Structural apelin analogues: mitochondrial ROS inhibition and cardiometabolic protection in myocardial ischaemia reperfusion injury

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BACKGROUND AND PURPOSE
Mitochondria-derived oxidative stress is believed to be crucially involved in cardiac ischaemia reperfusion (I/R) injury, although currently no therapies exist that specifically target mitochondrial reactive oxygen species (ROS) production. The present study was designed to evaluate the potential effects of the structural analogues of apelin-12, an adipocyte-derived peptide, on mitochondrial ROS generation, cardiomyocyte apoptosis, and metabolic and functional recovery to myocardial I/R injury.

EXPERIMENTAL APPROACH
In cultured H9C2 cardiomyoblasts and adult cardiomyocytes, oxidative stress was induced by hypoxia reoxygenation. Isolated rat hearts were subjected to 35 min of global ischaemia and 30 min of reperfusion. Apelin-12, apelin-13 and structural apelin-12 analogues, AI and AII, were infused during 5 min prior to ischaemia.

KEY RESULTS
In cardiac cells, mitochondrial ROS production was inhibited by the structural analogues of apelin, AI and AII, in comparison with the natural peptides, apelin-12 and apelin-13. Treatment of cardiomyocytes with AI and AII decreased cell apoptosis concentration-dependently. In a rat model of I/R injury, pre-ischaemic infusion of AI and AII markedly reduced ROS formation in the myocardial effluent and attenuated cell membrane damage. Prevention of oxidative damage by AI and AII was associated with the improvement of functional and metabolic recovery after I/R in the heart.

CONCLUSIONS AND IMPLICATIONS
These data provide the evidence for the potential of the structural apelin analogues in selective reduction of mitochondrial ROS generation and myocardial apoptosis and form the basis for a promising therapeutic strategy in the treatment of oxidative stress-related heart disease.
ABBREVIATIONS
A12, apelin-12; A13, apelin-13; CF, coronary flow; CO, cardiac output; DMPO, 1H,2H-dimethyl-2,6-dihydroxyphenol; EC, energy charge; EPR, electron paramagnetic resonance; H2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; H/R, hypoxia reoxygenation; I/R, ischaemia reperfusion; KHB, Krebs–Henseleit buffer; LVDP × HR, left ventricular developed pressure–heart rate product; MI, myocardial infarction; MitoPY1, mitochondria peroxyn yellow 1; MitoSOX™ Red, red mitochondrial superoxide indicator; mPTP, mitochondrial permeability transition pore; PCr, phosphocreatine; pGluA13, pyroglutamate form of apelin-13; ROS, reactive oxygen species; ΣAN, total adenine nucleotide pool (ATP + ADP + AMP)

INTRODUCTION
Ischaemic heart disease including myocardial infarction (MI) remains the major cause of mortality in the industrialized countries (Smith, 2001). The excessive generation of reactive oxygen species (ROS) and impaired cellular metabolism are closely linked with increased cell death and cardiac dysfunction (Korge et al., 2008; Circu and Aw, 2010). It is also reported that cardiac ischaemia reperfusion (I/R) is characterized by abnormal levels of adipocyte-derived circulating factors also known as adipokines (Kimura et al., 2009). Changes in the production of adipokines can affect key cellular pathways that serve to maintain metabolic and cardiovascular homeostasis (Schulze et al., 2003; Smith and Yellon, 2011). Although numerous adipokines have been identified (Van de Voorde et al., 2013), the effects of only a few in cardiometabolic ROS regulation have been extensively studied to date.

We have recently demonstrated that adipocyte-produced apelin plays an important role in metabolic and cardiovascular homeostasis (Dray et al., 2008; Pisarenko et al., 2010; Castan-Laurell et al., 2012; Pchejetsk et al., 2012). Apelin acts as an endogenous ligand for the G-protein coupled AP receptor. Both apelin and AP receptors are widely distributed in most tissues including lung, heart, brain, skeletal muscle, kidney and liver. Apelin is produced as a 77 amino acid prepropeptide, which is cleaved to shorter biologically active C-terminal fragments. Apelin-13 (A13), its pyroglutamate form [Pyr]-A13 [pyroglutamate form of apelin-13 (pGluA13)], and apelin-12 (A12) are the most potent apelin peptides corresponding to the sequence 65–77 (Lee et al., 2006; Shin et al., 2013). These peptides possess a high affinity for AP receptors and bioactivity in vivo and cause similar cellular effects. The apelin/AP system plays an important role in protection against I/R injury (Klein and Davenport, 2005; Smith and Yellon, 2011). Recent studies suggest that A13, pGluA13, A12 and, to a lesser extent, apelin-36 reduce infarct size and augment contractile function recovery in the heart of rodents after regional or global ischaemia (Simpkin et al., 2007; Klein and Baxter, 2008; Zeng et al., 2009; Rastaldo et al., 2011). Post-infarct treatment with pGluA13 decreases infarct size and attenuates cardiac tissue injury in rats in vivo (Azizi et al., 2013). Administration of exogenous A12 and A13 exerts potent positive inotropic effects on failing rat cardiac muscle and improves LV systolic function in dogs with advanced cardiac failure (Dai et al., 2006; Wang et al., 2013a).

