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Postconditioning with curaglutide, a novel GLP-1 analog, protects against heart ischemia-reperfusion injury in an isolated rat heart

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ABSTRACT

Aim: GLP-1(7–36)amide (GLP-1) is an intestinal hormone with effects on glucose metabolism and feeding behavior, including insulinotropic, insulinomimetic, glucagonostatic and anorectic actions. In experimental settings, GLP-1 has also been shown to diminish infarct size following heart ischemia-reperfusion. GLP-1 analogs with extended half-lives are continuously being developed against type 2 diabetes mellitus. Of these, only exendin-4 (exenatide, registered as Byetta) has been shown to mimic the infarct size-limiting effect of GLP-1 in a clinically relevant application as a postconditioning agent. The aim of this work was to test, in a postconditioning mode, a novel, proteolysis-resistant GLP-1 analog N-Ac-GLP-1(7–34)amide, herein termed curaglutide, for its cardioprotective ability.
Method: Global ischemia (35 min)-reperfusion (120 min) was applied in isolated, retrogradely perfused rat hearts. Peptides were present for 15 min at the onset of reperfusion. Cardiac function parameters (beats per minute, left ventricle developed and diastolic pressures, rate-pressure product) were measured. Infarct size was determined by 2,3,5-tripehyltetrazolium chloride staining and planimetry.
Results: Curaglutide did not affect any of the functional heart parameters when administered without preceding ischemia. Curaglutide 0.3 nM diminished significantly the postischemic hypercontracture, with no significant effect on the left ventricle developed pressure or rate-pressure product. Infarct size was reduced by curaglutide postconditioning from 24.8% (SEM 2.8, N = 14) to 11.4% (SEM 3.2, N = 8; $P<0.05$). These effects of curaglutide on postischemic hypercontracture and infarct size were similar in magnitude to corresponding effects of GLP-1 receptor agonist exendin-4. The cardioprotective effects of both agents were abolished in the presence of a GLP-1 receptor antagonist exendin(9–39).
Conclusion: Curaglutide is a new, proteolysis-resistant GLP-1 analog with a beneficial effect on reperfusion-injury in an isolated rat heart. Curaglutide was here shown to act through GLP-1 receptors. Based on the present results, more extensive experimental studies in vivo, comparing dose–response characteristics and efficacy of curaglutide and exendin-4 appear warranted.

1. Introduction

Ischemia-reperfusion injury (IRI) is a syndrome affecting the myocardium upon blood flow restoration following a sufficiently long interruption, such as those encountered in a coronary thrombosis or heart surgery. The major components of this syndrome include cardiomyocyte death, myocardial stunning, arrhythmias and no-reflow [1,2]. A large body of experimental research has accumulated aiming to elucidate the pathophysiology of IRI. A major clinically oriented goal of such studies has been to achieve a decreased final infarct size, in view of the well-established correlation between infarct size and a risk of left ventricle systolic dysfunction and/or heart failure [3–5]. Although a number of intervention modes *prior* to the onset of ischemia have proved effective against IRI experimentally [6], the unpredictable timing of that onset renders such approaches of little interest in acute clinical situations. In contrast, pharmacological *postconditioning*, in which a cardioprotective agent gains access to the ischemia-affected myocardium coincidentally with flow restoration, presents a highly relevant intervention from a clinical standpoint. Glucagon-like peptide-1 (GLP-1(7–36)amide), henceforth referred to as GLP-1, has been shown to be effective in limiting infarct size in animal experiments [7,8]. GLP-1 is an incretin hormone with a plasma half-life of 1–2 min, owing to a rapid degradation by a ubiquitous peptidase DPP-IV [9]. DPP-IV-resistant GLP-1 receptor agonists with slower elimination

Abbreviations: GLP-1, GLP-1(7–36)amide glucagon-like peptide-1; IR, ischemia-reperfusion; IRI, ischemia-reperfusion injury; LVS, left ventricle systolic pressure; LVD, left ventricle diastolic pressure; LVDEV, left ventricle developed pressure; BPM, beats per minute; IS, infarct size; AAR, area-at-risk (ischemic); AUC, area-under-the-curve; STEMI, ST-elevation myocardial infarction; PCI, percutaneous coronary intervention.

