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RESEARCH PAPER

Antagonism of endogenous nociceptin/orphanin FQ inhibits infarction-associated ventricular arrhythmias via PKC-dependent mechanism in rats

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BACKGROUND AND PURPOSE

Evidence indicates nociceptin/orphanin FQ (N/OFQ) may participate in the pathology of cardiac arrhythmias associated with myocardial infarction. But the role of N/OFQ in the arrhythmogenesis in acute myocardial infarction is unclear. The aim of this study was to investigate the effects of endogenous N/OFQ on infarction-associated arrhythmias.

EXPERIMENTAL APPROACH

The expression of N/OFQ, PKC activity and ventricular arrhythmias in presence and absence of UFP-101, a specific antagonist of N/OFQ receptor, were examined following permanent coronary artery occlusion in anaesthetized rats. The effect of N/OFQ on action potential duration was examined in isolated rat cardiomyocytes.

KEY RESULTS

It was observed that N/OFQ was increased by 41% in the myocardium after coronary artery occlusion ($P < 0.01$ vs. control). Pretreatment with UFP-101 (10^{-7} mol·kg^{-1}, i.v.) reduced the incidence of ventricular ectopic beats by 70% and ventricular tachycardia by 51% respectively (all $P < 0.05$ vs. control). Meanwhile, PKC activity was elevated in the rats treated with UFP-101 (by 35%, $P < 0.05$ vs. control). A selective PKC inhibitor, calphostin C, completely abolished the anti-arrhythmic effects of UFP-101 ($P < 0.01$). N/OFQ (at 10^{-11}, 10^{-9} and 1×10^{-7} mol·L^{-1}) shortened the action potential duration by 3% ($P > 0.05$), 10% ($P < 0.05$) and 22% ($P < 0.01$), respectively, via N/OFQ receptor.

CONCLUSIONS AND IMPLICATIONS

Antagonism of endogenous N/OFQ produces anti-arrhythmic effects on ventricular arrhythmias in acute myocardial infarction, possibly via modulating PKC activity and action potential of myocytes.

Abbreviations

AAR, area at risk of ischaemia; APD, action potential duration; CAO, coronary artery occlusion; MI, myocardial infarction; N/OFQ, nociceptin/orphanin FQ; UFP-101, N-(BZl)Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Gln-NH_2; VEB, ventricular ectopic beat; VF, ventricular fibrillation; VT, ventricular tachycardia

Introduction

Acute myocardial infarction (MI) induces cardiac arrhythmias (Lown *et al.*, 1977; Coronel *et al.*, 2002; Roger *et al.*, 2011) which could be observed in 1 h of MI (Coronel *et al.*, 2002). In the meantime, activation of sympathetic and cardiac sensory afferent nerves was observed, presenting marked increase in catecholamine (Schömig, 1990; Schömig *et al.*, 1991) in the heart and some neuropeptides (Franco-Cereceda *et al.*, 1993; Hua *et al.*, 2004; Guo *et al.*, 2007), including nociceptin/orphanin FQ (N/OFQ) in the sensory neurons (Guo *et al.*, 2008) innervating the heart. The findings may raise the possibility of co-participation or interaction of the two nervous systems and their nervous mediators in the pathology of acute MI. Over-activation of sympathetic mechanism is closely associated with the pathogenesis of cardiac arrhythmias at the early stage of MI (Schömig, 1990; Schömig *et al.*, 1991). Yeh and colleagues reported the effects of β-adrenergic stimulation in shortening action potential duration (APD) (Yeh *et al.*, 2007), which may be associated with the arrhythmogenic effects of β-adrenergic activity in MI (Tölg *et al.*, 1997; Zicha *et al.*, 2006). However, stimulation of α-adrenergic receptor produces anti-arrhythmic effects (Tölg *et al.*, 1997; Imani *et al.*, 2008), with up-regulation of PKC activity (Yeh *et al.*, 2007). A recent study of this group demonstrated that cardiac sensory nerves may play a cardioprotective role in acute MI, through the mediation of the constituent neuropeptides, substance P and calcitonin gene-related peptide (Zhang *et al.*, 2012).

N/OFQ is a 17-amino acid neuropeptide (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995), an endogenous ligand for N/OFQ receptor. Expression of the mRNA encoding N/OFQ in the heart of patients with coronary heart disease and heart failure was reported (McDonald *et al.*, 2010) and the high-affinity N/OFQ-binding sites in rat heart were demonstrated (Dumont and Lemaire, 1998). In addition, N/OFQ could reduce blood pressure and heart rate (HR) (Champion *et al.*, 1997; Giuliani *et al.*, 1997; Hashiba *et al.*, 2003) via inhibition of sympathetic activity (Giuliani *et al.*, 1997; Lai *et al.*, 2000). The findings may indicate a role of N/OFQ in cardiac physiology and pathophysiology. However, direct evidence demonstrating the role of N/OFQ in the arrhythmia associated with acute MI is still lacking, although the findings may imply that N/OFQ, as one of the containing mediators of sensory nerves, might participate in the pathology of acute MI.

Based on the findings and the clues, we designed this study to investigate the potential effect of endogenous N/OFQ on cardiac arrhythmia at early time of MI and its mechanism using a rat model. The changes in the expressions of N/OFQ in the myocardium in the area at risk of ischaemia (AAR) after ligation of the left anterior descending branch of coronary artery were examined. In the meantime, the difference in the morbidity and severity of the ventricular arrhythmias with and without the pretreatment with a specific antagonist of N/OFQ receptor during the 60 min of acute MI was analysed. The potential underlying mechanisms associated with PKC activation and APD were investigated in current study.