The beneficial effects of apelin are mediated by multicomponent cell signalling mechanisms that lead to inhibition of the mitochondrial permeability transition pore (mPTP) opening (Klein and Davenport, 2005; Simpkin et al., 2007) and activation of transmembrane Na+/H+ and Ca2+/Na+ exchange (Szkodi et al., 2002). Phosphorylation and activation of endothelial NOS are also implicated in myocardial protection afforded by apelin (Zeng et al., 2009; Rastaldo et al., 2011). We have recently demonstrated that apelin protects rat heart against I/R injury, prevents cardiac ROS-dependent hypertrophy and preserves cardiac function in cardiac remodelling (Foussal et al., 2010; Pisarenko et al., 2010). In contrast, apelin knockout animals exhibit increased myocardial infarction and dramatic suppression of prosurvival pathways resulting in greater cardiac dysfunction (Wang et al., 2013b). In parallel, experiments on APJ-deficient animals show abolition of cardiovascular effects of apelin (Hashimoto et al., 2006). These data indicate that the apelin/AP receptor system is a critical regulator of the myocardial response to I/R injury and may represent a possible drug target. A biological rationale for the potential use of apelin as
Cardiometabolic protection by apelin peptides

Methods

Animals

All animal care and experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the recommendations of the French Accreditation of the Laboratory Animal Care and was approved by the local Centre National de la Recherche Scientifique ethics committee. Studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010). A total of 58 animals were used in the experiments described here. Male Wistar rats weighing 310–340 g were housed in cages in groups of three, maintained at 20–30°C with a natural light–dark cycle. Rats were purchased from Pushchino Laboratory Animal Breeding (Pushchino, Moscow, Russia). All animals had free access to standard pelleted diet and tap water.

Heart isolation and perfusion

Rats were heparinized by i.p. injection (1600 IU·kg−1 body weight) and anaesthetized with urethane (1.3 g·kg−1 body weight). Hearts were excised and immediately placed into ice-cold Krebs–Henseleit bicarbonate buffer (KHB; composition, in mM; NaCl 118, KCl 4.7, CaCl2 3.0, Na2 EDTA 0.5, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25.0 and glucose 11.0; saturated with a mixture of 95% O2 and 5% CO2; pH 7.4 ± 0.1 at 37°C; the buffer was passed through a 5 μm Millipore filter before use) until contraction stopped. The aorta was then cannulated and Langendorff perfusion was performed at a constant pressure equivalent to 80 cm H2O for 15 min. Working perfusion was performed according to a modified method of Neely under constant left atrium pressure and aortic pressure of 20 and 100 cm H2O respectively. A needle was inserted into the left ventricular (LV) cavity to register LV pressure via a Gould Statham P50 transducer, SP 1405 monitor and a Gould Brush SP 2010 recorder (Gould, Oxnard, CA, USA). The contractile function intensity index was calculated as the LV developed pressure–heart rate product (LVDP × HR), where LVDP is the difference between LV systolic and LV end diastolic pressure. Cardiac pump function was assessed by cardiac output (CO), the sum of aortic output and coronary flow (CF).

Experimental design

Hearts were subjected to a preliminary perfusion in the working mode for KHB for 20 min, and the steady-state values of cardiac function and CF were recorded (Figure 1A). Then they were randomly assigned to five groups, as follows.

(1) Control (n = 10). A 5 min Langendorff perfusion with KHB was performed at a constant flow rate of 4 mL·min−1 for 5 min. The hearts were subjected to 35 min of normothermic global ischaemia and reperfused in Langendorff mode at a flow rate of 4 mL·min−1 for 5 min and then in the working mode for the next 25 min.

(2) A13 (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM A13 at a flow rate of 4 mL·min−1 for 5 min before 35 min of normothermic global ischaemia. Subsequent reperfusion protocol was the same as in control.

(3) A12 (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM A12 at a flow rate of 4 mL·min−1 for 5 min before ischaemia. Reperfusion was performed as in control.

(4) Analogue AI (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM AI at a flow rate of 4 mL·min−1 for 5 min prior to ischaemia. Then hearts were reperfused in the same manner as in control.

(5) Analogue AII (n = 10). A 5 min Langendorff perfusion was performed with KHB containing 140 μM AII at a flow rate of 4 mL·min−1 for 5 min before ischaemia. Subsequent reperfusion was the same as in control.

The myocardial effluent was collected in ice-cold tubes during both periods of Langendorff perfusion for immediate assessment of LDH activity. At the end of reperfusion, the hearts were freeze-clamped in liquid nitrogen for metabolite analysis. To determine the initial content of metabolites, the hearts were freeze-clamped in liquid nitrogen after steady state in separate series.

The optimal concentrations of peptides A13, A12, AI and AII were determined in preliminary experiments. Recovery of cardiac function was assessed using the protocol described above with the concentrations of the peptides in KHB of 70, 140 or 280 μM. Better recovery of the majority of cardiac function indices was observed with peptide concentration of 140 μM, which was used in further experiments.