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kinetics are available, including exendin-4, a constituent of a lizard *Heloderma suspectum* venom [10], as well as several derivatized GLP-1 forms [11]. While GLP-1, exendin-4 and liraglutide have all been shown to have an infarct size-limiting action [7,12–18], only GLP-1 and exendin-4 were tested using postconditioning-type, clinically relevant protocols [13,14,18]. However, unlike exendin-4, GLP-1 was only effective in the presence of a DPP-IV inhibitor [17]. A rapid conversion of GLP-1 to its metabolite GLP-1(9–36) has been demonstrated in isolated mouse hearts [15]. These results indicate that non-degradable GLP-1 analogs will be preferable for a GLP-1-receptor-mediated postconditioning against myocardial IRI. Here we report that a novel, N-terminally blocked and C-terminally truncated, DPP-IV resistant GLP-1 analog [19] (henceforth referred to as curaglutide) results in a limitation of IRI in an isolated rat heart when applied as a postconditioning agent. We also show this curaglutide action to be mediated by GLP-1 receptors.

2. Materials and methods

2.1. Chemicals

Curaglutide was manufactured by Polypeptide Laboratories (San Diego, USA). Exendin-4 and exendin(9–39) were purchased from Bachem AG (Switzerland).

2.2. Animals and experimental procedure

Male Sprague Dawley rats (330 to 370 g, Taconic, Denmark) were used. The animal studies conformed to the Guide for Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–23, revised 1996) and Danish legislation governing animal experimentation, 1987, and were carried out after permission had been granted by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

For anesthesia, a mixture of midazolam (2.5 mg/kg), fluanisone (2.5 mg/kg) and fentanyl citrate (0.08 mg/kg) was administered subcutaneously. Heparin (1000 i.e. per kg) was administered through the femoral vein. The animals were ventilated via a tracheotomy with a mixture of 35% O_2/65% N_2 and the chest cavity was opened. The excised heart was immediately placed in an ice-cold Krebs–Henseleit buffer and was quickly (within 120 s) mounted onto the Langendorff perfusion system (ADInstruments, UK) and perfused with modified Krebs–Henseleit solution (NaCl 118.5 mM, KCl 4.7 mM, $NaHCO_3$ 25.0 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.4 mM, glucose 11.1 mM), equilibrated to pH 7.4 with a gas mixture of 5% CO_2/95% O_2, at 37 °C. The left auricle was removed and a size 7 balloon (ADInstruments) was inserted into the left ventricle through the left atrium. The balloon volume was kept adjusted to a diastolic pressure of 4–10 mm Hg within the first 5 min following insertion, with no further adjustments during the remaining experimental period. Perfusion pressure was set to 70 mm Hg and maintained at this average value by a servo control system (ML175 STH Pump Controller, ADInstruments) with the peristaltic pump revolutions continually adjusted according to flow resistance. The hearts were allowed to stabilize for 20 min prior to recording of left ventricle functional parameter baseline values over the next 10 min: pressure (systolic, LVS; diastolic, LVD; developed, LVDEV = LVS − LVD), beats per minute (BPM) and rate-pressure product (RPP). Power Lab 8/30 system and Chart 5 Pro Software from ADInstruments were used for these recordings.

2.3. Exclusion criteria

Hearts were excluded if the average values for the last 10 min of the stabilization period failed to meet the following criteria: BPM: 210–350 min^{-1}, LVDEV: 80–150 mm Hg, RPP: >22,000 (mm Hg × min^{-1}). Hearts were also excluded if ventricular fibrillation lasting more than 5 min occurred during reperfusion. Based on these criteria, of the total 57 hearts used, 10 were excluded from the study.

2.4. Treatment groups

Fig. 1 outlines the time course for normoxic (A) and ischemia-reperfusion (B) experiments. Total perfusion time was always 185 min, consisting of 30 min stabilization, followed by 155 min of normoxic perfusion (Fig.1A) or 35 min global ischemia followed by 120 min reperfusion (Fig.1B). When present, peptides were added for 15 min, commencing at 35 min of normoxia or immediately after global ischemia. The experimental groups were: control normoxia, no peptide addition; normoxia, curaglutide 0.3 nM; control ischemia-reperfusion (IR), no peptide addition; IR, curaglutide 0.3 nM; IR, exendin-4 0.3 nM; IR, curaglutide 0.3 nM + exendin(9–39) 3 nM; IR, exendin(9–39) 3 nM.

