JPET Fast Forward. Published on April 23, 2007 as DOI: 10.1124/jpet.107.120394

Centrally-administered nociceptin/orphanin FQ (N/OFQ) evokes bradycardia, hypotension and diuresis in mice via activation of central N/OFQ peptide (NOP) receptors.

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JPET Fast Forward. Published on April 23, 2007 as DOI: 10.1124/jpet.107.120394
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Running title: Cardiovascular and renal responses to i.c.v. N/OFQ in mice

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Number of text pages: 32

Number of tables: 1

Number of figures: 4

Number of references: 29

Number of words in the Abstract: 247

Number of words in the Introduction: 690

Number of words in the Discussion: 1498

Abbreviations: I.C.V., intracerebroventricular; I.V., intravenous; HR, heart rate; MAP, mean arterial pressure; N/OFQ, nociceptin/orphanin FQ; NOP receptor, nociceptin/orphanin FQ peptide receptor; V, urine output; UNaV, urinary sodium excretion; UKV, urinary potassium excretion; C_{H20}, free water clearance; UFP-101, [Nphe^{1},Arg^{14},Lys^{15}]N/OFQ-NH_2.

Recommended section assignment: Gastrointestinal, Hepatic, Pulmonary and Renal

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Abstract

The present studies examined the cardiovascular and renal responses produced by activation of central NOP receptors in conscious mice. To assess this, we examined changes in heart rate (HR), mean arterial pressure (MAP), urine output (V), urinary sodium excretion (UNaV) and free-water clearance (CH_20) produced by acute intracerebroventricular (i.c.v.) injection of N/OFQ (0.03, 0.3, 1 or 3 nmol) or isotonic saline vehicle (2 µl) in conscious telemetered ICR-CD1 mice. Following i.c.v. injection, N/OFQ, but not vehicle, dose-dependently decreased HR and MAP and increased V. At 3 nmol, N/OFQ reduced HR (C, 672±23 beats/min; 20-min, 411±30 beats/min) and MAP (C, 108±4 mmHg; 20-min, 62±6 mmHg). In the same telemetered mice i.c.v. N/OFQ significantly elevated V (0.65±0.03 cc/2hr) as compared to levels for the vehicle-treated group (0.15±0.09 cc/2hr). Central N/OFQ/vehicle did not alter UNaV or C_{H20}. In separate studies, 2-hr i.c.v. pretreatment with the NOP receptor antagonist [$NPhe^1$,Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101) (10 or 30 nmol) markedly, but transiently, reduced HR but not MAP, V, UNaV or CH_20. Following 2-hr UFP-101 (10 or 30 nmol) pretreatment, subsequent i.c.v. injection of N/OFQ (1 or 3 nmol) failed to alter cardiovascular or renal function. In contrast, in separate mice 2-hr pretreatment with N/OFQ (1 or 3 nmol) or vehicle failed to prevent the cardiodepressor and diuretic responses to a subsequent i.c.v. injection of the same dose of N/OFQ. Together, these findings demonstrate that in conscious mice the central administration of N/OFQ evokes marked bradycardia, hypotension and diuresis by selective activation of central NOP receptors.

Introduction

Nociceptin/orphanin FQ (N/OFQ) is an endogenous neuropeptide (FGGFTGARKSARKLANQ) that selectively binds to and activates an opioid-like receptor called the N/OFQ peptide (NOP) receptor (previously called opioid receptor-like 1, ORL1). The NOP receptor shares high sequence homology (~60%) to the mu-, kappa- and delta-opioid receptors (Bunzow et al., 1994; Chen et al., 1994) and, like them, is negatively coupled to adenylyl cyclase (Reinscheid et al., 1995); activates inwardly rectifying K^+ conductance (Vaughan et al., 1997; Chiou 2000); and inhibits voltage-sensitive Ca^{+2} channels (Connor and Christie, 1998; Larsson et al., 2000). Although N/OFQ is most structurally homologous to dynorphin A(1-17), the endogenous agonist of the kappa-opioid peptide (KOP) receptor (Meunier et al., 1995; Reinscheid et al., 1995), it does not display appreciable binding affinity to the KOP receptor and vice versa (Lachowicz et al., 1995; Mollereau et al., 1999).

There is a moderate to high density of prepro-N/OFQ (the polypeptide precursor of N/OFQ), N/OFQ and NOP receptor mRNA and protein throughout the brain in rats (Anton et al., 1996; Neal et al., 1999a; Neal et al., 1999b; Neal et al., 2001) and mice (Ikeda et al., 1998; Houtani et al., 2000), particularly in regions known to participate in the central control of blood pressure and fluid/electrolyte balance. Accordingly, N/OFQ evokes novel cardiovascular and renal excretory responses following its injection into the brain (reviewed by Kapusta, 2000 and Salis et al., 2000). The intracerebroventricular (i.c.v.) injection of N/OFQ, for instance, concurrently decreases heart rate (HR) and mean arterial pressure (MAP) in conscious (Kapusta et al., 1997; Kapusta and Kenigs, 1999; Zhang et al., 1999) and anesthetized (Chen et al., 2002) rats by a mechanism that

involves inhibition of central sympathetic outflow (Kapusta and Kenigs, 1999; Shirasaka et al., 1999). These findings are of particular interest since the central N/OFQ-evoked reduction in MAP is associated with a concurrent reduction in HR rather than a baroreflex-induced tachycardia (Kapusta and Kenigs, 1999; Shirasaka et al., 1999). In addition to cardiovascular depressor responses, central N/OFQ has also been shown to produce unique changes in renal excretory function and renal sympathetic nerve activity (RSNA) (reviewed by Kapusta, 2000). In conscious rats, i.c.v. N/OFQ concomitantly increases urine output (V) and decreases both urinary sodium excretion (UNaV) (Kapusta et al., 1997; Kapusta and Kenigs, 1999) and RSNA (Kapusta and Kenigs, 1999; Shirasaka et al., 1999). These diuretic and antinatriuretic responses to i.c.v. N/OFQ occur independently of a renal nerve mechanism since chronic bilateral renal dennervation does not alter the renal responses produced by central N/OFQ (Kapusta and Kenigs, 1999).

While the cardiovascular and renal responses to i.c.v. N/OFQ have been well characterized in the rat, those evoked by this peptide in conscious mice remain largely unknown. In related but separate studies, we observed that N/OFQ produces a dose-dependent increase in urine output when injected centrally to conscious mice (Rizzi et al., 2004). Thus, the purpose of the present investigations was to extend these initial observations and more critically examine the cardiovascular and renal responses produced by the i.c.v. injection of N/OFQ in conscious mice. Having a basic understanding of central N/OFQ's effects in mice (wild-type) also provides a foundation for comparison of results from future pharmacological and physiological studies that will explore the role of this peptide-receptor system using transgenic NOP receptor (or

N/OFQ) knockout mice. Therefore, the present studies examined the cardiovascular and renal excretory responses to increasing i.c.v. doses of N/OFQ in conscious mice. For these investigations, cardiovascular and renal excretory function was assessed simultaneously by using radio-telemetric and metabolic function methods, respectively. Additional studies were performed to determine whether the cardiovascular and renal responses to i.c.v, N/OFQ were mediated by selective activation of central NOP receptors. For these studies, the cardiovascular and renal responses to i.c.v. N/OFQ were examined in conscious mice pretreated centrally with the NOP receptor antagonist, [Nphe^{1},Arg^{14},Lys^{15}]N/OFQ-NH_2 (UFP-101). UFP-101 has nanomolar affinity and high selectivity for NOP receptors and antagonizes the biological effects of N/OFQ in several *in vivo* and *in vitro* assays (Calo' et al., 2005). Here we report that activation of central NOP receptors via centrally administered N/OFQ markedly reduces HR and MAP in conscious mice at doses that also produce diuresis.