Figure 1B and C illustrates the effects of varying peptide concentration of 140 μM A12 on myocardial ROS generation, structural analogues of A12 on myocardial ROS generation, metabolic status and cell apoptosis in myocardial I/R injury. However, apelin peptides are rapidly cleared from the circulation with a t1/2 of no longer than several minutes because of hydrolysis by various peptidases including ACE2, a carboxypeptidase cleaving the C-terminal phenylalanine residue (Vickers et al., 2002; Japp et al., 2010). In this context, enhancement of the stability of apelin structure by chemical modification could be an option for addressing new therapeutic challenges.
Spin trap measurements in perfusate

5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO), to give a final concentration of 100 mM, was added to aliquots of the coronary effluents collected at the end of the steady state and at 1, 3, 5 and 30 min of the reperfusion. The effluent samples were rapidly frozen and stored in liquid nitrogen until electron paramagnetic resonance (EPR) measurements. Addition of DMPO to the effluent perfusate was used to avoid improvement of cardiac function and CF by the spin trap during reperfusion (Tosaki et al., 1990).

Varian E-109 E X-band electron spin resonance spectrometer (Varian Inc., Palo Alto, CA, USA) was used for registration of EPR spectra of perfusate samples. EPR signals of DMPO spin adducts were recorded in a glass capillary tube at room temperature. Spectrometer settings were the following: magnetic field modulation of 0.1 mT, modulation of frequency of 100 kHz, microwave power of 10 mW and microwave frequency of 9.15 GHz. DMPO–OH spin adduct concentrations were calculated as described previously using a standard solution of the spin label 2,2,6,6-tetramethylpiperidine-N-oxyl.

Tissue processing, metabolite analysis and assay of LDH activity

Frozen tissue was quickly homogenized in cooled 6% perchloric acid (10 mL·g⁻¹) using an Ultra-Turrax T-25 homogenizer (IKA-Labortechnik, Staufen, Germany), and the homogenates were centrifuged at 2500 × g for 10 min at 4°C. The supernatants were then neutralized with 5 M K₂CO₃ to pH 7.4. The protein-free extracts were centrifuged after cooling to remove the precipitated KClO₄. Tissue dry weights were determined by weighing a portion of the pellets after extraction with perchloric acid and drying overnight at 110°C. Concentrations of ATP, ADP, AMP, phosphocreatine (PCr) and lactate in neutralized tissue extracts were determined spectrophotometrically by enzymic methods (Bergmeyer, 1974). LDH activity in the myocardial effluent was measured according to the method of Bergmeyer and Bernt (1974) using pyruvate as substrate.

Cultured rat cardiomyocytes

Rat ventricular myocardial H9C2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells
were cultured in 12-well cell culture plates with DMEM (Gibco, Invitrogen, Cergy-Pontoise, France) containing 10% FBS (Invitrogen), 100 U·mL⁻¹ penicillin and 100 μg·mL⁻¹ streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and were used at less than 80% of confluence.

**Isolation of adult mouse cardiomyocytes**

Cardiomyocytes were isolated from 10-week-old male C57BL/6j mice using enzymatic digestion and mechanical dispersion methods previously described (Zhang et al., 2009). In brief, after retrograde perfusion with Ca²⁺-free Krebs–Ringer buffer (35 mM NaCl, 4.75 mM KCl, 1.19 mM KH₂PO₄, 16 mM Na₂HPO₄, 134 mM sucrose, 25 mM Na₂CO₃, 10 mM glucose, 10 mM HEPES, pH 7.4, with NaOH) and digestion with collagenase solution (Collagenase II, 8 mg·mL⁻¹), the left ventricular myocytes were separated using a fine scalpel and scissors. After gentle trituration, cells were kept in KB solution (10 mM taurine, 70 mM glutamic acid, 25 mM KCl, 10 mM glucose, 10 mM HEPES, pH 7.4, with KOH) and studied within 6 h at room temperature.

**Measurement of ROS formation in cardiomyocytes**

The cellular levels of ROS were determined in isolated cardiomyocytes or H9C2 subjected to hypoxia (1% O₂, 5% CO₂) for 2 h followed by 5 min of reoxygenation (95% O₂, 5% CO₂) using H₂DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate] and red mitochondrial superoxide indicator (MitoSOX™ red). In brief, cells were washed once with 37°C HBSS/Ca/Mg buffer and incubated in 5 μM carboxy-H₂DCFDA, or 1 μM MitoSOX red for 30 min at 37°C followed by three washes with 37°C HBSS/Ca/Mg buffer. The fluorescence was then measured at the excitation wavelength of 488 nm for carboxy-H₂DCFDA and 505 nm for MitoSOX. An emission wavelength of 535 nm was used for MitoSOX. In each case, three randomly selected fields in each well were selected for examination. Levels of mitochondrial H₂O₂ were detected by measuring the intensity of the activated mitochondria peroxy yellow 1 (MitoPY1), a novel fluorescent probe for imaging H₂O₂ specifically within the mitochondria of living cells (Dickinson and Chang, 2008).

**Apoptosis**

TUNEL assays were performed using the kit manufacturer’s protocol (Promega, Lyon, France) and as previously described (Bianchi et al., 2005). H9C2 cells were exposed to hypoxia for 16 h and fixed in 2% paraformaldehyde at −20°C. Fixed cells were incubated with TUNEL reaction mixture (terminal deoxynucleotidyl transferase and nucleotide mixture) for 1 h at 37°C. The results were expressed as the mean percentage of TUNEL-positive cells.