Methods

Protocol

The study was approved by the Institutional Animal Care and Use Committee of Shanxi Medical University and conformed to the guidelines for the care and use of laboratory animals (National Institute of Health Guide for the Care and Use of Laboratory Animals, NIH Publications No. 80-23, revised 1996) (http://grants.nih.gov/grants/olaw/olaw.htm). Male Sprague–Dawley rats, weighing 250–280 g, were employed in the experiments. Efforts were made to minimize the number of the animals used in the study. At the end of experiments, the animals were killed by decapitation under additional anaesthetic administration (sodium pentobarbital, 100 $mg \cdot kg^{-1}$, i.v.).

In this study, the *in vivo* experiments (Table 1) were performed to investigate (i) the changes in the ventricular arrhythmias and PKC activity during MI in the presence and the absence of a specific antagonist of N/OFQ receptor, UFP-101 (N-(BZl)Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Arg-Lys-Gln-NH_2) (Calo *et al.*, 2005) and (ii) the effects of a selective PKC inhibitor calphostin C (Okamura *et al.*, 1999) on the cardiac effects of UFP-101. The *in vitro* study was carried out to examine the expressions of N/OFQ and its receptor in the myocardium and the effect of N/OFQ on the APD of the isolated cardiomyocytes.

Experimental model of acute MI

The coronary artery occlusion (CAO) and the sham surgery were carried out under anaesthesia (induction with sodium pentobarbital 65 $mg \cdot kg^{-1}$, i.p., and maintained with 15 $mg \cdot kg^{-1} \cdot h^{-1}$, i.v., through caudal vein) and additional local anaesthetic infiltration (0.25% bupivacaine, with total volume less than 1 mL) in the surgical sites, as we previously reported (Guo *et al.*, 2007). The adequate anaesthesia was monitored by observation of the changes in the depth and the pattern of respiration upon nociceptive stimulation. The MI was induced by permanent CAO of the left anterior descending branch of coronary artery for 60 min. The CAO was confirmed by changes in the ECG and the colour of myocardium connected to the occluded artery. The haemodynamic changes were monitored during the CAO and analysed offline. To facilitate collection of the samples of the myocardium in the AAR 2 mL of Even's blue (2%; Sigma, St. Louis, MO, USA) was injected via caudal vein of the animal at the end of the experiments. The hearts were harvested from the animals in each CAO group (including the saline-treated group and the three groups given different doses of UFP-101, Table 1) at the end of infarction and sectioned into 2 mm slices. AAR was defined as the ratio of the area unstained by the Even's blue to that of the total left ventricle, as we previously reported (Zhang and Guo, 2009). All the samples for measurements of N/OFQ and PKC activity were collected from AAR for the animals underwent CAO (CAO group) and the position matched myocardium of the animals in sham surgery group.

Examination of N/OFQ and its receptor

Immunohistochemical assay was carried out as previously reported (Guo *et al.*, 2007; 2008) with three hearts (n = 3,

Table 1
Protocol of the study

	Adult male Sprague–Dawley rats			
	In vivo experiments		*In vitro* experiments	
Experiments	**CAO**	**Sham**	**Myocardium**	**Myocytes**
AAR	$n = 3 \times 4$	–	–	–
IHC	–	–	$n = 3$	–
EIA	$n = 6$	$n = 6$	–	–
Arrhythmia analysis				
Saline	$n = 6$	$n = 6$	–	–
UFP 10^{-11} mol·L^{-1}	$n = 6$	–	–	–
UFP 10^{-9} mol·L^{-1}	$n = 6$	–	–	–
UFP 10^{-7} mol·L^{-1}	$n = 6$	–	–	–
UFP 10^{-7} mol·L^{-1} + CC	$n = 5$	–	–	–
PKC activity	$n = 6$	–	–	–
APD	–	–	–	$n = 3 \times 6$

IHC, immunohistochemical assay; EIA, enzyme immunoassay; UFP, UFP-101; –, not carried out.

Table 1) collected from animals without any surgical manipulation and any drug (or agent) treatment. The sections of the heart were separately incubated with antiserum for N/OFQ (rabbit anti-rat, 1:250 dilution; Sigma) or N/OFQ receptor (rabbit anti-rat, 1:250 dilution; Sigma) and then processed with Alexa Fluor 488 (presenting N/OFQ in green colour) or Alexa Fluor 568 (presenting N/OFQ receptor in red colour) conjugated lgG (donkey anti-rabbit, 4 μg·mL^{-1}; Invitrogen, Grand Island, NY, USA). The specificity of the immunoreactive staining was verified by omitting the primary antibodies with the other procedure the same as the descriptions above.

Enzyme immunoassay was carried out for the quantitative analysis of changes of N/OFQ in the myocardium using a commercial N/OFQ-Kit (Cayman Chemical, Ann Harbor, MI, USA), as previously reported (Guo *et al.*, 2008). The samples of myocardium were collected from the AAR in the CAO group (pretreated with saline, $n = 6$) or the position-matched myocardium in the sham surgery group ($n = 6$, Table 1). The contents of the samples were finally made 100 mg of myocardium per mL. The results were presented as pg·mL^{-1} (i.e. pg per 100 g of myocardium).

Ventricular arrhythmias

Cardiac ventricular ectopic beats (VEBs) were defined as identifiable premature QRS complexes, ventricular tachycardia (VT) as four or more consecutive VEBs at the rate faster than the resting sinus rate, and ventricular fibrillation (VF) as unidentifiable QRS complexes of low voltage (please see the ECG online) respectively. The arrhythmias were analysed according to the Lambeth Conventions (Walker *et al.*, 1988). The severity of the ventricular arrhythmias was presented as the morbidity and number of episodes of VEB, VT and VF in the 60 min of CAO. The changes in the haemodynamics were monitored throughout the experiments via a catheter implanted in the right carotid artery.

Thirty animals were randomly assigned to five groups (Table 1), the four treated groups were i.v. injected with UFP-101 (Tocris Bioscience, Bristol, UK) at a range of doses of 10^{-11}, 10^{-9} and 10^{-7} mol·kg^{-1}, or 0.9% saline in a volume of 0.1 mL at 5 min before the ligation of the coronary artery and the sham surgery group (sham).