Methods

Subjects

Age-matched ICR-CD1 (Harlan Sprague Dawley, Inc., Indianapolis, IN) weighing 20-25g were used in these studies. Animals were housed and tested in a climate-controlled room on a 12-hr dark/light cycle. All animals were fed a standard rodent chow diet and allowed food and tap water *ad libitum*. All experimental procedures were performed in accordance with LSU Health Sciences Center and NIH guidelines for the care and use of experimental animals.

Surgical procedures

Mice were instrumented with a stainless steel cannula into the right lateral cerebral ventricle under anesthesia (ketamine, 200 mg/kg i.p., in combination with: xylazine, 10 mg/kg i.p.). The coordinates used for cannula implantation were derived from the atlas of the mouse brain by Paxinos and Franklin (Paxinos and Franklin, 2003): 0.3 mm posterior to the bregma; 1.0 mm lateral to the midline; and 3.1 mm below the skull surface. Custom cut and fabricated guide, dummy (obturator) and internal cannula were purchased from Plastics One (Roanoke, VA). The guide cannula was fixed into position by jeweler's screws and cranioplastic cement. Verification of cannula position in the lateral cerebroventricle was made by observing spontaneous flow of cerebrospinal fluid from the tip of the cannula after removal of the obturator (after cannula implantation and before experimentation) and after the completion of the experiment by intracerebroventricular dye injection and subsequent postmortem brain section verification of dye placement.

Following a five day recovery period, mice were anesthetized (ketamine, 200 mg/kg i.p. in combination with xylazine, 10 mg/kg i.p.) and instrumented with a TA11PC20 radio telemetry probe (Data Sciences International, Arden Hills, MN), which was implanted into and secured to the left common carotid artery. Proper catheter placement was verified by the quality of the audio output signal on an FM radio. The body of the probe was placed in a subcutaneous pocket created in the right flank and the wound closed and sutured. Body temperature was maintained at 37°C using a heat lamp until sternal recumbency had been recovered. After surgery, penicillin (60,000 units/kg, i.m.) and yohimbine (0.1 mg/kg) were administered to prevent infection and facilitate recovery from surgery, respectively. Mice remained undisturbed in their home cages for seven days before the initiation of cardiovascular and renal excretory function studies. Prior to testing basal cardiovascular function was observed in all mice and shown to have characteristic circadian rhythm indicative of recovery from surgery.

Experimental protocols

Concurrent cardiovascular and renal function studies: In the first set of experiments, cardiovascular and renal excretory function studies were performed simultaneously. This was achieved by placing the telemetered mice individually in metabolic cages that sat directly on top of the telemetry receiver plates, which allowed for the simultaneous measurement of MAP and HR as well as collection of spontaneously voided urine. The metabolic cages were constructed of Plexiglas (walls) and stainless steel (row of bars positioned horizontally that the animal sat upon) and were designed to allow spontaneously voided urine to be collected onto a large plastic

weigh boat placed underneath the animal. On the day prior to experimentation, each mouse was removed from its home cage and “trained” by being placed individually in a metabolic cage for 20 minutes. The animals were then removed and the lower abdominal region was gently messaged to void any urine already present in the bladder. Each animal was then touched in the region of the i.c.v. cannulae and then returned to the metabolic cages for two and a half hours. On the day of the experiment, mice were again placed in metabolic cages that were positioned on top of the telemetry plates. The telemetry probes were then turned on with the least possible disturbance to the animals for the measurement of baseline MAP and HR (sampling frequency = 500 Hz; sampling interval = 2-sec). Following baseline sampling, the mice were removed from the metabolic cages and urine was voided from the bladder of each animal by massaging the abdominal area as described. An i.c.v. injector was then securely placed into each animal’s indwelling i.c.v. guide cannula. The i.c.v. injector was attached via polyethylene (PE-10) tubing to a Hamilton syringe for injection of drug/vehicle. Animals were then returned to the metabolic cages. The PE tubing was extended outside of the cages to keep disturbance to the animals to a minimum and to prevent it from being gnawed and damaged. After stabilization and additional baseline sampling (i.e., two 10-min pre-drug control periods), N/OFQ (0.03, 0.3, 1, 3 nmol) or isotonic saline vehicle (2 µl) was delivered i.c.v. via the Hamilton syringe injector assembly. Then, HR and MAP were measured continuously during a 120-min experimental period (twelve consecutive 10-min periods). Parameters analyzed were the MAP and HR responses to N/OFQ and isotonic saline control. Renal excretory studies involved the hourly collection of spontaneous urine voided immediately following vehicle/drug administration for two

hours. Parameters analyzed were urine flow rate, urinary excretion of sodium and potassium, urine osmolality and free-water clearance**.**

Cardiovascular antagonist studies: Studies were performed to determine the cardiovascular responses to i.c.v. N/OFQ in conscious mice that were pretreated centrally for 2-hours with either UFP-101 or N/OFQ. In these studies, mice that were chronically instrumented with both radio-telemeters and indwelling i.c.v. cannulae received a 2-hr i.c.v. pretreatment with either UFP-101 (10 or 30 nmol) or N/OFQ (1 or 3 nmol), and drug-evoked changes in HR and MAP were assessed using the experimental protocol described above. Renal function was not measured. After 2-hr drug pretreatment animals then received a second i.c.v. injection, this time with N/OFQ (1 or 3 nmol). HR and MAP were then measured for an additional 2 hours. Following all testing, animals were disconnected from their injectors and returned to their home cages.

Renal antagonist studies: Studies were performed to determine the renal excretory responses to i.c.v. N/OFQ in conscious mice that were pretreated centrally for 2-hours with either vehicle or UFP-101. For these studies, mice that were instrumented with chronic i.c.v. cannulae (i.e., no radio telemeters) received a 2-hr i.c.v. pretreatment with either isotonic saline (2 µl) or UFP-101 (10 or 30 nmol). Drug-evoked changes in urine output were then assessed during the pretreatment period using the experimental protocol described above. Following the 2 hour pretreatment, animals then received a second i.c.v. injection, this time of N/OFQ (1 or 3 nmol). Urine output was then measured hourly during a 2-hr experimental period. In other experiments urine output was also assessed in additional groups of mice that received simultaneous

administration (i.e., co-treatment) of N/OFQ (1 or 3 nnmol) and UFP-101 (10 or 30 nmol, respectively). Following all testing, animals were disconnected from their injectors and returned to their home cages.

Analytical techniques

Urine volume was determined gravimetrically. Urine sodium and potassium concentrations were measured by flame photometry (model 943, IL Labs). Urine osmolality was analyzed using a vapor pressure osmometer (Wescor, model 5500). Telemetry data was compiled and analyzed using Dataquest A.R.T. Software (DSI, v. 2.3) and Microsoft Excel. The output from the radio telemetry probes was recorded (500 Hz) using receiver plates placed beneath the home or metabolic cages. Data was sent to a consolidation matrix before being stored on a personal computer. Results were plotted and graphed using GraphPad Prism software (v. 4.00).

Statistics

Results are expressed as the mean ± SEM. The magnitude of the changes in cardiovascular and renal excretory parameters at different time points after i.c.v. injection of drugs were compared with respective group control values by a one-way repeated-measures analysis of variance (ANOVA) with subsequent Dunnett's test. Differences occurring between treatment groups (e.g., multiple doses of drug) were assessed by two-way repeated measures ANOVA with treatment being one fixed effect and time the other, with the interaction included. The time (minutes) was the repeated factor. Post-hoc analysis was performed using Bonferonni's test. Where appropriate, a Student's t-test was also used to compare means between two groups. In each case, statistical significance was defined as $p<0.05$.