**Data analysis**

All data are presented as means ± SEM. Comparison of multiple groups was performed by two-way ANOVA followed by a Bonferroni’s post hoc test for in vivo studies using GraphPad Prism version 4.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA). All other statistical analyses were performed by one-way ANOVA followed by the Bonferroni post hoc test. Statistical significance was defined as P < 0.05.

**Materials**

The analogues of apelin, Al and AlII, and A12 were synthesized by the automatic solid phase method using a 431A peptide synthesizer (Applied Biosystems, Berlingen, Germany) and Fmoc technology. In order to increase the chemical stability of A12 and its resistance to aminopeptidase cleavage, for analogue I (AI), we replaced the oxidized methionine by norleucine and included N-terminal α-methylarginine residue and included N-Methylarginine residue in the N-terminus of the peptide. For analogue II (AlII), the N-terminal arginine residue was methylated, the methionine residue was changed to l-norleucine and the C-terminal phenylalanine was amidated. They were purified by preparative HPLC and identified by 1H-NMR spectroscopy and mass spectrometry (Sidorova et al., 2012). The peptide pGuA13 was a commercial product (Phoenix Pharmaceutical, Belmont, CA, USA). The structure of peptides utilized in our experiments is presented in Table 1. Other enzymes and chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Solutions were prepared using deionized water (Milli-pore Corp., Bedford, MA, USA).

**Results**

**Recovery of cardiac function of isolated perfused rat heart**

Effects of A13, A12, Al or AlII infusion before global ischaemia on functional recovery of rat isolated perfused hearts at the end of reperfusion are shown in Table 2. In control, recovery of CF, LVDP × HR product and CO was 76, 46 and 59% as...
The metabolic state of isolated rat hearts after reperfusion

Changes in the myocardial metabolite contents at the end of reperfusion are compared with the steady-state values in Table 3. The control group exhibited a dramatic decrease in ATP content (to 31% of the pre-ischaemic value) and a profound build-up of ADP and AMP. The total adenine nucleotide pool (ATP + ADP + AMP) (ΣAN) and the energy charge (EC) of post-ischaemic cardiomyocytes were reduced by almost 40% of the steady-state values. PCr content was less than 50% of the steady-state value, while myocardial lactate was four times higher. These shifts reflected a poor recovery of aerobic metabolism in reperfused hearts.

Pre-ischaemic infusion of peptides A13, A12, Al or All reduced myocardial lactate content to the initial value. This effect was combined with enhanced restoration of ATP and the EC in reperfused hearts. All peptides improved preservation of the ΣAN pool compared with control; in case of analogue Al this effect was statistically significant. Analogues Al and All preserved ATP and the EC in reperfused hearts better than peptide A13. Additionally, analogue Al significantly improved ATP restoration compared with peptide A12. The peptides did not significantly affect the recovery of PCr or preservation of the ΣCr pool in reperfused hearts compared with control. These data suggest that these apelin peptides compared with the steady-state values, respectively; LV diastolic pressure was significantly higher than the initial value (10 ± 1 and –3 ± 1 mmHg respectively). Infusion of peptides A13, A12, Al or All enhanced recovery of LVDP × HR product, CO and CF on average 1.6, 2.2 and 1.2 times compared with control respectively. These effects were accompanied by a reduction of LV diastolic pressure to 3 ± 1 mmHg at the end of reperfusion, thus suggesting attenuation of reperfusion contracture. No significant differences were found in the effects of the peptides on recovery of cardiac function indices or CF.

Table 2
Effects of infusion of 140 μM A13, A12, Al or All before global ischaemia on recovery of cardiac function and coronary flow of rat isolated perfused heart at the end of reperfusion

| Coronary flow (mL·min⁻¹) | LVDP × HR (mmHg·min⁻¹) | CO (mL) | LV diastolic pressure (mmHg) |
|--------------------------|------------------------|---------|-----------------------------|
| **Steady state**         | 17 ± 2*                | 30 897 ± 511* | 22 ± 2* | –3 ± 1* |
| Control                  | 13 ± 1*                | 14 252 ± 588* | 13 ± 1* | 10 ± 1* |
| A13                      | 16 ± 1*                | 22 387 ± 723* | 29 ± 1* | 3 ± 1*  |
| A12                      | 15 ± 1                | 22 032 ± 934* | 28 ± 2* | 3 ± 1*  |
| Al                       | 16 ± 1*                | 22 493 ± 876* | 29 ± 9* | 3 ± 1*  |
| All                      | 16 ± 1*                | 22 797 ± 866* | 30 ± 1* | 3 ± 1*  |

The values are expressed as means ± SEM for 10 experiments. *P < 0.05, significantly different from steady state; †P < 0.05, significantly different from control.