PKC activity assay

The PKC activity was examined and compared between the animals treated with the saline ($n = 6$) and UFP-101 (10^{-7} mol·kg^{-1}, $n = 6$, Table 1) at 60 min of CAO. The samples were collected and put into liquid nitrogen for further process. The assay for PKC activity was carried out using a PepTag® non-radioactive protein kinase assays kit (Promega Corporation, Fitchburg, WI, USA) as we previously reported (Zhao *et al.*, 2010). The result was presented as percentage of the positive control (an agent provided by the manufacture, indicating where is the positively activated PKC in the gel after electrophoresis, and used for calculating how much PKC was activated).

PKC activity and ventricular arrhythmias

Calphostin C (Calbiochem, Darmstadt, Germany), a specific inhibitor of PKC, was i.v. injected (0.1 mg·kg^{-1} in 0.1 mL, given at 5 min before injection of UFP-101 of 10^{-7} mol·kg^{-1}) in five animals ($n = 5$, Table 1) before the CAO and then the ventricular arrhythmias were analysed.

Measurements of APD

The effect of N/OFQ on the APD was examined with a range of concentrations of N/OFQ (10^{-11}, 10^{-9} and 10^{-7} mol·L^{-1}), tested on six myocardial strips ($n = 6$) from three rats, for each dose. The UFP-101 was used to verify the specificity of the action. The APD was measured using patch-clamp technique

as we previously reported (Yuan and Guo, 2011). Briefly, isolated myocytes were allowed to settle in the bath (0.8 mL) for 5 min before being perfused with external solution [containing (in mmol·L^{-1}): NaCl 142.0, KCl 5.4, NaH_2PO_4 0.4, $MgCl_2$ 0.5, HEPES 5.0 (Amresco Inc., Solon, OH, USA), $CaCl_2$ 1.8 and glucose 5.5 (pH adjusted to 7.4 with NaOH)] at a rate of 1 mL·min^{-1}. Patch electrode with a tip resistance of 2–5 MΩ and filled with internal solution [containing (in mmol·L^{-1}): KCl 120, $CaCL_2$ 1, $MgCL_2$ 5, Na_2-ATP 5, EGTA 11, HEPES 10, glucose 10.0 (pH adjusted to 7.3 with KOH)] was used in the experiment. After obtaining a gigaseal (2–10 GΩ), suction pulses were applied to establish the whole cell mode. Command pulse (4 mA, 2 ms) was delivered to induce the action potential and data were acquired with an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA, USA) loaded with the software, pCLAMP version 9.0 (Axon Instruments), connected to a PC computer.

Statistical analysis

Values are presented as mean and SD. The Student's *t*-test and one-way ANOVA with *post hoc* Bonferroni's test were performed to analyse the differences between groups. The morbidities of ventricular arrhythmias were compared by chi-square analysis. In each case, a *P*-value of <0.05 was considered significant.

Results

Sixty-eight rats were employed in the *in vivo* study and 62 of them fulfilled the experiments, while 6 animals were discarded from the study, among which 3 died of bleeding and 2 died after CAO (before the end of the test) and 1 was withdrawn from the study showing continuous abnormal cardiac performance after setting up the experimental model.

Animal model

The myocardium connecting to the occluded vessel turned into pale while the elevation of ST-T segment and increase of ventricular arrhythmias, as shown in the ECG (online), were observed immediately following the CAO. The areas at risk of ischaemia occupying 33 ± 4% of left ventricles at the end of 60 min of CAO were observed. Slight changes in systolic and diastolic blood pressures (DBPs) (less than 20% of baseline) and HR (less than 15% of baseline) were observed after the CAO (Figure 1). In the range of the doses of UFP-101, only the dose of 10^{-7} mol·kg^{-1} produced significant reduction in systolic blood pressure [by (average value of) 13–22% from the baseline, n = 6, P < 0.05] and DBP [by (average value of) 40–49% from the baseline, n = 6, P < 0.05] during the 60 min of MI. No significant change in the HR was observed during the time of CAO (n = 6, P > 0.05, Figure 1).

Expressions of N/OFQ and its receptor

The immunoreactive materials for the N/OFQ (Figure 2B) and its receptors (Figure 2D) were located in the myocytes of the ventricles, including the anterior wall of the left ventricles. The concentration of N/OFQ was significantly greater in the myocardium (20.7 ± 2.5 pg·mL^{-1}, n = 6) from the animals of the CAO group, compared with that of the sham group (14.7 ± 1.5 pg·mL^{-1}, n = 6, P < 0.05) at 60 min of CAO.