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Drugs

N/OFQ (FGGFTGARKSARKLANQ) and UFP-101 ([Nphe^{1},Arg^{14},Lys^{15}]N/OFQ-NH_2) were synthesized and graciously provided by Drs. Remo Guerrini, Severo Salvadori and Girolamo Calo' (University of Ferrara, Ferrara, Italy). Stock solutions of peptides were prepared fresh and stored frozen. All drugs/vehicle were administered i.c.v. in a volume of 2 µl.

Results

The cardiovascular responses produced by the i.c.v. administration of N/OFQ in conscious ICR-CD1 mice are presented in Figure 1a (typical tracing), Figure 1b (time-course) and Figure 1c (peak delta). In Figure 1b, mean values for each parameter are depicted during control (C, 10-min) and consecutive 10-min experimental periods (time 10-120 min) beginning immediately after the i.c.v. injection of N/OFQ (0.03, 0.3, 1 or 3 nmol) or isotonic saline vehicle. As shown (Fig. 1b), the i.c.v. administration of isotonic saline did not alter HR or MAP at any point throughout the study. Following i.c.v. injection of N/OFQ at 1 or 3 nmol, however, HR and MAP began to decrease within 30-sec to 1-min (Figs. 1a and 1b). At 3 nmol, the highest dose tested, N/OFQ decreased HR from 672 ±23 (C) to 411±30 beats/min (20-min) and MAP from 108±4 (C) to 62±6 mmHg (20-min) (Fig. 1b). The peak reductions in each parameter occurred 20 minutes after central drug administration, with each response returning to baseline by 50- and 40-minutes, respectively, post-injection (Fig 1b). Moreover, the peak cardiovascular depressor responses elicited by central N/OFQ in conscious mice were dose-dependent (Fig. 1c), with responses in HR being significantly different than the vehicle-treated group at 1 nmol and maximal at 3 nmol. Similarly, the central N/OFQ-evoked reduction in MAP was rapid in onset (Figs. 1a and 1b) and significantly different than control values at 0.3, 1 and 3 nmol doses (Figs. 1b and 1c); maximal reduction in MAP was produced by 1 nmol of N/OFQ.

Figure 2 illustrates the cumulative urine output produced by i.c.v. N/OFQ, which was sampled during a 2-hr period in the same telemetered animals for which cardiovascular data is presented in Figs. 1b and 1c. Concurrent with the cardiovascular

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depressor responses, i.c.v. N/OFQ produced a dose-dependent diuresis over the course of 120-min (Fig. 2). An ANOVA confirmed that the peak diuresis produced by i.c.v. injection of 1 and 3 nmol N/OFQ was significantly ($p<0.01$) greater than that elicited by the saline vehicle-treated group**.** However, there were no statistical differences in urine output produced between any of the doses of N/OFQ tested. At the 3 nmol dose, i.c.v. N/OFQ increased urine output over the 2-hr period to 0.65±0.03 cc as compared to 0.15±0.09 cc in the saline-treated group. In contrast to these higher doses, i.c.v. injection of 0.03 and 0.3 nmol N/OFQ did not significantly alter 2-hr urine output as compared to vehicle treatment. Doses of N/OFQ greater than 3 nmol were not fully tested since they elicited sedation in the animals. In regards to time-course (Table 1), the 3 nmol dose of N/OFQ increased urine output during both the first and second hours of the protocol as compared to levels of urine output measured during the same time periods in the isotonic saline vehicle group. In contrast, the 1 nmol dose increased urine output only during the first hour as compared to animals treated centrally with isotonic saline (Table 1). However, i.c.v. doses of N/OFQ that significantly increased urine output had no significant effects on urinary sodium or potassium excretion or free water clearance (Table 1).

In separate studies, changes in HR and MAP were examined in telemetered mice that were pretreated centrally with the purported NOP receptor antagonist UFP-101 (2-hr pretreatment period) and then challenged with a subsequent i.c.v. injection of N/OFQ. As shown in Figure 3a (data for the pretreatment period), i.c.v. injection of 10 or 30 nmol UFP-101 alone significantly decreased HR in conscious mice (C: 697±59 and 628±22 beats/min, resp. vs. 30-min: 508±73 and 383±28 bpm, resp.). At the lower

dose the bradycardia persisted for 90-min, whereas at the 30 nmol dose HR did not fully recover over the 2-hr pretreatment period. In contrast, UFP-101 did not appreciably alter MAP. These two doses of UFP-101 were selected so as to maintain a 10:1 antagonist to agonist ratio which other investigators have reported is optimal for the antagonist action of UFP-101 at NOP receptors *in vivo* (Nazzaro et al., 2006; Hashiba et al., 2003; Vitale et al., 2006). Figure 3b (left panel) illustrates the peak changes in HR and MAP produced by the subsequent i.c.v. injection of N/OFQ (1 or 3 nmol) observed in these same groups of mice that were pretreated centrally with UFP-101. As shown (Fig. 3b, left panel), 2-hr i.c.v. pretreatment with 10 nmol UFP-101 completely abolished the bradycardic and hypotensive responses to i.c.v. injection of 1 nmol N/OFQ (C: 600±45 beats/min vs. N/OFQ 20-min: 628±33 beats/min and C: 122±6 mmHg vs. N/OFQ 20-min: 121±5 mmHg, resp.). Similarly, the bradycardia and hypotensive effects typically produced by i.c.v. injection of 3 nmol N/OFQ were completely prevented in mice that received 2-hr i.c.v. pretreatment with 30 nmol UFP-101 (Fig. 3b; C: 605±31 beats/min vs. N/OFQ 20-min: 600±29 beats/min and C: 108±3 mmHg vs. N/OFQ 20-min: 104±4 mmHg, resp.). Note that this antagonist action of UFP-101 (30 nmol) occurred despite the fact that in this group the control level for HR measured prior to N/OFQ injection had not fully recovered by the end of the 2-hr pretreatment period (see Fig. 3a). In contrast to these findings, Fig. 3b (right panel) shows that i.c.v. N/OFQ produced marked reductions in HR and MAP in conscious mice that had instead been pretreated centrally for 2-hrs with N/OFQ (1 or 3 nmol). The magnitudes of these depressor responses with the 1 nmol (C: 674±31 bpm and 112±6 mmHg; N/OFQ 20-min: 515±51 bpm and 89±11 mmHg) and 3 nmol dose (C: 699±31 bpm and 110±6 mmHg; N/OFQ 20-min: 516±31

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bpm and 81±2 mmHg) were similar to those observed when N/OFQ was initially given as a pretreatment (data not shown).

Figure 4 illustrates the levels for cumulative urine output produced by the i.c.v. injection of N/OFQ (1 or 3 nmol) in groups of conscious mice pretreated centrally for 2-hr with UFP-101 or vehicle and in which cardiovascular function was not measured (i.e., non-telemetered). As shown, the diuretic responses to i.c.v. N/OFQ at 1 and 3 nmol were completely abolished by a 2-hr pretreatment with UFP-101 at 10 nmol (V=0.27±0.06 cc/2hr) and 30 nmol (V=0.24±0.06 cc/2hr), respectively, whereas that following a 2-hr pretreatment with isotonic saline vehicle was not (V=0.67±0.08 and 0.62±0.14 cc/2hr, resp.). Moreover, pretreatment with UFP-101 alone had no significant effect on urine output (10 nmol, V=0.28±0.11 cc/2hr; 30 nmol, 0.45±0.07 cc/2hr). UFP-101 also blocked the diuresis elicited by i.c.v. N/OFQ when the two compounds were administered simultaneously as a co-treatment (10 nmol UFP-101:1 nmol N/OFQ, V=0.23±0.12 cc/2hr; 30 nmol UFP-101:3 nmol N/OFQ, V=0.29±0.040 cc/2hr).