2.5. Determination of infarct size

Following reperfusion, the hearts were processed for 2,3,5-triphenyltetrazolium chloride staining and planimetric infarct size determination, as described earlier [13]. Quantitation was done by an investigator blind to the experimental conditions. Infarct size (IS) was expressed as a percentage of the total ischemic area at risk (AAR) (% IS/AAR).

2.6. Statistical analysis

All values are presented as means, with the SEM given in parentheses. One-way ANOVA with Dunnett's post hoc test (GraphPad Prism® 5) was used to compare treatment results to control conditions. $P<0.05$ was considered significant.

3. Results

3.1. Functional parameters

Baseline parameter values were (N = 14 in all cases): BPM 291 (7) min^{-1}, LVDEV 105.2 (5.4) mm Hg, LVD 8.3 (0.7) mm Hg, RPP 30302 (1434) mm Hg min^{-1}. At the end of normoxic perfusion, the values were (N = 5): BPM 215 (14) min^{-1}, LVDEV 75.2 (8.6) mm Hg, LVD 19.2 (5.2) mm Hg and RPP 16060 (1740) mm Hg min^{-1}. This degree of deterioration of the functional values observed at the end of normoxic perfusion was typical of a standard Langendorff preparation, attributable in part to the use of a crystalloid perfusion buffer with an attendant edema development [20]. The time profiles of these parameters in normoxic experiments were not affected by the presence of curaglutide between 35 and 50 min of perfusion, i.e. during the period corresponding to peptide administration in the ischemia-reperfusion experiments.

The effects of ischemia-reperfusion on LVD, LVDEV and RPP are shown in Fig. 2A–C. LVD rose sharply after flow interruption, declining somewhat toward the end of the ischemic period, and rising sharply again at the onset of reperfusion (Fig. 1A). Peak values were reached

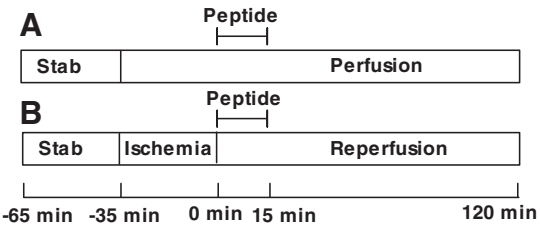

Fig. 1. A scheme illustrating perfusion periods for normoxic perfusion (A) and ischemia-reperfusion (B). "Stab" indicates a 30 min stabilization period. When present, peptides were added for 15 min, from the beginning of the last 120 min of perfusion (A) or reperfusion (B).