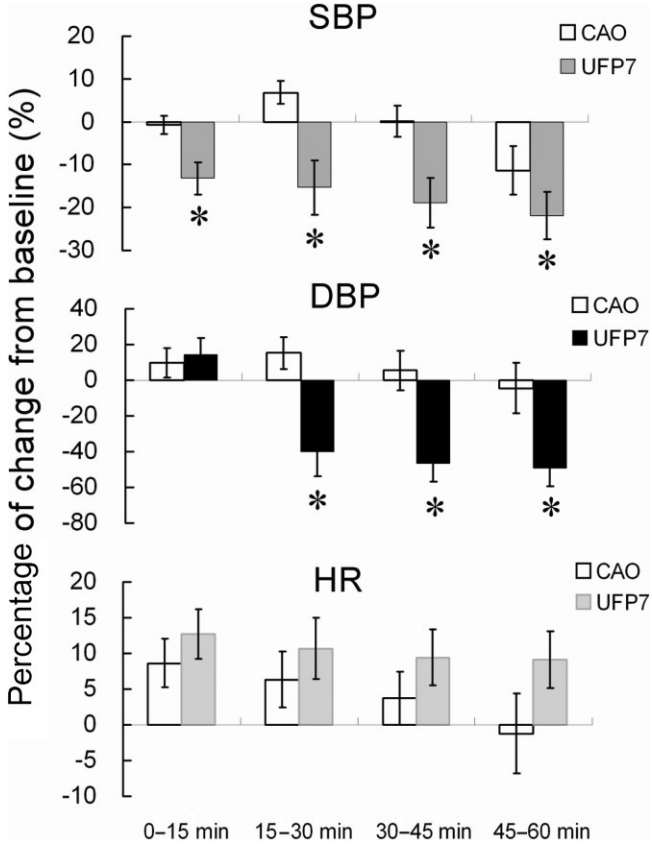

Figure 1

Effects of UFP-101 on haemodynamics. UFP-101 (10^{-7} mol·kg^{-1}) produced significant reduction in SBP and DBP with no significant effect on HR during the CAO. SBP, systolic blood pressure; UPF7, UFP-101 at 10^{-7} mol·kg^{-1}. *difference (P < 0.05 vs. CAO).

Ventricular arrhythmias

The ventricular arrhythmias were rare and not significantly different among the groups before the CAO in the anaesthetized rats. However, high incidences of VEB, and VT and VF (Figure 3A, B and D) were observed in the animals of the CAO group, compared with the sham surgery group.

Pretreatment with UFP-101, at doses of 10^{-11}, 10^{-9} and 10^{-7} mol·kg^{-1} (presented as UFP11, UFP9 and UFP7 in Figure 3), resulted in a dose-dependent reduction of VEB by –4 ± 14% (n = 6, P > 0.05), 54 ± 16% (n = 6, P < 0.01) and 71 ± 3% (n = 6, P < 0.01), respectively (Figure 3C and D), compared with the saline-treated animals (CAO, n = 6) after the CAO. The effects of UFP-101 lasted throughout the time of the arrhythmia (at least for 37 min). UFP-101 at 10^{-7} mol·kg^{-1} effectively reduced VT morbidity, 33% versus 67% in control (saline-treated CAO group, P < 0.05), and number of episodes of VT, 2 ± 0.4 episodes/rat versus 3 ± 1 episodes/rat in control (saline-treated CAO group, P < 0.05, Figure 4). While the UFP-101, at 10^{-11} and 10^{-9} mol·kg^{-1}, failed to produce any significant effect on VT. However, one episode of VF was observed in an animal from the CAO group (CAO, n = 6) and in one animal treated with 10^{-7} mol·kg^{-1} of UFP-101 (n = 6), while VF was seen in three animals treated with UFP-101 (10^{-7} mol·kg^{-1}) plus calphostin C (UFP7 + CC, n = 5).