Discussion

N/OFQ and NOP receptors are abundantly expressed in murine CNS cardiovascular regulatory sites (Ikeda et al., 1998; Houtani et al., 2000). However, in contrast to the rat, the cardiovascular and renal responses produced by activation of the N/OFQ-NOP system in the CNS of mice have not been fully characterized. In related studies, Rizzi et al. (2004) reported that i.c.v. N/OFQ elicits dose-dependent diuresis (UFP-101- sensitive) in conscious mice; however, this peptide's cardiovascular effects were not explored in these studies. Extending these findings, the results of the present investigations demonstrate that, in conscious telemetered ICR-CD1 mice, the i.c.v. administration of N/OFQ markedly alters HR (bradycardia) and MAP (hypotension) at doses that produce diuresis. The cardiovascular and renal responses to central N/OFQ occurred in a dose-dependent manner and were shown to be mediated by a selective pathway involving NOP receptors.

In comparison to findings observed in conscious rats (Kapusta and Kenigs, 1999; Kapusta, 2000), the pattern of the changes in cardiovascular function produced by i.c.v. N/OFQ in conscious mice was similar in direction and duration but remarkably different in magnitude. More specifically, at maximally effective doses N/OFQ lowers HR and MAP in both conscious male ICR-CD1 mice and Sprague-Dawley rats, with peak responses in each species occurring 20-min following the peptide's i.c.v. administration. However, compared to respective pre-drug control values, the 10-min peak HR response to i.c.v. N/OFQ (3 nmol) was considerably greater in mice (Δ=-261±27 bpm, nadir 20-min, Fig. 1c of present study) than that produced by N/OFQ (5.5 nmol) in rats (Δ=-82±11 bpm, nadir 20-min; Kapusta and Kenigs, 1999). The 10-min peak MAP

response to i.c.v. N/OFQ was also greater in mice (Δ=-46±5 mmHg, 3 nmol, Fig. 1c) than in rats (Δ=-16±5 mmHg, 5.5 nmol; Kapusta and Kenigs, 1999). Note that the i.c.v. doses for this comparison are based on dose-response studies that established the lowest i.c.v. dose of N/OFQ which produced maximal cardiovascular and renal responses in each species; 3 nmol for mouse (current study) and 5.5 nmol for rat (Kapusta et. al., 1997). The observation that i.c.v. N/OFQ evoked greater bradycardic and hypotensive responses in conscious mice is likely due to the increased contribution of the sympathetic nervous system to basal autonomic tone in mice versus rats. In this setting, in mice the N/OFQ-mediated inhibition of central sympathetic outflow (and possibly activation of parasympathetic outflow) would, therefore, be anticipated to elicit greater magnitudes of cardio-depressive responses.

In addition to marked cardiovascular depressor responses, the results of the present studies demonstrate that the central administration of N/OFQ to conscious mice significantly increased urine output. This finding is in agreement with those of a separate but related study (Rizzi et al., 2004) in which the renal excretory responses produced by the central and peripheral administration of N/OFQ and a novel NOP receptor ligand UFP-102 were examined in mice under conditions in which cardiovascular function was not measured. In the present study, as compared to control levels in mice treated with vehicle, i.c.v. N/OFQ (3 nmol) increased cumulative urine output over the 2-hr study by 333% (Fig. 2). Interestingly, the N/OFQ-evoked diuresis occurred in both the first and second hour periods of the protocol; thus, the diuresis continued well after the N/OFQ-evoked bradycardia and hypotension had returned to baseline (approximately 40-50 min). As previously reported (Kapusta and Kenigs, 1999),

the central administration of N/OFQ (5.5 nmol) to conscious rats also increased urine output by approximately 254%. In these rat studies it was observed that the peak diuresis produced by central N/OFQ also occurred at a time (40-min post drug injection) in which the cardiovascular depressor responses to central N/OFQ had fully recovered. In this and other rat studies, however, the duration of the central N/OFQ-evoked diuresis was shorter and lasted only for approximately 1 hour (Kapusta et al., 1997; Kapusta and Kenigs, 1999) as compared to persisting for 2 hours in the present studies in mice. Another important difference is that in rats i.c.v. N/OFQ has been shown to produce a marked decrease in urinary sodium excretion that occurs concurrent with the diuresis; thus, in rats central N/OFQ produces diuresis, antinatriuresis and an increase in free-water clearance (i.e., a water diuresis) (Kapusta et al., 1997; Kapusta and Kenigs, 1999). Yet in contrast, in the present studies i.c.v. N/OFQ did not alter urinary sodium excretion or free-water clearance in conscious telemetered mice. In addition to species differences, it is likely that variations in the experimental methods and protocols used in the rat versus mouse studies contributed to the differences in the renal excretory responses observed between these two species. Of merit, in the rat studies urine was collected from chronically implanted bladder catheters from animals that were continuously infused i.v. with isotonic saline (55 µl/min) (Kapusta et al., 1997; Kapusta and Kenigs, 1999). Under these conditions (i.e., i.v. isotonic saline infusion) drug-induced changes (e.g., increases or decreases) in urine flow rate and/or urinary sodium excretion can readily be detected (Kapusta et al., 1997; Kapusta and Kenigs, 1999). This is in contrast to the present studies in which urine was spontaneously collected from euvolemic mice (i.e., no volume load) that were placed in individual metabolic

cages. In this latter case, because basal levels for urine output and urinary sodium excretion were substantially low it is likely that a bona fide antinatriuretic effect of central N/OFQ could not be detected in the present studies in mice. Regardless, it is clear from these investigations that in both species the central administration of N/OFQ markedly enhanced the renal excretion of water. Although not tested, it is likely that in mice i.c.v. N/OFQ evokes diuresis via inhibiting the secretion/release of vasopressin into the systemic circulation as this peptide has been shown to do in the rat (Kapusta et al., 1997; Kakiya et al., 2000).

UFP-101 has been demonstrated to behave as a selective and pure NOP receptor antagonist in many species and biological systems (reviewed by Calo et al., 2005). Furthermore, i.c.v. UFP-101 effectively blocks the diuretic response to i.c.v. N/OFQ in conscious mice when the two compounds are administered as a co-treatment (Rizzi et al., 2004). Here we report that, in conscious telemetered mice, a 2-hr pretreatment with i.c.v. UFP-101 effectively blocked the diuretic response to a subsequent i.c.v. N/OFQ injection. UFP-101 itself did not alter urine output, thereby confirming that UFP-101 is a selective NOP receptor antagonist in preventing N/OFQ's action on renal excretory function. However, when administered i.c.v. either alone or as a co-treatment with N/OFQ (data not presented), UFP-101 profoundly and selectively lowered HR. Despite this residual agonist-like activity of UFP-101, it was shown that once HR recovered to/toward baseline (≥2 hrs) the bradycardia and hypotensive responses typically elicited by i.c.v. N/OFQ injection were completely blocked (Fig. 3b). This antagonism was most likely due to a receptor antagonist action of UFP-101 at NOP receptors and not attributable to tachyphylaxis. This possibility is suggested since in

other mice a 2-hr i.c.v. pretreatment with N/OFQ did not block the cardiovascular depressor responses to a subsequent (i.e., 2nd) i.c.v. N/OFQ injection (Fig. 3b).

Thus, while the mechanism by which UFP-101 affects HR has yet to be determined, these findings strongly support the notion that in mice i.c.v. N/OFQ produces cardiovascular depressor and diuretic responses via selective activation of central NOP receptors. This hypothesis is further supported by pilot data in which we have shown that the cardiovascular depressor (i.e., decreases in HR and MAP) responses to i.c.v. N/OFQ, but not the bradycardia to i.c.v. UFP-101, are abolished in transgenic NOP receptor knockout mice (personal observation).