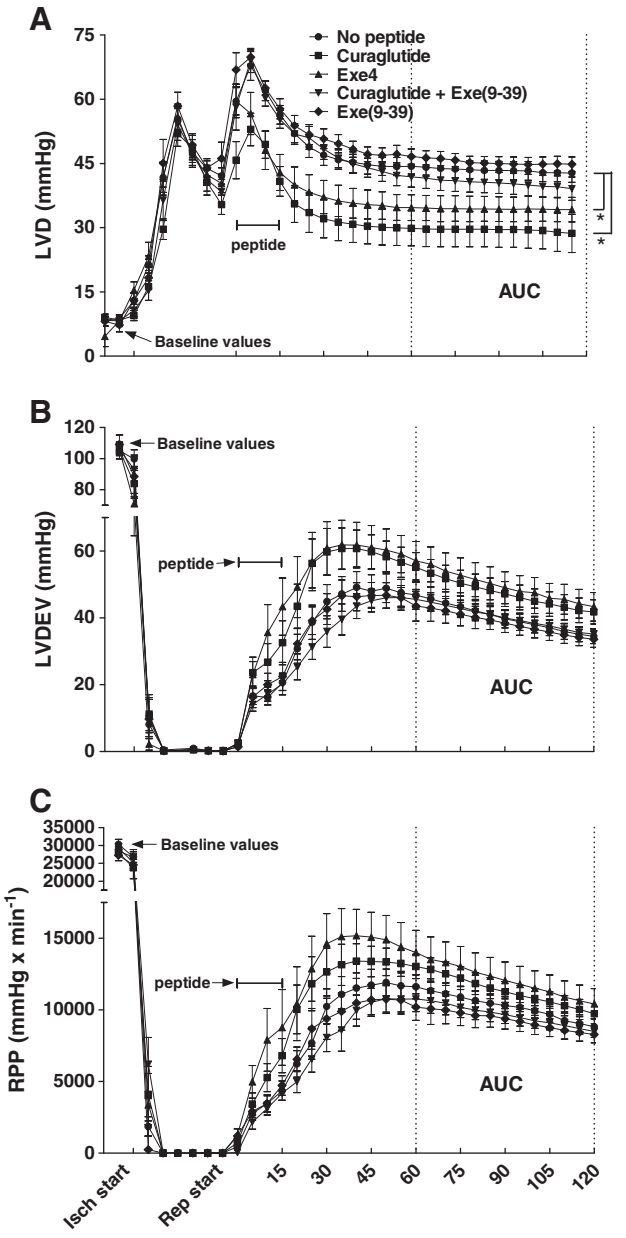

Fig. 2. Time courses of the left ventricle diastolic pressure (LVD) (A), left ventricle developed pressure (LVDEV) (B) and rate-pressure product (RPP) (C) in the ischemia-reperfusion experiments. Points represent means of 7–14 experiments, with bars indicating SEM. Start of the ischemia and reperfusion periods, the period of peptide administration and the period for which the area under-the-curve (AUC) was calculated are indicated. The following treatment groups are indicated by symbols (same symbols in A–C): control ischemia—no peptide present; curaglutide 0.3 nM; Exe-4 (exendin-4) 0.3 nM; curaglutide 0.3 nM + Exe(9–39) (exendin(9–39)) 3 nM; Exe(9–39) 3 nM. Points marked "baseline values" represent means for each group during the last 10 min of the stabilization period. * indicates $P<0.05$ compared to the "no peptide" condition.

some 5–10 min after reperfusion started, declining to a near-plateau approximately 60 min later. Area-under-the-curve (AUC) was used as a time-integrated measure of functional parameter values over the last 60 min of reperfusion. As in our earlier work, this last 60 min period was chosen to secure a complete peptide washout over the preceding 45 min, thus ensuring that any changes of functional parameter values within the analyzed period would reflect postconditioning immediately after ischemia, as opposed to a direct, continuing presence of the peptide in the coronary circulation. In addition, we had previously observed the smallest inter-heart variability in this late reperfusion period. These AUC-values for LVD were significantly decreased compared to control ischemia (Fig. 2A), following postconditioning with either curaglutide or exendin-4. They were not affected either by postconditioning with curaglutide in the presence of GLP-1 receptor antagonist exendin(9–39) [21,22] or when using exendin(9–39) alone (Fig. 2A).

Postconditioning with either curaglutide or exendin-4 did not increase AUC values for LVDEV or RPP significantly (Fig. 2B and C, respectively). A trend towards an LVDEV increase may have been apparent for both peptides.

3.2. Infarct size

In the absence of postconditioning (control ischemia-reperfusion), infarct size was 24.8% (2.8%, N = 14) (Fig. 3). Postconditioning with curaglutide 0.3 nM reduced the infarct size to 11.4% (3.2%, N = 8, $P<0.05$), close to the value 12.6% (3.2%, N = 8, $P<0.05$) obtained when exendin-4 0.3 nM was used in a similar manner. Exendin(9–39) has been shown to abolish infarct-limiting actions of GLP-1 [23] and exendin-4 [13,15]. Postconditioning with curaglutide in the presence of exendin(9–39) resulted in infarct size 21.4% (2.4, N = 8), not different from control ischemia or from the value in the presence of exendin(9–39) alone (21.7%;3.6, N = 9).