Table 3
Effects of infusion of 140 μM A13, A12 and analogues Al and All before global ischaemia on myocardial metabolite contents (in μmol·g⁻¹ dry weight) and EC in rat heart at the end of reperfusion

| Metabolite | Steady state | Control | A13 | A12 | Al | All |
|------------|-------------|---------|-----|-----|----|-----|
| ATP        | 22.42 ± 2.06 | 7.04 ± 0.92* | 10.39 ± 1.08** | 12.10 ± 0.57*** | 14.32 ± 0.78**** | 14.59 ± 1.03***** |
| ADP        | 2.80 ± 0.12  | 6.13 ± 0.44* | 5.91 ± 0.36*  | 5.66 ± 0.22*  | 5.02 ± 0.32**  | 6.52 ± 0.39*  |
| AMP        | 0.71 ± 0.01  | 3.73 ± 0.43* | 3.07 ± 0.77*  | 1.96 ± 0.07*  | 1.80 ± 0.45*  | 2.46 ± 0.39*  |
| ΣAN        | 25.93 ± 1.45 | 16.90 ± 0.99* | 19.36 ± 1.44* | 19.73 ± 0.63* | 21.14 ± 1.19 | 23.56 ± 0.95* |
| EC         | 0.91 ± 0.02  | 0.58 ± 0.03* | 0.69 ± 0.03**  | 0.76 ± 0.01*** | 0.80 ± 0.02*** | 0.75 ± 0.02*** |
| PCr        | 24.30 ± 2.30 | 12.69 ± 1.59* | 13.26 ± 1.63* | 15.48 ± 1.17* | 15.85 ± 1.77* | 15.20 ± 1.41* |
| Cr         | 34.96 ± 2.13 | 45.23 ± 2.55* | 46.02 ± 4.32* | 44.89 ± 2.14* | 43.68 ± 3.66 | 44.77 ± 3.89* |
| ΣCr        | 59.26 ± 1.87 | 57.92 ± 1.98 | 58.68 ± 3.16 | 59.65 ± 1.56 | 59.53 ± 3.31 | 60.64 ± 3.03 |
| Lactate    | 1.72 ± 0.19  | 6.93 ± 1.29* | 1.91 ± 0.44*  | 1.67 ± 0.38*  | 1.86 ± 0.48*  | 1.47 ± 0.24*  |

Values are the mean ± SEM of eight experiments. *P < 0.05, significantly different from steady state; †P < 0.05, significantly different from control; ‡P < 0.05, significantly different from A13. ΣAN = ATP + ADP + AMP. ΣCr = PCr + Cr. The EC = (ATP + 0.5ADP)/ΣAN.
enhanced ATP formation in reperfused hearts, stimulating glucose utilization.

**DMPO–OH adduct formation in coronary effluent of isolated perfused rat heart**

The effluent DMPO–OH concentrations did not differ significantly between the groups before ischaemia (Figure 2A), thus indicating that infusion of peptides does not initiate ROS formation. A significant increase in DMPO–OH concentration was observed at the first, third and fifth minutes of reperfusion in the control group, compared with the steady-state value. Pre-ischaemic administration of A13, A12, AI or AII decreased DMPO–OH formation during reperfusion compared with control. This effect was statistically significant for all peptides at the third and fifth minutes of reperfusion. In this case, DMPO–OH concentrations did not differ from the pre-ischaemic values. The DMPO–OH adduct could be formed from a decomposition of the superoxide radical adduct DMPO–OOH or from direct trapping of OH radicals generated in the Haber–Weiss and the Fenton reactions (Tosaki et al., 1990). Thus the detection of DMPO–OH adduct in the coronary effluent did not directly reflect ROS formation in the heart and the data obtained indicated that the peptides reduced the release of ROS-generating systems and hydrogen peroxide from myocardial tissue.

**Effects of peptides on LDH leakage from isolated perfused rat heart**

The ability of A13, A12, AI and AII to reduce cell membrane damage was assessed by changes in LDH release into the myocardial effluent before and after global ischaemia (Figure 2B). LDH leakage did not differ significantly between the peptide groups and control for 5 min of perfusion before ischaemia. Therefore, infusion of peptides by itself did not...
cause damage to the sarcolemma of non-ischaemic cardiomyocytes. The release of LDH for 5 min of reperfusion in control increased by more than twofold compared with the value before ischaemia, indicating I/R membrane damage. The post-ischaemic LDH leakage was significantly attenuated in all peptide groups compared with control suggesting fewer membrane defects.

Apoptosis
In order to determine whether apelin analogues affect cell death in pathological situation, we examined the effects of A12, AI and AII on hypoxia-induced apoptosis in H9C2 cells using the TUNEL labelling. Control H9C2 cells showed spontaneous apoptosis (7.3 ± 0.5%) when examined in normoxic conditions (Figure 3). When subjected to hypoxia for 16 h, significant apoptosis (37.5 ± 2.8%) was observed. As shown in Figure 4, apoptotic cells had classic condensation and fragmentation of their nuclei. Assessment of TUNEL-positive cells with A12, AI and AII revealed a dose-dependent decrease in the number of apoptotic H9C2 cells (Figure 3B). As shown in Figure 4, the anti-apoptotic effects of AI and AII at concentrations of 10^-6 to 10^-7 M were comparable with those of A12 (10^-7 M) and pGluA13 (10^-7 M).