In addition to characterizing the cardiovascular and renal responses produced by exogenous administration of N/OFQ, it is of physiological importance to understand how the endogenous N/OFQ-NOP receptor system participates in the central (and peripheral) control of cardiovascular and/or renal function. The findings of the current studies with UFP-101 indicate that the native N/OFQ-NOP receptor system in mice is not involved in the tonic regulation of MAP or the renal excretion of water/sodium. However, given that UFP-101 did affect HR (produced bradycardia) following acute i.c.v. administration, this conclusion must be carefully interpreted. Considering this point, it is likely that other investigations that incorporate transgenic NOP receptor (or N/OFQ ligand) knockout mice as well as small interfering RNA (siRNA) targeted against the NOP receptor may serve as additional approaches for investigating N/OFQ's endogenous physiological role(s) in cardiovascular and renal function during health and stress/pathology. With this likelihood, the findings of the present studies, which were performed in wild-type mice, will provide a foundation for comparison of results from investigations that will examine

N/OFQ's role in the central control of cardiovascular and renal function using these transgenic or siRNA model systems.

In summary, our results show that in conscious mice the central administration of the opioid-like peptide, N/OFQ, produces marked cardiovascular depressor responses at doses that also elicit diuresis. These responses were completely prevented by i.c.v. pretreatment of mice with UFP-101, thus, demonstrating that the central effects of N/OFQ on cardiovascular function and urine output are mediated by a NOP receptor pathway.

Acknowledgements

The authors would like to extend their thanks to Professors Girolamo Calo', Domenico Regoli, Severo Salvadori and Remo Guerrini at the University of Ferrara, Ferrara, Italy, for graciously providing the N/OFQ and UFP-101 peptides used in these studies. We also thank Dr. Daniela Rizzi for technical assistance and discussion of data.

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JPET Fast Forward. Published on April 23, 2007 as DOI: 10.1124/jpet.107.120394
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Footnotes

This research was supported by grants to D.R.K. from the National Institute of Diabetes and Digestive and Kidney Diseases (DK-43337 and DK-02605) and the Heart Blood and Lung Institute (HL71212). Additional funding for this work was provided to the Cardiac and Vascular Core Facility in the Department of Pharmacology and Center of Excellence in Cardiovascular Research at Louisiana State University Health Sciences Center by COBRE Grant P20 RR018766 (D.R.K.) from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH).

Legends for figures

Figure 1a. Representative tracing of the cardiovascular responses produced by the i.c.v. injection of N/OFQ in a conscious telemetered ICR-CD1 mouse. The tracing illustrates the typical systemic cardiovascular depressor responses produced by the i.c.v. injection of 3 nmol N/OFQ in mice. Heart rate and mean arterial pressure were measured via telemetry during control (C, 5-min) and immediately after i.c.v. N/OFQ injection for 120-min (500 Hz, consecutive 2-sec sampling interval). HR, heart rate; MAP, mean arterial pressure.

Figure 1b. Time-course of the cardiovascular responses produced by the i.c.v. injection of N/OFQ in conscious, telemetered ICR-CD1 mice. The values are mean ± S.E.M. and illustrate the systemic cardiovascular responses produced by the i.c.v. injection of 0.03 nmol N/OFQ (□, n=7), 0.3 nmol N/OFQ (◊, n=6), 1 nmol N/OFQ (■, n=9), 3 nmol N/OFQ (♦, n=7) or isotonic saline vehicle (2 µl, ○, n=8). Heart rate and mean arterial pressure were measured during control (C, 10-min) and immediately after i.c.v. drug/vehicle injection for 120-min denoted as time periods 10 to 120 min (consecutive 10-min periods). HR, heart rate; MAP, mean arterial pressure. *, $p<0.05$ when compared to respective pre-drug control (time C).

JPET Fast Forward. Published on April 23, 2007 as DOI: 10.1124/jpet.107.120394
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Figure 1c. Peak cardiovascular responses produced by the i.c.v. injection of N/OFQ in conscious, telemetered ICR-CD1 mice. The values are mean ± S.E.M. and illustrate the peak systemic cardiovascular responses produced by the i.c.v. injection of 0.03 nmol N/OFQ (n=7), 0.3 nmol N/OFQ (n=6), 1 nmol N/OFQ (n=9), 3 nmol N/OFQ (n=7) or isotonic saline vehicle (2 µl; n=8) measured within the first 20 min (0-20 min) following vehicle/drug administration. Note that data depicted in this figure are from the same groups of telemetered mice for which cardiovascular data is presented in Fig. 1b. HR, heart rate; MAP, mean arterial pressure. *, $p<0.05$ when compared to the saline-treated group.

Figure 2. Changes in urine output produced by the i.c.v. injection of N/OFQ in conscious, telemetered ICR-CD1 mice. The values are mean ± S.E.M. and illustrate the changes in cumulative urine output produced by the i.c.v. injection of 0.03 nmol N/OFQ (n=8), 0.3 nmol N/OFQ (n=7), 1 nmol N/OFQ (n=14), 3 nmol N/OFQ (n=12) or isotonic saline vehicle (2 µl; n=13). Note that data depicted in this figure are from the same groups of telemetered mice in which cardiovascular data is presented in Figs. 1b and 1c. Data shown in Fig. 2 represents mean values for cumulative urine collected throughout the entire 120-min experimental protocol beginning immediately after vehicle/drug administration. V, urine output. **, $p<0.01$ compared to isotonic saline vehicle-treated mice.

Figure 3a. Time-course of the cardiovascular responses produced by i.c.v. pretreatment of conscious, telemetered ICR-CD1 mice with the purported NOP receptor antagonist, UFP-101. The values are mean ± S.E.M. and illustrate the systemic cardiovascular responses produced by the i.c.v. injection of 10 nmol UFP-101 (■, n=5), 30 nmol UFP-101 (▲, n=5) or isotonic saline vehicle (2 µl, ○, n=8). Heart rate and mean arterial pressure were measured during control (C, 10-min) and immediately after i.c.v. drug/vehicle injection during a 120-min "pretreatment" period denoted as time periods 10 to 120 min (consecutive 10-min samples). HR, heart rate; MAP, mean arterial pressure. *, P<0.05 when compared to respective pre-drug control (time C).

Figure 3b. Peak cardiovascular responses produced by i.c.v. N/OFQ in conscious ICR-CD1 mice that were pretreated centrally for 2-hr with either UFP-101 or N/OFQ. The values are mean ± S.E.M. and illustrate the peak systemic cardiovascular responses produced by the i.c.v. injection of N/OFQ (1 or 3 nmol) following a 2-hr i.c.v. pretreatment with either UFP-101 (left panel; 10 nmol, n=6; 30 nmol; n=5) or N/OFQ (right panel; 1 nmol, n=5; 3 nmol, n=4). An antagonist**:**agonist ratio of 10**:**1 was used for studies with UFP-101 and N/OFQ. Data shown are the peak changes in cardiovascular function that occurred within the first 20 min (0-20 min) following i.c.v. drug/vehicle administration. HR, heart rate; MAP, mean arterial pressure. *, p<0.05 when compared to baseline value immediately prior to N/OFQ.