4. Discussion

We show here for the first time the cardioprotective effect of a N-Ac-GLP-1(7–34)amide, for which we here propose the name curaglutide. Curaglutide is an N-terminally acetylated, C-terminally truncated analog of GLP-1 (Table 1), synthesized as part of an effort to develop new GLP-1 analogs resistant to DPP-IV-mediated proteolysis [19]. Curaglutide has been shown to be resistant to DPP-IV-catalyzed degradation over 2 h in vitro, under the conditions when the native peptide was proteolyzed with a half-time of 23 min [19]. Here we tested curaglutide for its cardioprotective action in a clinically relevant administration mode as a postconditioning agent.

4.1. Functional parameters

Curaglutide had a beneficial effect both at the level of myocardial performance and infarct size. LVD was the functional parameter affected most strongly, showing a significant decrease following curaglutide postconditioning (Fig. 2A). Importantly, a postischemic LVD increase, or hypercontracture, is one of the chief mechanisms contributing to cardiomyocyte death at reperfusion through mechanical stress leading to sarcolemmal rupture [24,25]. The LVD-lowering effect of curaglutide was equal to that of exendin-4, a naturally occurring, DPP-IV resistant peptide with a 53% sequence identity to GLP-1 [10]. Cardioprotective

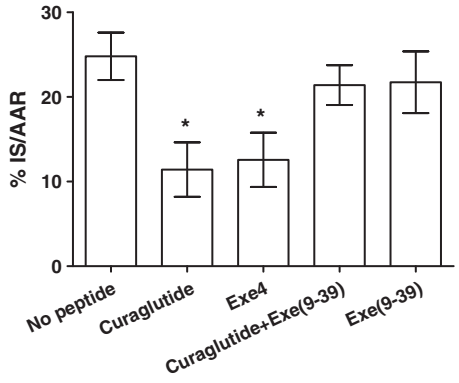

Fig. 3. Effect of curaglutide on infarct size. Columns represent the mean infarct size (N = 7–14) calculated as the percentage of area at risk (%IS/AAR), with bars indicating SEM. Treatment groups designated as in Fig. 2 are indicated. * indicates $P<0.05$ compared to the "no peptide" condition.

Table 1
Amino acid sequences of GLP-1 (GLP-1(7–36)amide) and curaglutide (N-Ac-GLP1(7–34) amide).

Peptide	Sequence	MW_{av} [Da]
GLP-1 (GLP-1(7–36) amide)	HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR-CONH2	3297.7
Curaglutide (N-Ac-GLP-1(7–34) amide)	Ac-HAEGTFTSDVSSYLEGQAAKEFIAWLVK-CONH2	3126.4

MW_{av}, molecular weight.

properties of exendin-4 have earlier been demonstrated by us [13] as well as by others [14–16] in animal models.

4.2. Infarct size

In addition to the hypercontracture-limiting effect of curaglutide early after ischemia, curaglutide postconditioning was associated with a significant decrease in the infarct size as assessed after 2 h of reperfusion (Fig. 3). Similar to the observations concerning the hypercontracture, the magnitudes of the curaglutide-induced and exendin-4-induced infarct limiting effects (a relative decrease of approximately 54%) were comparable (Fig. 3). Infarct size reduction by exendin-4 of a similar relative magnitude (~56%) was observed in our earlier study [13]. It may be noted that in earlier work we obtained somewhat larger control infarcts (approximately 33% of myocardial mass, compared to ~25% here), possibly owing to ischemia duration of 45, rather than 35 min. That difference may in part explain why in the present work, unlike previously, neither curaglutide nor exendin-4 was seen to exert a statistically significant effect on LVDEV (Fig. 2B). (A trend towards LVDEV improvement was apparent, and was abolished in the presence of exendin(9–39) (Fig.2B)). We have shown that any LVDEV improvement due to postconditioning would mostly be derived from a diminished total extent of myocyte death, as opposed to a direct inotropic action through GLP-1 receptors [23]. Thus, any such effect on LVDEV would tend to decrease with a smaller control infarct, as seen here. Consistent with this interpretation, here as well as previously [13,23] we did not observe any inotropic effects of curaglutide during normoxic perfusion. Some of the earlier in vitro studies on GLP-1-mediated limitation of heart reperfusion injury have produced either negative [7] or positive [16] results regarding the GLP-1-mediated improvement of postischemic functional parameters.