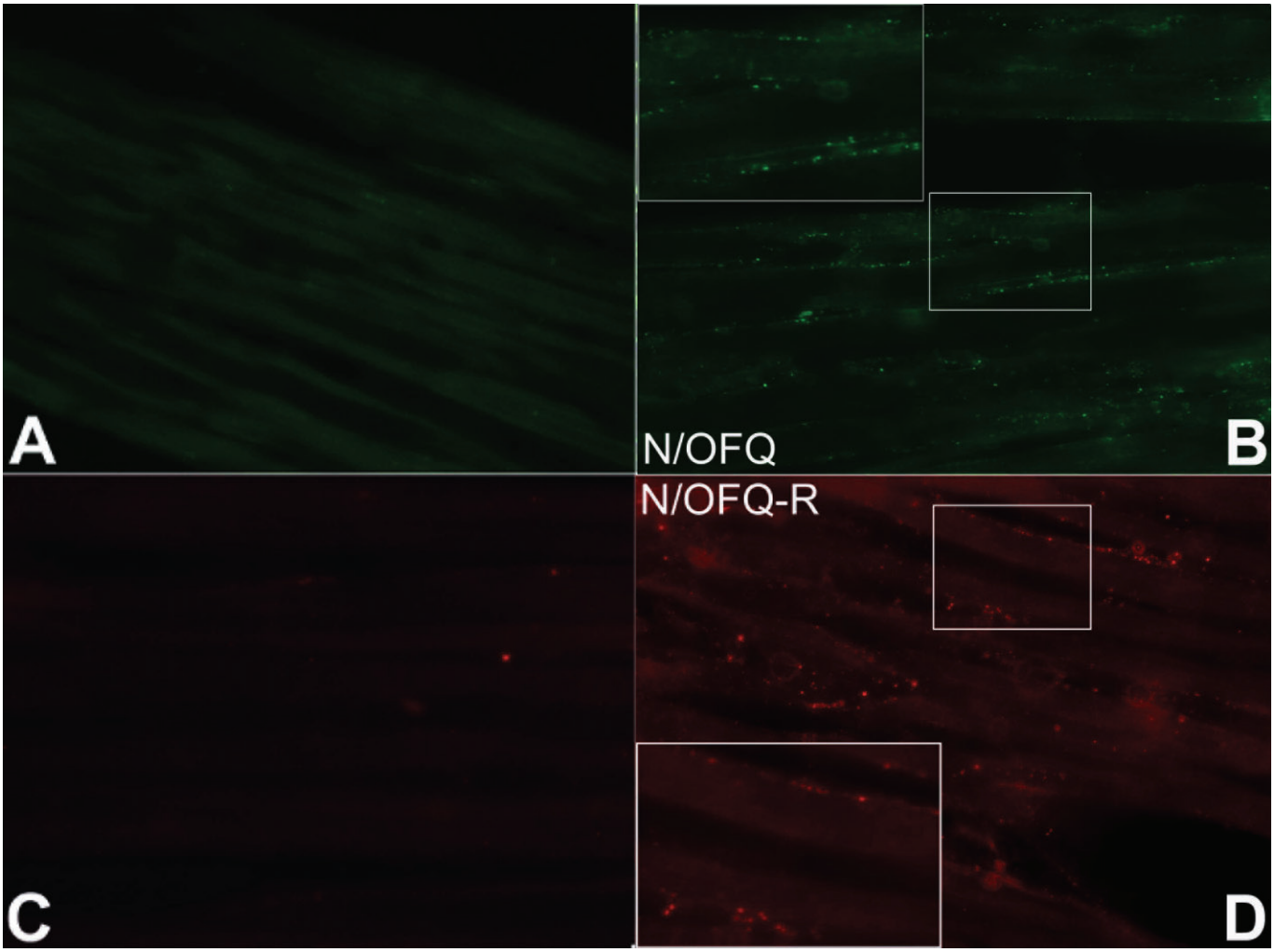

Figure 2

Expressions of N/OFQ and N/OFQ receptor. B and D present the immunoreactive materials for N/OFQ (B) and N/OFQ receptor (NOFQ-R, D) in the myocytes of the anterior wall of left ventricle. A and C were negative controls (processed without the primary antibodies) for the N/OFQ and N/OFQ-R respectively. N/OFQ-R, nociceptin/orphanin FQ receptor.

PKC activity

Greater PKC activity was observed in the animals treated with UFP-101 at 10^{-7} mol·kg^{-1} (UFP7, n = 6) compared with the saline-treated animals (n = 6) after the CAO (50 ± 7% vs. 37 ± 7%, P < 0.05, Figure 5).

Furthermore, it was observed that the anti-arrhythmic effects of UFP-101 (10^{-7} mol·kg^{-1}, n = 6) were completely abolished by calphostin C (n = 5), resulting in the VEB, 117 ± 15 beats per rat versus 15 ± 4 beats per rat (UFP7 + CC vs. UFP7, P < 0.01, Figure 3B and D). The PKC inhibitor also abolished the anti-arrhythmic action of UFP-101 on the morbidity of VT (80% vs. 33%, UFP7 + CC vs. UFP7, P < 0.01) and the number of episodes of VT (34 ± 9 episodes/rat vs. 2 ± 0.4 episodes/rat (UFP7 + CC vs. UFP7, P < 0.01, Figures 3 and 4).

Action potential

N/OFQ at the concentrations of 10^{-11}, 10^{-9} and 10^{-7} mol·L^{-1} shortened the APD, in a dose-dependent manner, in the isolated cardiomyocytes (Figure 6). The effect of N/OFQ (10^{-9} mol·L^{-1}) on the APD was completely reversed when the myocytes were pretreated with UFP-101 (10^{-8} mol·L^{-1}, n = 6), while UFP-101 (10^{-8} mol·L^{-1}, n = 6) alone did not show any effect on APD (Figure 6B, inset).

Discussion and conclusions

A potential anti-arrhythmic role of endogenous N/OFQ had been expected before the start of this experiment, which was based on the evidence showing N/OFQ exerts the action reducing blood pressures via attenuating catecholamine release (Giuliani *et al.*, 1997) or inhibiting sympathetic synaptic transduction (Lai *et al.*, 2000), indicating an inhibitory effect of N/OFQ on sympathetic action, but not at the level of any of the subtypes of adrenergic receptor. The results of current study demonstrate a β-adrenergic activity-like effect