Figure 4. Changes in urine output produced by the i.c.v. injection of N/OFQ in conscious mice pretreated i.c.v. for 2-hr with either isotonic saline vehicle or UFP-101. The values are mean ± S.E.M. and illustrate the changes in cumulative urine output produced by i.c.v. injection of N/OFQ (1 or 3 nmol) in mice that received a 2-hr pretreatment (pt) with isotonic saline vehicle (2 µl; n=8/group) or UFP-101 (10 nmol, n=8; 30 nmol, n=8). An antagonist**:**agonist ratio of 10**:**1 was used for studies with UFP-101 and N/OFQ. Data shown are mean values for N/OFQ-evoked changes in cumulative urine output measured during a 2-hr experimental period, which began after completion of the 2-hr pretreatment. Values (mean ± S.E.M.) are also illustrated (hatched bars) for the changes in 2-hr cumulative urine output produced by the co-treatment of UFP-101 and N/OFQ (1 and 10 nmol, respectively, n=8; 3 and 30 nmol, respectively, n=8). V, urine output. *, $p<0.05$ compared to isotonic saline vehicle pretreatment; †, $p<0.05$ compared to N/OFQ in animals pretreated with isotonic saline.

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Table 1: Time-course renal excretory responses produced by the i.c.v. injection of N/OFQ in conscious, telemetered ICR-CD1 mice.

	FIRST HOUR				SECOND HOUR			
ICV TREATMENT	**V (cc/hr)**	**UNaV (µEq/hr)**	**UKV (µEq/hr)**	**C_{H20} (µl/hr)**	**V (cc/hr)**	**UNaV (µEq/hr)**	**UKV (µEq/hr)**	**C_{H20} (µl/hr)**
Saline (n=13)	0.07±0.06	27±16	29±13	-0.93±0.43	0.08±0.04	27±9	24±4	-0.82±0.09
N/OFQ, 0.03 nmol (n=8)	0.20±0.07	31±4	46±13	-1.35±0.39	0.13±0.05	20±3	20±6	-0.96±0.23
N/OFQ, 0.3 nmol (n=7)	0.29±0.10	28±17	28±6	-0.99±0.20	0.02±0.02[a]	13±13 [a]	13±13 [a]	-0.38±-0.38 [a]
N/OFQ, 1 nmol (n=14)	0.30±0.06*	23±6	33±7	-1.02±0.18	0.18±0.05	29±7	32±8	-1.67±0.31
N/OFQ, 3 nmol (n=12)	0.34±0.08*	20±6	23±4	-0.71±0.08	0.31±0.06*	36±10	31±10	-1.09±0.28

[a,] denotes that only one of seven mice tested in this group excreted urine in the second hour.

Figure 1a

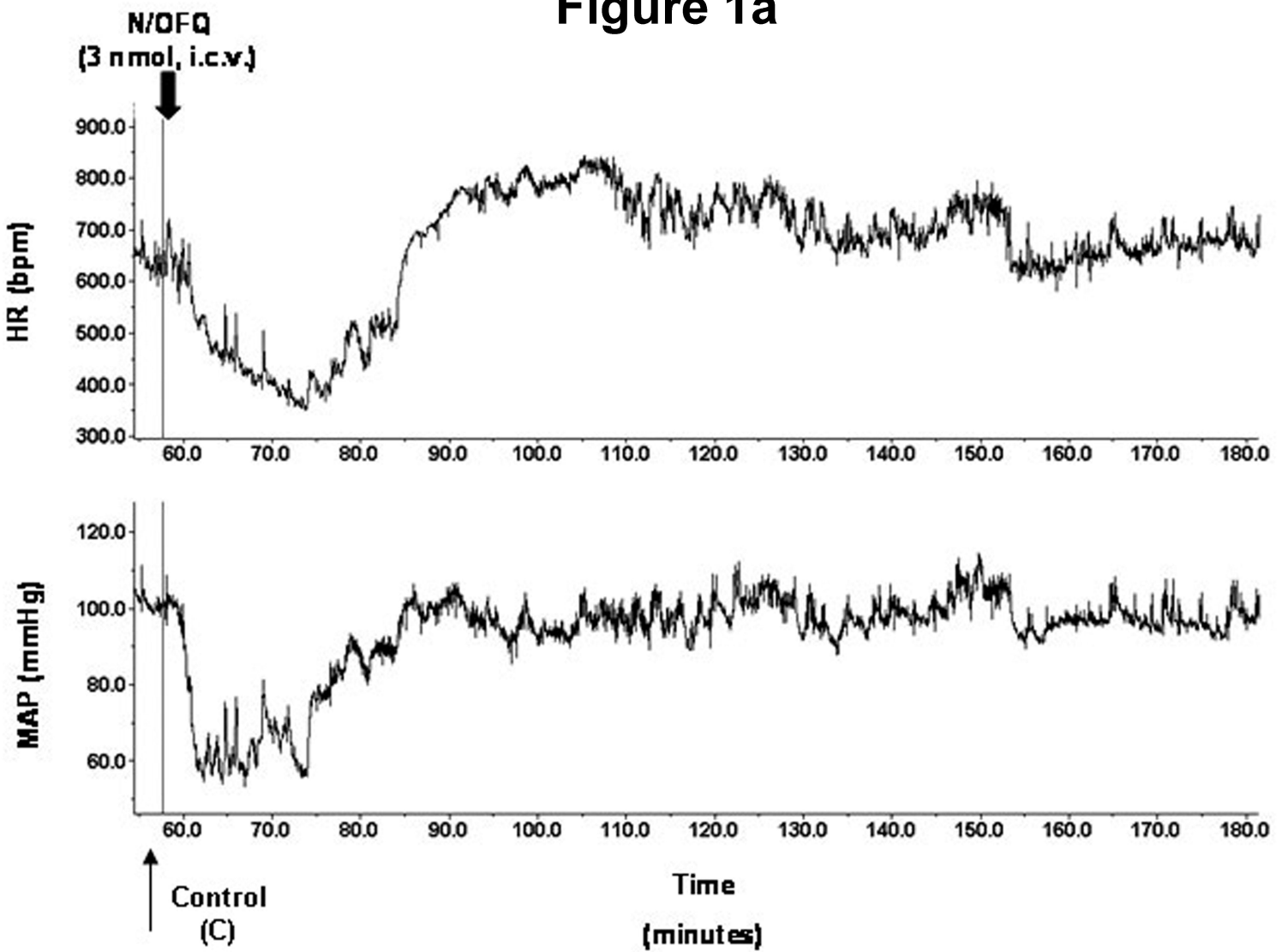
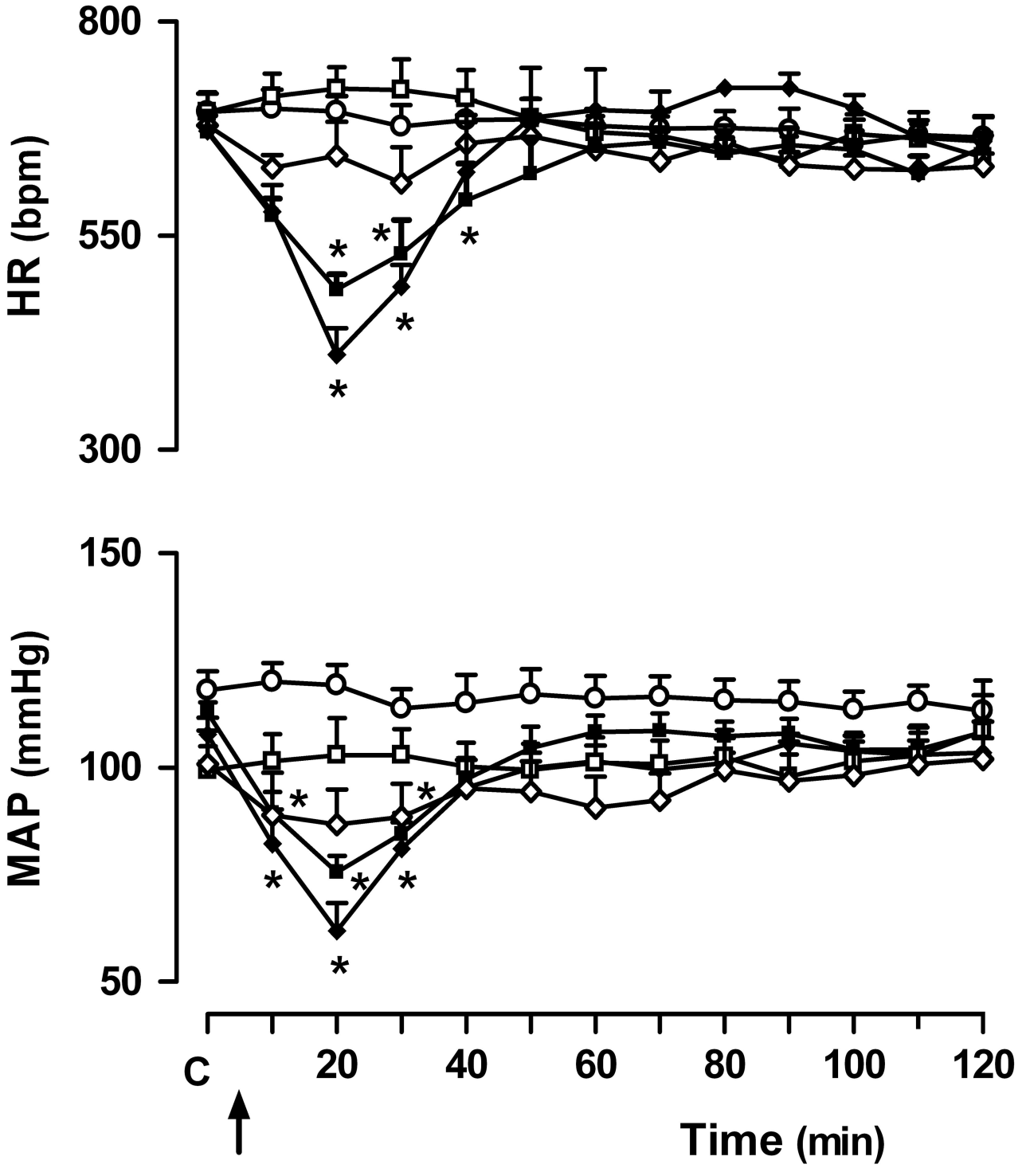