Effects of peptides on ROS formation in cardiomyocytes
To test whether apelin analogues regulate ROS homeostasis in pathophysiological conditions, we measured the effects of
pGluA13, A12, AI and AII on ROS generation in cells subjected to hypoxia reoxygenation (H/R) using different fluorescent probes. Analysis of dichlorodihydrofluorescein-detectable ROS revealed an increase in fluorescence intensity in cultured adult mouse cardiomyocytes subjected to H/R (Figure 4A and B). Treatment of cardiomyocytes with A12, AI and AII markedly decreased H/R-induced ROS generation. As a positive control pGluA13 (10^{-7} M) was used. Consistent with these results, confocal microscopic imaging demonstrated significantly decreased fluorescence intensity of MitoSOX Red in A12-, AI- and AII-treated H9C2 cells (Figure 5). In addition, as shown in Figure 6, both analogues AI and AII markedly decreased H/R-induced ROS generation. As a positive control pGluA13 (10^{-7} M) was used. Consistent with these results, confocal microscopic imaging demonstrated significantly decreased fluorescence intensity of MitoSOX Red in A12-, AI- and AII-treated H9C2 cells (Figure 5). In addition, as shown in Figure 6, both analogues AI and AII markedly decreased H/R-induced ROS generation.

Discussion

Myocardial ischaemia and reperfusion involves complex pathophysiological events that lead to increased oxidative stress, cardiometabolic dysfunction and cell apoptosis (Turer and Hill, 2010). Because mitochondria are key regulators for energy metabolism, ROS production and cell fate, targeting mitochondria might be a promising strategy for selective protection of cardiac cells in limiting myocardial I/R injury. In the present study, we show for the first time that the physiologically active, native peptides pGluA13 and A12 decreased mitochondrial ROS generation in cardiomyocytes subjected to hypoxia and reoxygenation. In addition, pre-ischaemic infusion of A13 and A12 reduced functional and metabolic responses of isolated perfused heart to global ischaemia and reperfusion. These beneficial effects were accompanied by decreased formation of the hydroxyl radical adduct DMPO–OH and decreased LDH leakage in the myocardial effluent during the early reperfusion. We also explored the action of the novel structural analogues of A12 on mitochondrial ROS production, cardiomyocyte apoptosis and myocardial metabolic status. Our results confirm the potent inhibitory activity of the A12 analogues against mitochondrial ROS formation, ATP depletion and apoptotic cell death. These experimental data indicate the therapeutic potential of A12 analogues in attenuating oxidative and metabolic damage induced by myocardial I/R injury.
The present results agreed with the earlier reports in cell culture and animal models where peptides A13, pGuA13 and A12 increased cellular survival in different models of I/R injury (Simpkin et al., 2007; Zeng et al., 2009; Zhang et al., 2009; Azizi et al., 2013). One of the principal findings of our study is that enhanced cardiomyocyte viability afforded by natural apelins was associated with decreased generation of short-lived mitochondrial ROS. This phenomenon may be related to an increase in enzymatic antioxidant activity induced by the peptides or to their direct antioxidant action. Indeed, we have previously demonstrated that pGuA13 treatment enhances myocardial catalase activity and prevents oxidative stress-linked hypertrophy in cardiomyocytes and pressure overload-induced cardiac remodelling (Foussal et al., 2010). In a recent study, we have observed stimulation of Cu,Zn superoxide dismutase (SOD), catalase and GSH peroxidase (GSH-Px) activities by A12 in rat isolated heart during reperfusion (Pisarenko et al., 2014). However, reasons for the up-regulation of antioxidant enzymes by apelin peptides remain unexplored. In this respect, the only finding was the enhancement of catalase mRNA expression induced by pGuA13 in cultured rat cardiomyocytes (Foussal et al., 2010). Further studies are needed to evaluate the ability of these peptides per se to scavenge ROS or affect catalytic activity of antioxidant enzymes.

The potential mechanisms of reducing mitochondrial ROS production by apelin peptides are closely related to up-regulation of antioxidant enzymes. Although mitochondria express protective antioxidants, the oxidation of proteins and the accumulation of DNA lesions may contribute to the
 angiogenesis and decreasing permeability of microvascular protective role in ischaemic myocardium through promoting autocrine or paracrine factors, apelin analogues may play a H2O2 formation. Another possibility involves mobilization of may be of critical importance for reducing lipid peroxides and mitochondrial superoxide formation may be of critical importance for reducing lipid peroxides and Mn SOD activity of several nuclear transcription factors such as nuclear respiratory factors 1 and 2 and activates the genes involved in the respiratory chain, mitochondrial import machinery and transcription factors of mtDNA. Precisely because of this, apelins may regulate the induction of antioxidant defences including SODs, catalase and GSH-Px, and subsequently lead to cell death in the form of apoptosis or necrosis opening and the release of proapoptotic proteins, and subsequently lead to cell death in the form of apoptosis or necrosis opening and the release of proapoptotic proteins, and consequently have a longer half-life in the circulation, along with better storage stability (Sidorova et al., 2012). We have evaluated proteolytic stability of analogues A1 and AII using 3H-NMR techniques in model experiments with human plasma. Stability of both analogues was on average 15 times greater than that of the natural peptides A12 and A13 at the same incubation conditions (unpublished data). In accordance with this, we assume that the t1/2 of analogue A1 or AII in blood circulation may be prolonged up to 1 h. We therefore propose that chemical modification of C-terminal fragments of apelin is a useful approach to design new therapeutic tools for the treatment of cardiovascular diseases.

