document that exendin-4 treatment preserves myocardial function and prevents cardiac remodeling through MKK3/Akt-1. The improvement in functional recovery and the decreased remodeling observed by exendin-4 were abolished by genetic elimination of MKK3 and/or Akt-1. Exendin-4 treatment mitigated cardiac hypertrophy and interstitial fibrosis and attenuated scar size, but these effects were abrogated in mice deficient of MKK3 and Akt-1. Furthermore, exendin-4 treatment induced an angiogenic response, but deletion of MKK3 and Akt-1 diminished the angiogenic response induced by exendin-4.

A plethora of experimental data has been generated concerning the role of GLP-1 in diabetes, but very limited evidence has focused on its cardiovascular effects. Both GLP-1(7–36) amide and the GLP-1 receptor agonist, exendin-4, are shown to increase heart rate and blood pressure in both anesthetized and conscious restrained rats. Although the mechanisms are controversial (6, 38), initial observation suggests that GLP-1 has the potential to modulate the cardiovascular system other than lowering glucose level (30). Promising clinical data showed that GLP-1 infusion improved regional and global function in patients with acute myocardial ischemia and severe systolic dysfunction after successful primary angioplasty (33). Sokos et al. (34) reported that a long-term infusion of GLP-1 improves both LV ejection fraction and functional capacity in human patients with advanced heart failure (34). This is in agreement with another pilot study showing that treatment with GLP-1 (7–36) improved LV function in patients with coronary artery disease (28).

The stimulation of GLP signaling with GLP-1 has also been demonstrated to improve cardiac performance in conscious
dogs with dilated cardiomyopathy and rat ischemia/reperfusion injury (24, 42). Evidence from Yellon’s observations suggests that GLP-1 added before ischemia caused a significant reduction of infarct size (10). This is consistent with our observation that exendin-4 treatment remarkably preserved myocardial function improvement in post-MI.

Our previous studies have suggested that activation of p38 is associated with the protection induced by adenosine receptor, histone deacetylase inhibition, and GLP-1 (41, 42). Deletion of MKK3 suppressed the preconditioning effect induced by histone deacetylase inhibition (41). MKK3 overexpression protected the heart against ischemia/reperfusion injury al-

Fig. 11. Exendin-4 treatment promotes angiogenesis in the infarcted myocardium. A–H: quantitative analyses in CD-31- and α-smooth muscle actin (α-SMA)-positive capillary density in WT (A and E), Akt-1−/− (B and F), MKK3−/− (C and G), and Akt-1−/−;MKK3−/− (D and H) mice that underwent different treatments, as described in MATERIALS AND METHODS. Total number of vessels was calculated and normalized to the tissue area. Values are shown as means ± SE (n = 3/per group). *P < 0.05 vs. MI + saline.
though MKK3 was reported to be involved in the early cardio-depressant action of tumor necrosis factor (7, 19). Moreover, it was reported that GLP-1 induced phosphorylation of MKK3/MKK6 in CHO/GLPR and RIN 1046-38 cells (21). In the present study, our data indicated that exendin-4 treatment resulted in a marked increase in phosphorylation of both MKK3 and p38, but the exendin-4-induced improvement in cardiac function was rendered completely absent by deletion of MKK3. These findings further address an involvement of p38 signaling in exendin-4-produced beneficial effect.