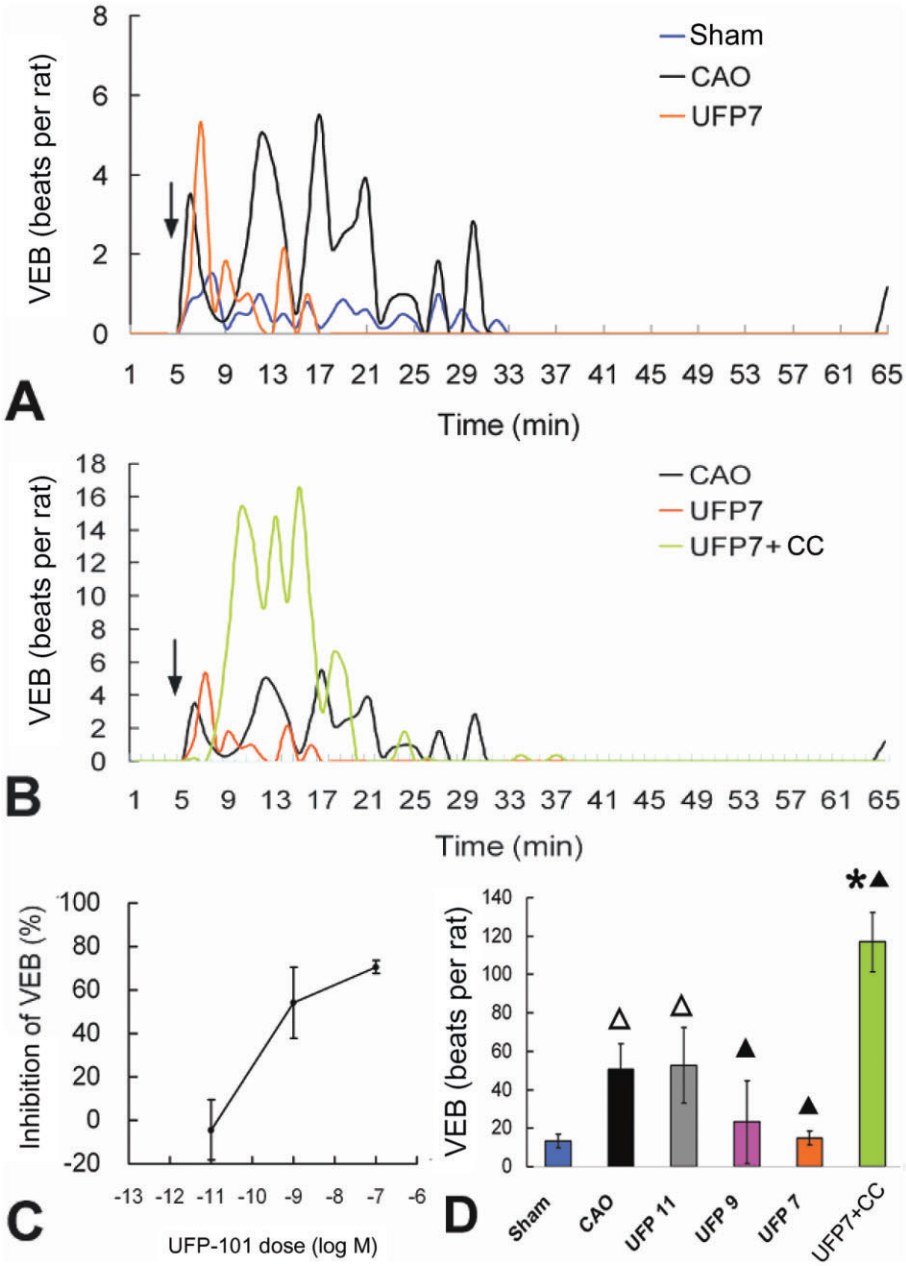

Figure 3

Effects of UFP-101 and calphostin C on VEB. UFP-101 at 10^{-7} mol·kg^{-1} (UFP7) reduced the ventricular arrhythmias (A, $n = 6$ for each group). CC at 0.1 mg·kg^{-1} abolished the effect of UFP7 (B, in group of UFP7, $n = 6$, and UFP7 + CC group, $n = 5$). For clarity, the bars showing SD were omitted. The arrows indicate the timing of onset of CAO. (C) The dose-dependent anti-arrhythmic effects of UFP-101 ($n = 6$ for each dose); (D) the profile of episodes of VEB in the 60 min of CAO in the experimental groups. △difference ($P < 0.01$ vs. sham); ▲difference ($P < 0.01$ vs. CAO); *difference ($P < 0.01$ vs. UFP7); UFP11, UFP-101 at 10^{-11} mol·kg^{-1}; UFP9, UFP-101 at 10^{-9} mol·kg^{-1}; UFP7, UFP-101 at 10^{-7} mol·kg^{-1}; UFP7 + CC, UFP-101 at 10^{-7} mol·kg^{-1} plus calphostin C at 0.1 mg·kg^{-1}.

(Yeh *et al.*, 2007) of N/OFQ in arrhythmogenesis and in modulation of (shortening) the APD, while the pretreatment with UFP-101 (a specific antagonist of N/OFQ receptor) resulted in activation of PKC and inhibition of infarction-induced arrhythmias, presenting an α-adrenergic activity-like action (Tölg *et al.*, 1997; Yeh *et al.*, 2007; Imani *et al.*, 2008) in acute MI. Therefore, the results of current study provided a correction of the original expectation.

In this study, we located N/OFQ and its receptors in the cardiomyocytes, which are the important basis for cardiac actions of N/OFQ. In addition, it was observed the up-regulation of N/OFQ in the myocardium at risk of ischaemia 60 min after acute MI, during which marked increase in ventricular arrhythmias was observed. Unexpectedly, antagonism of endogenous N/OFQ action with UFP-101, a specific antagonist of N/OFQ receptor, resulted in a significant inhi-

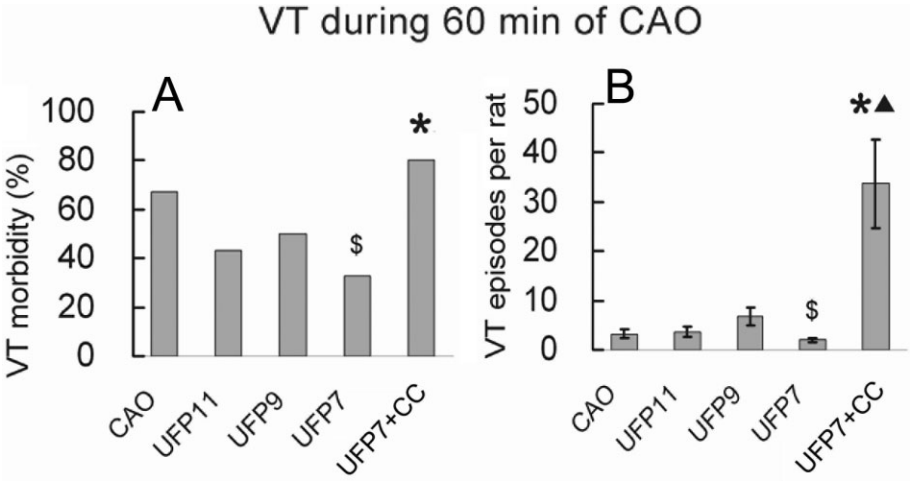

Figure 4

Effects of UFP-101 and calphostin C on VT andVF. Significant reductions of the morbidity (A) and number of episodes of VT (B) in UFP7 group ($n = 6$). Pretreatment with CC ($n = 5$) reversed the effects of UFP7 (UFP7 + CC). $ difference ($P < 0.05$ vs. CAO); *difference ($P < 0.01$ vs. UFP7); ▲difference ($P < 0.01$ vs. CAO).

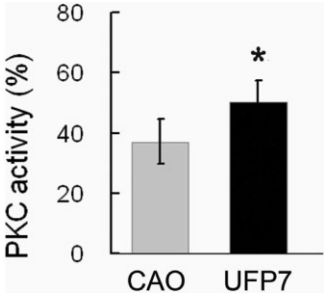

Figure 5

Up-regulation of PKC activity, shown as percentage of the positive control (an agent provided by the manufacture, indicating where is the positively activated PKC, in the gel after electrophoresis and used for calculating how much PKC was activated) in the animals pre-treated with UFP-101 (UFP7 group, $n = 6$), compared with that of CAO group ($n = 6$). *difference ($P < 0.05$ vs. CAO). UFP7, UFP-101 at 10^{-7} mol·kg^{-1}.

bition of the ventricular arrhythmias. The finding suggests an arrhythmogenic role of the endogenous N/OFQ. The results obtained here were exactly opposite the original expectation and clearly indicate a pro-arrhythmic effect of endogenous N/OFQ on the infarction-associated ventricular arrhythmias.