In conclusion, the results of this study provide the first evidence that structural analogues of apelin attenuate excessive mitochondrial ROS production and preserve myocardial metabolic status in cultured cardiomyocytes and rat isolated heart models of ischaemia and reperfusion. Further, we show that a selective inhibition of mitochondrial ROS formation by apelin analogues is associated with inhibition of apoptosis, reduction of cell membrane damage and improvement of cardiac function. Thus, these peptides can be considered as prototypes of drugs to regulate the mitochondrial oxidative stress, apoptosis and metabolic status in ischaemic heart disease.

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Author contributions

O. P., A. P., P. V. and O. K. conceived and designed the experiments. V. S., I. S., V. P., A. T. and O. K. performed the experiments. O. P., V. S., R. A. and O. K. analyzed the data. R. A. and I. S. contributed reagents/materials/analysis tools. O. K. and O. P. wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest.
References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013a). The Concise Guide to PHARMACOLOGY 2013/14: G Protein-Coupled Receptors. Br J Pharmacol 170: 1459–1581.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013b). The Concise Guide to PHARMACOLOGY 2013/14: Transports. Br J Pharmacol 170: 1706–1796.

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M et al. (2013c). The Concise Guide to PHARMACOLOGY 2013/14: Enzymes. Br J Pharmacol 170: 1797–1867.

Ames BN, Shigenaga MK, Hagen TM (1995). Mitochondrial decay in aging. Biochim Biophys Acta 1271: 165–170.

Azizi Y, Faghihi M, Imani A, Roghani M, Nazari A (2013). Post-infarct treatment with [Pyr1]-apelin-13 reduces myocardial damage through reduction of oxidative injury and nitric oxide enhancement in the rat model of myocardial infarction. Peptides 46: 76–82.

Baines CP (2011). The mitochondrial permeability transition pore and the cardiac necrotic program. Pediatr Cardiol 32: 258–262.

Bergmeyer HU (1974). Methods of Enzymatic Analysis. Academic Press: New York, pp. 1510–2129.

Bergmeyer HU, Bernt E (1974). Lactate dehydrogenase. UV-assay with pyruvate and NADH. In: Bergmeyer HU (ed.). Methods of Enzymatic Analysis. Academic Press: New York, pp. 574–578.

Bianchi P, Kunduzova O, Masini E, Cambon C, Bani D, Raimondi L et al. (2005). Oxidative stress by monoamine oxidase mediates receptor-independent cardiomyocyte apoptosis by serotonin and postischemic myocardial injury. Circulation 112: 3297–3305.

Castan-Laurell I, Dray C, Knauf C, Kunduzova O, Valet P (2012). Apelin, a promising target for type 2 diabetes treatment? Trends Endocrinol Metab 23: 234–241.

Chen JK, Chow SE (2005). Antioxidants and myocardial ischemia: reperfusion injuries. Chang Gung Med J 28: 369–377.

Circu ML, Aw TY (2010). Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic Biol Med 48: 749–762.

Dai T, Ramirez-Correa G, Gao WD (2006). Apelin increases contractility in failing cardiac muscle. Eur J Pharmacol 553: 222–228.

Dickinson BC, Chang CJ (2008). A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells. J Am Chem Soc 130: 11561–11562.

Dray C, Knauf C, Daviaud D, Waget A, Boucher J, Buleon M et al. (2008). Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. Cell Metab 8: 437–445.

Foussal C, Lairez O, Calise D, Pathak A, Guilbeau- Frugier C, Valet PH et al. (2010). Activation of catalase by apelin prevents oxidative stress-linked cardiac hypertrophy. FEBS Lett 584: 2363–2370.

Hashimoto T, Kihara M, Ishida J, Imai N, Yoshida S, Toya Y et al. (2006). Apelin stimulates myosin light chain phosphorylation in vascular smooth muscle cells. Arter Thromb Vasc Biol 26: 1267–1272.

Japp AG, Cruden NL, Barnes G, van Gemeren N, Mathews J, Adamson J et al. (2010). Acute cardiovascular effects of apelin in humans: potential role in patients with chronic heart failure. Circulation 121: 1818–1827.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kimura K, Miura S, Iwata A, Sugihara M, Arimura T, Nishikawa H et al. (2009). Association between cardiac function and metabolic factors including adiponectin in patients with acute myocardial infarction. J Cardiol 53: 65–71.

Kleinz MJ, Baxter GF (2008). Apelin reduces myocardial reperfusion injury independently of PI3K/Akt and P70S6 kinase. Regul Pept 46: 271–277.

Kleinz MJ, Davenport AP (2005). Emerging roles of apelin in biology and medicine. Pharmacol Therap 107: 198–211.

Korge P, Ping P, Weiss JN (2008). Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circ Res 103: 873–880.

Lee DK, George SR, O’Dowd BF (2006). Unravelling the roles of the apelin system: prospective therapeutic applications in heart failure and obesity. Trends Pharmacol Sci 27: 190–194.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Bunenman OP et al.; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucleic Acids Res 42 (Database Issue): D1098–D1106.