4.3. Involvement of GLP-1 receptors and mechanism of cardioprotection

Exendin(9–39) is a well-established antagonist at GLP-1 receptors [21,22], and was seen to abolish the cardioprotective effects of exendin-4 in isolated hearts [23] and in cultured cardiomyocytes [15]. GLP-1 receptors have been demonstrated in the cardiomyocytes and myocardial vasculature [16], consistent with the exendin-4-mediated, exendin(9–39)-sensitive stimulation of cAMP production in cultured cardiomyocytes [15]. In the present work, exendin(9–39) blocked the beneficial effect of curaglutide on the hypercontracture (Fig. 2A) as well as the infarct size (Fig. 3), in parallel with its similar blocking effect on corresponding actions of exendin-4. This data provide a strong pharmacological evidence of curaglutide acting as a GLP-1 receptor agonist. Mechanistic basis of curaglutide-mediated cardioprotection was not addressed in this work. The GLP-1 receptor is a G protein-coupled, class B receptor, known to activate adenylate cyclase-dependent and PI3K-dependent signaling [26]. Curaglutide has been shown to stimulate cAMP production in rat insulinoma cells [19]. Bose et al. demonstrated that both cAMP-dependent and PI3K-dependent pathways were involved in GLP-1-mediated infarct limitation [7], likely promoting mitochondria stabilization (through the inhibition of permeability transition) and inhibition of apoptosis [1,27]. However, in addition to mitochondrial stabilization and/or an antiapoptotic effect, the present results would also be consistent with curaglutide ameliorating cardiomyocyte death through the lessening of postischemic hypercontracture (Fig. 2A). The relative contributions of hypercontracture and severe mitochondrial dysfunction (mitochondrial permeability transition), respectively, to the lethal myocardial injury upon reperfusion are a matter of debate [28]. Recently, evidence has been presented suggesting that the relative importance of these phenomena may depend on ischemia duration, with cell death primarily triggered by the mitochondrial permeability transition being more prevalent at longer ischemia periods [28].

In addition, GLP-1-mediated glucose uptake, GLUT-1 glucose transporter sarcolemmal expression and stimulation of p38 MAP kinase following a low-flow ischemia in an isolated rat heart were reported [29].

4.4. Concluding remarks

This work has demonstrated a heart reperfusion injury-ameliorating effect of curaglutide, a novel, DPP-IV resistant GLP-1 analog. While the family of long-acting GLP-1 analogs for the treatment of type 2 diabetes presently comprises at least five agents [11], among such analogs only exendin-4 has been demonstrated to be effective in a *postconditioning* mode against heart reperfusion injury in experimental settings [13,14]. However, important issues remain regarding the administration of putative postconditioning agents, including optimal dosage, timing and potential adverse effects. For instance, a biphasic dose–response relationship for exendin-4 was found in an ex vivo rat model [13], suggesting a somewhat narrow window of desirable exendin-4 concentrations and complicating any clinical protocol. Exenatide (structurally identical to exendin-4 and registered as Byetta for the treatment of diabetes type 2) has been shown to possess antigenic properties, with 41–49% of patients demonstrating a development of antibodies [30]. Exenatide administration may also be associated with side effects such as nausea or indigestion. Some of these issues might be avoided when using analogs such as curaglutide, which is 100% identical to the human GLP-1(7–34)amide sequence. Thus, continued testing of novel GLP-1 receptor agonists, of which curaglutide is a recent example, remains justified in the context of heart ischemia-reperfusion, with a goal of finding an optimal combination of drug efficacy and clinical applicability.

It should be noted that ex vivo studies such as the present one do not allow the assessment of the long-term effects of postconditioning with GLP-1 receptor agonists. While in vivo, long-term experiments are necessary to address this issue, we have obtained encouraging results in a proof-of-concept patient trial, in which exenatide postconditioning of STEMI patients undergoing primary PCI resulted in an increased myocardial salvage index from 0.62 to 0.71 (a reduction of IS/AAR from 0.39 to 0.30) at 3 months after the intervention [31,32].

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