Previous reports indicate that the N/OFQ receptor mediates the cardiovascular effects of N/OFQ (Champion *et al.*, 1997; Giuliani *et al.*, 1997; Dumont and Lemaire, 1998; Burmeister *et al.*, 2008) and UFP-101. UFP-101 can reverse central and peripheral actions of N/OFQ (Calo *et al.*, 2005). However, the effect of UFP-101 on the haemodynamics after MI observed in this study was not consistent with previous reports (Champion *et al.*, 1997; Giuliani *et al.*, 1997; Hashiba *et al.*, 2003). Previous findings demonstrated that activation of N/OFQ receptor resulted in decrease in blood pressure and HR. The result seems to oppose the findings of this study presenting a reduction in blood pressures after antagonism of N/OFQ receptor. The precise mechanism underlying the finding is unknown at this time. The speculation we can make now is that the difference in the experimental models used in this study (MI) and in previous reports (normal animals) may be associated with the difference in the reaction to the exogenously given N/OFQ (previous reports) or by the endogenous N/OFQ (current study) when the animals were under different circumstance (pathophysiological or physiological). Obviously, further study is needed to clarify the speculation. The non-adequate coronary perfusion associated with the inhibition of the haemodynamics may evoke cardiac arrhythmia. Anyhow, the haemodynamic changes in the animals pretreated with UFP-101 in this study did not suggest an association of the anti-arrhythmic action of UFP-101 with the haemodynamic changes. On the contrary, the results may indicate the multitude and complexity of the actions of the endogenous N/OFQ in the pathology of acute MI.

The current results showed that UFP-101 produced the anti-arrhythmic effects at such a low dose as 1 nmol·kg^{-1} and the effects induced by 100 nmol·kg^{-1} lasted at least for about 37 min, presenting the efficacy of the antagonist (Figure 3), as reported by others (Calo *et al.*, 2002). The increase of N/OFQ in the myocardium at the early time of CAO and the action of N/OFQ on the APD of myocytes as well as up-regulation of PKC activity by UFP-101 in the myocardium may imply a cardiac site of the actions of N/OFQ and UFP-101. However, current and previous reports suggest multiple activation of pathways for different activities of N/OFQ on cardiac performance (Champion *et al.*, 1997; Giuliani *et al.*, 1997; Dumont and Lemaire, 1998; Lai *et al.*, 2000), on which further investigation is needed.

PKC plays important roles in cardioprotection (Sakamoto *et al.*, 1995; Ikonomidis *et al.*, 1997) and anti-arrhythmia (Matsumura *et al.*, 2000; Vegh and Parratt, 2002). Lee and colleagues reported that enhancement of connexin43, through activation of PKC pathway, attenuates the