Pchejetski D, Foussal C, Alfarano C, Lairez O, Calise D, Guilbeau-Frugier C et al. (2012). Apelin prevents cardiac fibroblast activation and collagen production through inhibition of sphingosine kinase 1. Eur Heart J 33: 2360–2369.

Pisarenko OI, Shulzhenko VS, Pelogeykina YA, Studneva IM, Khatri VN, Dvoryantsev SN (2012). Synthesis and cardioprotective analogues of apelin 12 in acute myocardial infarction in rats. J Pharmacol Pharmacother 4: 198–203.

Pisarenko OI, Lankin VZ, Konovalova GG, Serebryakova LI, Shulzhenko VS, Timoshin AA et al. (2014). Apelin-12 and its structural analogue enhance antioxidant defense in experimental myocardial ischemia and reperfusion. Mol Cell Biochem 391: 241–250.

Rastaldo R, Cappello S, Folino A, Berta GN, Sprio AE, Losano G et al. (2011). Apelin-13 limits infarct size and improves cardiac postischemic mechanical recovery only if given after ischemia. Am J Physiol Heart Circ Physiol 300: H2308–H2315.

Schulze PC, Kratzsch J, Linke A, Schoene N, Adams V, Gielen S et al. (2003). Elevated serum levels of leptin and soluble leptin receptor in patients with advanced chronic heart failure. Eur J Heart Fail 5: 33–40.

Shin K, Pandey A, Liu XQ, Anini Y, Jan K, Rainey JK (2013). Preferential apelin-13 production by the proprotein convertase PCSK3 is implicated in obesity. FEMS Bio Bio 3: 328–333.

Sidorenko MV, Az’muko AA, Pa’ikeeva ME, Molokoedov AS, Bushuev VN, Dvoryantsev SN et al. (2012). Synthesis and cardioprotective properties of apelin-12 and its structural analogues. Russ J Bioorg Chem 38: 40–45.

Simpkin JC, Yellon DM, Davidson SM, Lim SY, Wynne AM, Smith CC (2007). Apelin-13 and apelin-36 exhibit direct cardioprotective activity against ischemia-reperfusion injury. Basic Res Cardiol 102: 518–528.

Smith CC, Yellon DM (2011). Adipocytokines, cardiovascular pathophysiology and myocardial protection. Pharmacol Ther 129: 206–219.
Smith SC (2001). Primary and secondary prevention of heart disease: can we curb the global epidemic? Trans Am Clin Climatol Assoc 112: 89–95.

Szokodi I, Tavi P, Foldes G, Voutilainen-Myllyla S, Ilves M, Tokola H et al. (2002). Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. Circ Res 91: 434–440.

Tempel D, de Boer M, van Deel ED, Haasdijk RA, Duncker DJ, Cheng C et al. (2012). Apelin enhances cardiac neovascularization after myocardial infarction by recruiting apln+ circulating cells. Circ Res 111: 585–598.

Than A, Zhang X, Leow MK, Poh CL, Chong SK, Chen P (2014). Apelin attenuates oxidative stress in human adipocytes. J Biol Chem 28: 4763–4774.

Tosaki A, Blasig IE, Pali T, Ebert B (1990). Heart protection and radical trapping by DMPO during reperfusion in isolated working rat hearts. Free Radic Biol Med 8: 363–372.

Turer AT, Hill JA (2010). Pathogenesis of myocardial ischemia-reperfusion injury and rationale for therapy. Am J Cardiol 106: 360–368.

Tycinska AM, Sobkowicz B, Mroczko B, Sawicki R, Musial WJ, Dobrzychcki S et al. (2010). The value of apelin-36 and brain natriuretic peptide measurements in patients with first ST-elevation myocardial infarction. Clin Chim Acta 411: 2014–2018.

Van de Voorde J, Pauwels B, Boydens C, Decaluwé K (2013). Adipocytokines in relation to cardiovascular disease. Metabolism 62: 1513–1521.

Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J et al. (2002). Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem 277: 14838–14843.

Wang M, Gupta RC, Rastogi S, Kohli S, Sabbah MS, Zhang K et al. (2013a). Effects of acute intravenous infusion of apelin on left ventricular function in dogs with advanced heart failure. J Card Fail 19: 509–516.

Wang W, McKinnie SM, Patel VB, Haddad G, Wang Z, Zhabyeyev P et al. (2013b). Loss of apelin exacerbates myocardial infarction adverse remodeling and ischemia-reperfusion injury: therapeutic potential of synthetic apelin analogues. J Am Heart Assoc 2: e000249.

Weir RA, Chong KS, Dalzell JR, Petrie CJ, Murphy CA, Steedman T et al. (2009). Plasma apelin concentration is depressed following acute myocardial infarction in man. Eur J Heart Fail 11: 551–558.

Zeng XJ, Zhang LK, Wang HX, Lu LQ, Ma LQ, Tang CS (2009). Apelin protects heart against ischemia/reperfusion injury in rat. Peptides 30: 1144–1152.

Zhang Z, Yu B, Tao G (2009). Apelin protects against cardiomyocyte apoptosis induced by glucose deprivation. Chin Med J 122: 2360–2365.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site: http://dx.doi.org/10.1111/bph.13038

Table S1 Effects of peptide infusion before global ischaemia on recovery of LVDP (in mmHg) during reperfusion of rat isolated perfused heart.