Exenatide was demonstrated to reduce myocardial infarction in association with an increase of Akt-1 phosphorylation (35). However, in dilated cardiomyopathy independent of an insulinotropic effect, GLP-1 did not show an increase in the phosphorylation of Akt-1 at Ser473 (8). GLP-1-induced cardioprotection against acute ischemia/reperfusion in the rat was not in association with Akt-1 Ser473 (42). However, in this observation, exendin-4 treatment induced a profound increase in Akt-1 phosphorylation at Ser129; this is a phosphorylation of Akt-1 associated with Akt-1 Ser473 (42). However, in this observation, exendin-4 treatment induced a profound increase in Akt-1 phosphorylation at Ser129; this is a phosphorylation of Akt-1 that was not examined previously in response to GLP-1R stimulation. These studies are well characterized by the observation in which the functional role of Akt-1 depends on the extent to which an activated Akt-1 is induced (32). The discrepancy of GLP-1 on Akt-1 is likely to be associated with the differences in animal models used as well as pharmacological approaches (GLP-1 vs. GLP-1R agonist). GLP-1R agonist caused an increase in the phosphorylation of Akt-1 and induced cardioprotection in MI, which extends our knowledge of the relationship between GLP-1R and Akt-1 in modulating myocardial remodeling. This is consistent with a recent report demonstrating an improved cardiac function in Akt-1 transgenic mice (2). Inhibition of DPP4 increased angiogenic responses in diabetic cardiomyopathy (31). Human mesenchymal stem cells engineered with GLP-1 also activate a proangiogenic signaling pathway in limb ischemia (15). This is in line with a report showing that GLP-1 promotes angiogenesis in human endothelial cells through the Akt pathway (3). In our study, exendin-4 treatment significantly promoted capillary density, but this effect was abolished by the elimination of MKK3 and Akt-1, suggesting that Akt-1 and MKK3 mediate the GLP-1R-mediated-proangiogenic effect. The increased MKK3 phosphorylation following exendin-4 treatments was not attenuated in Akt-1−/− mice. However, exendin-4-induced Akt-1 phosphorylation was lost in MKK3−/− myocardium, indicating that Akt-1 phosphorylation was secondary to MKK3 following exendin-4 treatment. Noticeably, the synergistic effect of MKK3 and Akt-1 was not demonstrated in MI hearts following exendin-4 treatment, which supports a cascade sequence of MKK3 and Akt-1.

Antiapoptosis is regarded as one of the most important cytoprotections among the pleiotropic actions reported for GLP-1 (17, 27). Exendin-4 treatment resulted in a decrease in cleaved PARP and active caspase-3; these decreases were abrogated by deletion of MKK3 and Akt-1. LV ablation-induced heart failure in rats treated with the DPP4 inhibitor, sitagliptin, attenuated cardiac remodeling and cardiomyocyte apoptosis (11). Delivery of GLP-1 encapsulating genetically modified mesenchymal stem cells in a post-MI pig improved LV function and reduced epicardial infarct size, which was associated with increased angiogenesis and an altered remodeling response (37). Exendin-4 and DPP4 attenuated cardiac remodeling in rat diabetic cardiomyopathy by reducing cardiac fibrosis (5). It was reported that GLP-1 and the exendin-4 analog AC3174 prevented cardiac remodeling in post-MI rat heart (18), which is supported by a recent clinical investigation showing that circulating dipeptidyl peptidase-4 activity is well correlated with cardiac dysfunction in human heart failure (11). The present study demonstrated that exendin-4 mitigates the hypertrophic response and prevented the deposition of collagen content, which was abrogated by depletion of MKK3 and Akt-1. However, in a long-term post-MI cardiac remodeling model, vildagliptin, an inhibitor of DPP4, produced no substantial protective effects on cardiac function or preservations in cardiac remodeling (39). This is likely due to the effects of different pharmacological agents and a different animal model. A previous report indicated that a large dose of exendin-4 could still produce protective effects in an isolated GLP-1R−/− mouse heart, implying that a GLP-1R-independent pathway mediates the cardioprotective effects of exendin-4 (4).

Conclusion. The present study is the first to demonstrate that exendin-4 preserves cardiac functional recovery, reduces scar size, and attenuates myocardial remodeling in postinfarcted hearts. The improvement in cardiac function, prevention of myocardial remodeling, and reduction in scar size following exendin-4 treatment are mitigated by depletion of MKK3 and Akt-1. Exendin-4 treatment induces a robust neovascularization in the postinfarcted myocardium, which is dependent on MKK3 and Akt-1. Furthermore, exendin-4 treatment remarkably attenuated apoptotic protein levels, which were also diminished by the elimination of MKK3 and Akt-1. Taken together, our study reveals that exendin-4 treatment-induced cardioprotective effects in the infarcted hearts are dependent on both MKK3 and Akt-1.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: J.D., L.Z., Z.W., and N.Y. performed experiments; J.D., L.Z., Z.W., and N.Y. analyzed data; J.D., L.Z., Z.W., and N.Y. wrote the manuscript; Z.W., P.D.-S., S.Z., G.Q., and T.C.Z. interpreted results of experiments; L.W., P.Y.L., G.Q., and T.C.Z. conception and design of research; L.W. and T.C.Z. edited and revised manuscript; T.C.Z. drafted manuscript; T.C.Z. approved final version of manuscript.

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