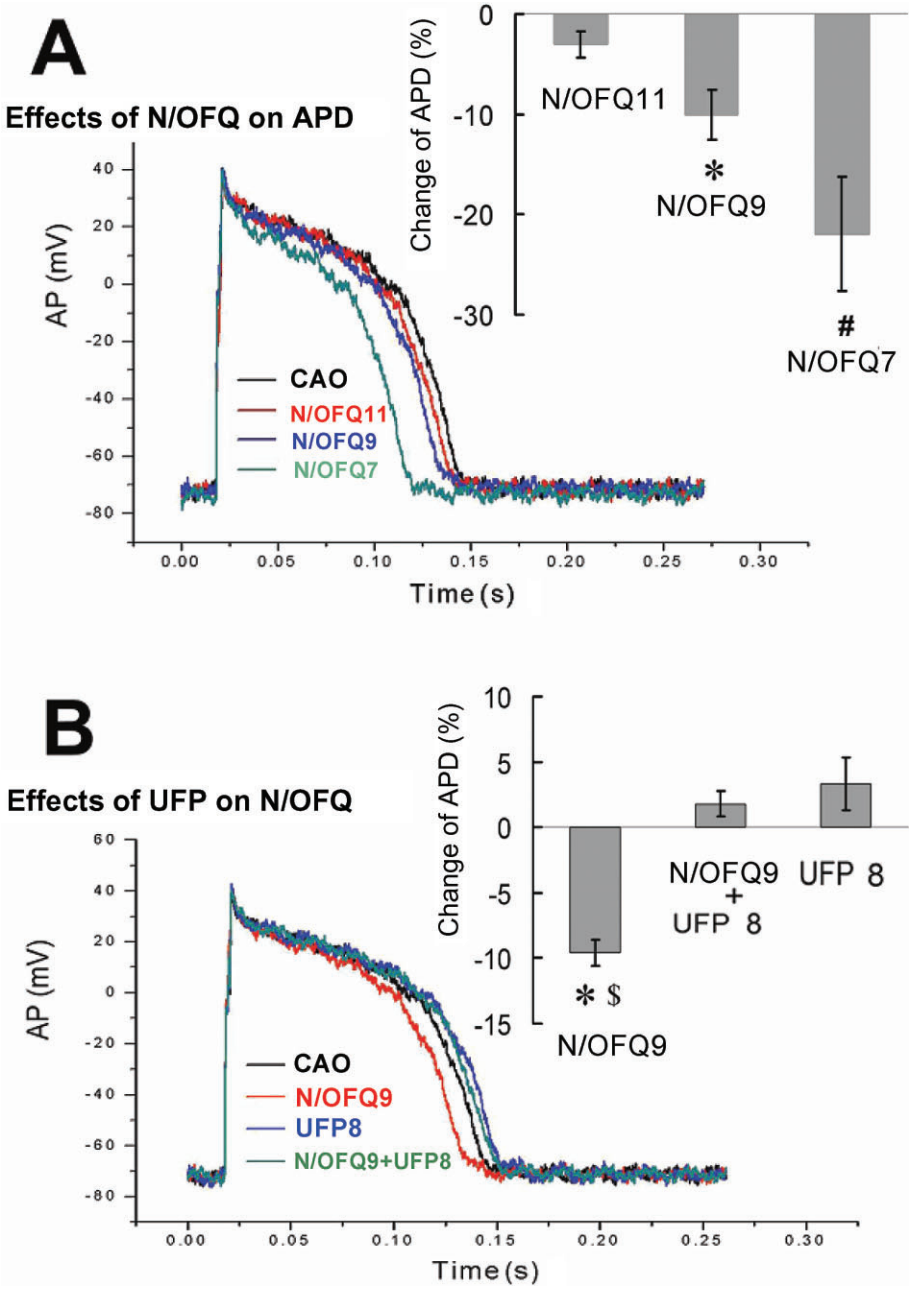

Figure 6

Effects of N/OFQ on APD. N/OFQ at the concentrations of 10^{-11}, 10^{-9} and 10^{-7} mol·L^{-1} shortened the APD by 3 ± 1% (n = 6, P > 0.05), 10 ± 3% (n = 6, P < 0.05) and 22 ± 6% (n = 6, P < 0.01) respectively (A). N/OFQ at 10^{-9} mol·L^{-1} (N/OFQ9, n = 6) shortened APD versus baseline (A, B). The effect was reversed by UFP-101 10^{-8} mol·L^{-1} (N/OFQ9 + UFP8, n = 6, B). UFP8 alone did not produce significant effect on the APD (B). N/OFQ11, N/OFQ at 10^{-11} mol·L^{-1}; N/OFQ9, N/OFQ at 10^{-9} mol·L^{-1}; N/OFQ7, N/OFQ at 10^{-7} mol·L^{-1}; UFP8, UFP-101 at 10^{-8} mol·L^{-1}. *difference (P < 0.05 vs. 0); #difference (P < 0.01 vs. 0); $difference ($P$ < 0.05 vs. OFQ9 +UFP8).

arrhythmogenic response to programmed electrical stimulation (Lee *et al.*, 2012). Here, we found that antagonism of endogenous N/OFQ activity led to significant up-regulation of PKC activity along with a significant inhibition of the ventricular arrhythmias induced by MI. The findings may imply an inhibition of PKC activity by the endogenous N/OFQ in acute MI, which might be associated with the increase in the cardiac arrhythmia. UFP-101 abolished the effect of the endogenous N/OFQ on PKC, producing the anti-arrhythmia, which mimics the effect of activation of α-adrenergic activity (Tölg *et al.*, 1997; Yeh *et al.*, 2007; Imani *et al.*, 2008). Further investigation in current study demonstrated that calphostin C, a selective inhibitor of PKC, completely abolished the anti-arrhythmic effects of UFP-101, which may support the notion and indicates that up-regulation of PKC is a downstream mechanism of the

anti-arrhythmic action of UFP-101. The effects of UFP-101 on the cardiac arrhythmia and on the action potential in this study were consistent with the effects of stimulation of α-adrenergic receptors, producing anti-arrhythmic effects (Tölg *et al.*, 1997; Imani *et al.*, 2008) and up-regulating PKC activity (Yeh *et al.*, 2007). In current study, the effects of exogenous N/OFQ on the APD were similar to the effect induced by β-adrenergic stimulation (Yeh *et al.*, 2007), which is associated with arrhythmogenic effects of infarction-associated arrhythmias (Tölg *et al.*, 1997; Zicha *et al.*, 2006). However, the reduction of blood pressures seen in the animals pretreated with UFP-101 (10^{-7} mol·kg^{-1}) did not suggest an α-adrenergic activation-like effect of UFP-101. The observation may imply the multitude of characteristics of UFP-101 in cardiovascular action, about which the underlying mechanism needs to be further studied.

In this study, the changes in arrhythmia, PKC, APD and haemodynamics were carefully watched in the investigation of the potential effect and the underlying mechanism of endogenous N/OFQ on cardiac arrhythmias in MI. The parameters were closely related to the known adrenergic activities in cardiac arrhythmia, as described above, which served in current study as guideposts in the journal searching for the mechanism of action of endogenous N/OFQ on cardiac arrhythmias. The findings may imply the association of the anti-arrhythmic effect of UFP-101 with modulation of PKC activity and APD. However, the implication and the relationship of N/OFQ and adrenergic mechanisms need to be further clarified in future study.

Current findings carry potential clinical implications that antagonism of endogenous N/OFQ action produces anti-arrhythmic effects, implying a novel pharmacological target for prevention of cardiac arrhythmias at an early time of acute MI.

Limitation of the study

The potential molecular mechanisms of the interaction of N/OFQ with the α- and β-adrenergic mechanisms in the pathophysiology of arrhythmogenesis and anti-arrhythmias were not investigated in this study.

Conclusion

The results of this study suggest that antagonism of endogenous N/OFQ action produces anti-arrhythmic effects on the infarction-associated ventricular arrhythmias, possibly via modulating PKC activity and action potential of myocytes, which indicates a novel target for anti-arrhythmia strategy at an early time of acute MI.

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 ECG following CAO. Elevation of ST segment (B, C and D) versus the pre-CAO baseline (A), VEB (B and C), VT (D) and VF (E) following the CAO. The figures show a single representative experiment from a single animal. ECG, electrocardiogram; VEB, ventricular ectopic beat; VT, ventricular tachycardia; VF, ventricular fibrillation; CAO, coronary artery occlusion.