N/OFQ
(3 nmol, i.c.v.)
HR (bpm)
900.0
800.0
700.0
600.0
500.0
400.0
300.0
60.0
70.0
80.0
90.0
100.0
110.0
120.0
130.0
140.0
150.0
160.0
170.0
180.0
MAP (mmHg)
120.0
100.0
80.0
60.0
Control
(C)
Time
(minutes)

Figure 1b


800
550
300
HR (bpm)
150
100
50
MAP (mmHg)
C
20
40
60
80
100
120
Time (min)
i.c.v.:
Saline (n=8)
N/OFQ (0.03 nmol) (n=7)
N/OFQ (0.3 nmol) (n=6)
N/OFQ (1 nmol) (n=9)
N/OFQ (3 nmol) (n=7)

JPET Fast Forward. Published on April 23, 2007 as DOI: 10.1124/jpet.107.120394

Figure 1c

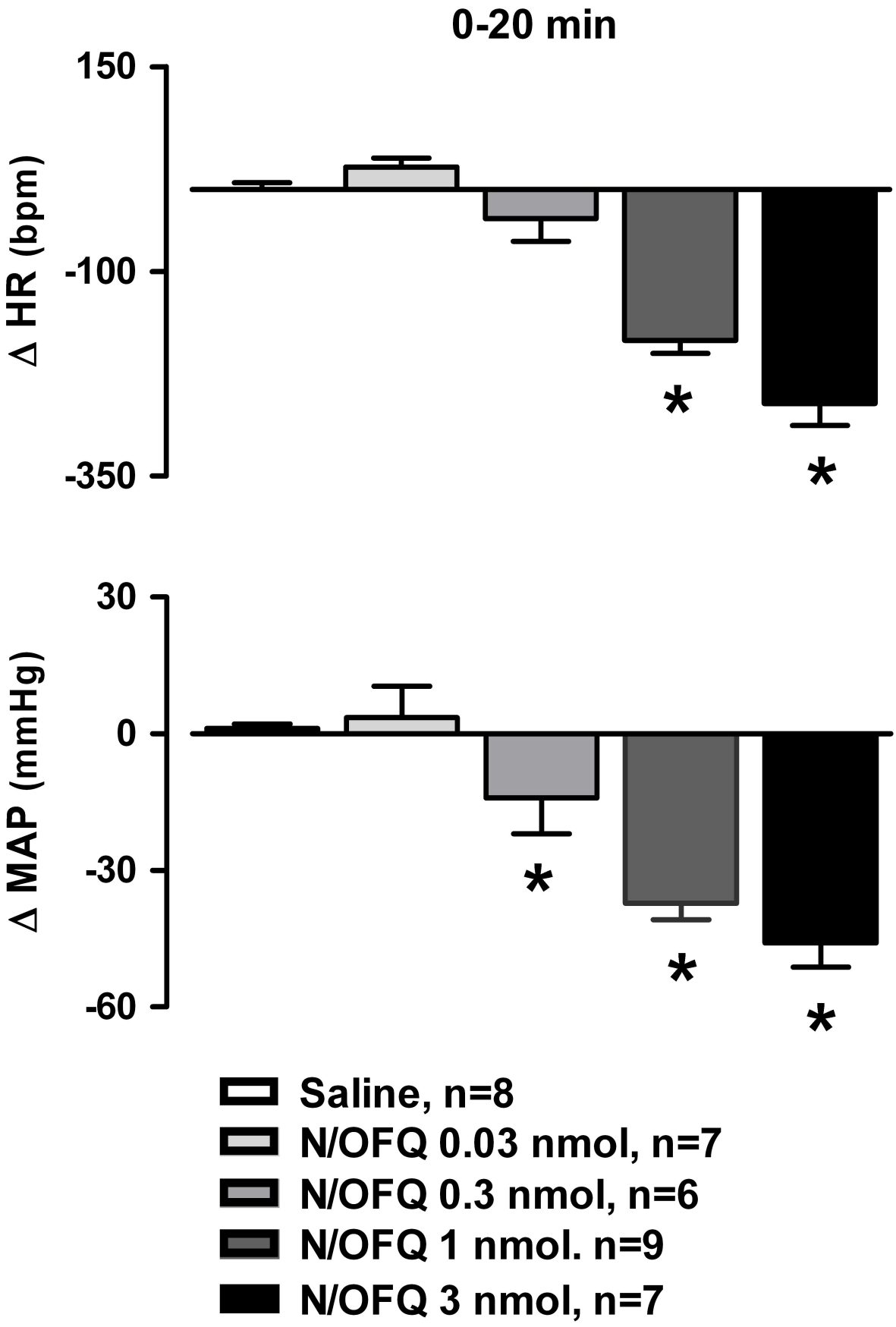


Figure 2

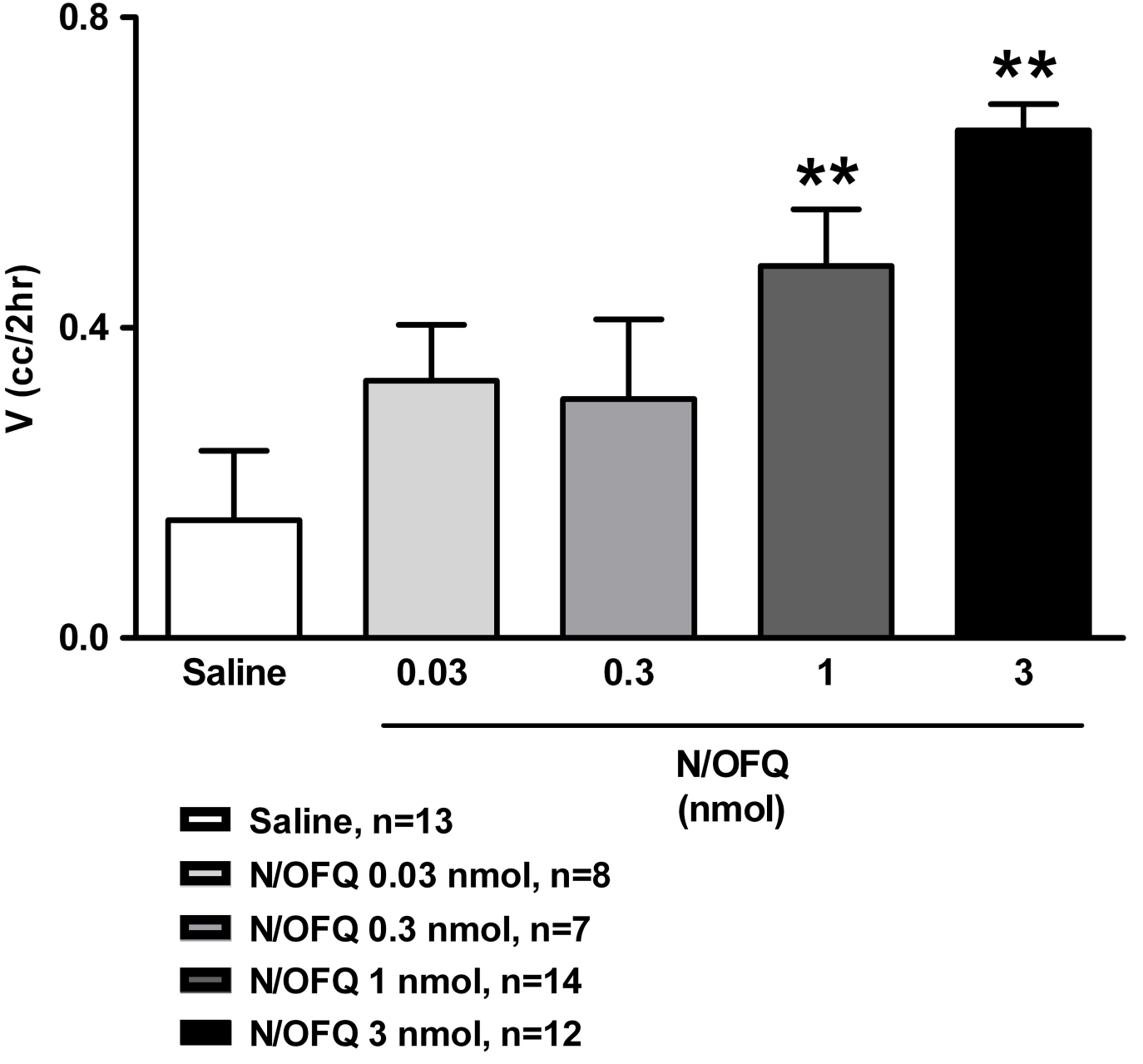

0.8
0.4
0.0
V (cc/2hr)
**
**
Saline
0.03
0.3
1
3
N/OFQ
(nmol)
Saline, n=13
N/OFQ 0.03 nmol, n=8
N/OFQ 0.3 nmol, n=7
N/OFQ 1 nmol, n=14
N/OFQ 3 nmol, n=12

Figure 3a

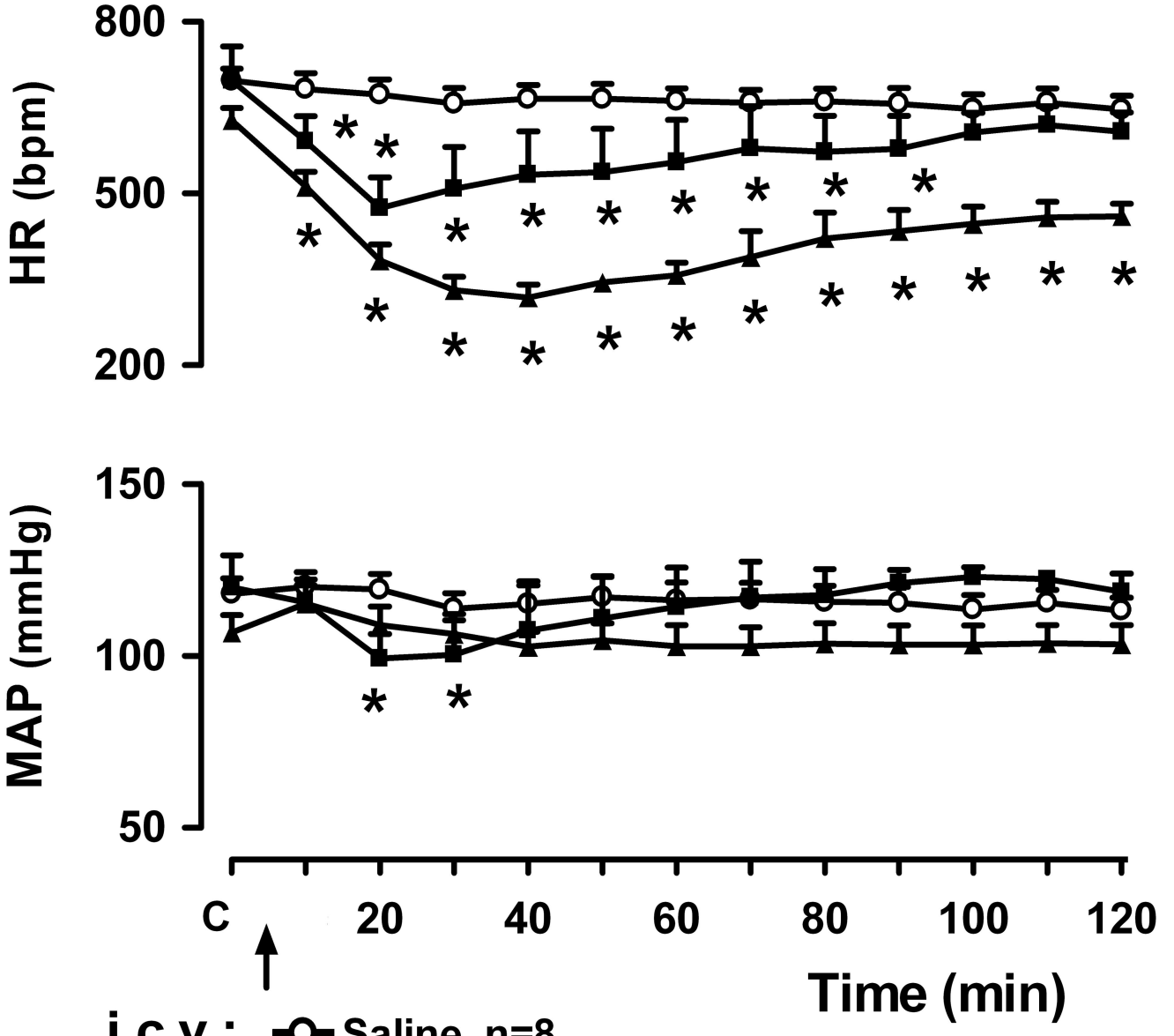

800
500
200
HR (bpm)
150
100
50
MAP (mmHg)
C
20
40
60
80
100
120
Time (min)
i.c.v.:
Saline, n=8
UFP-101 (10 nmol), n=5
UFP-101 (30 nmol), n=5

Figure 3b

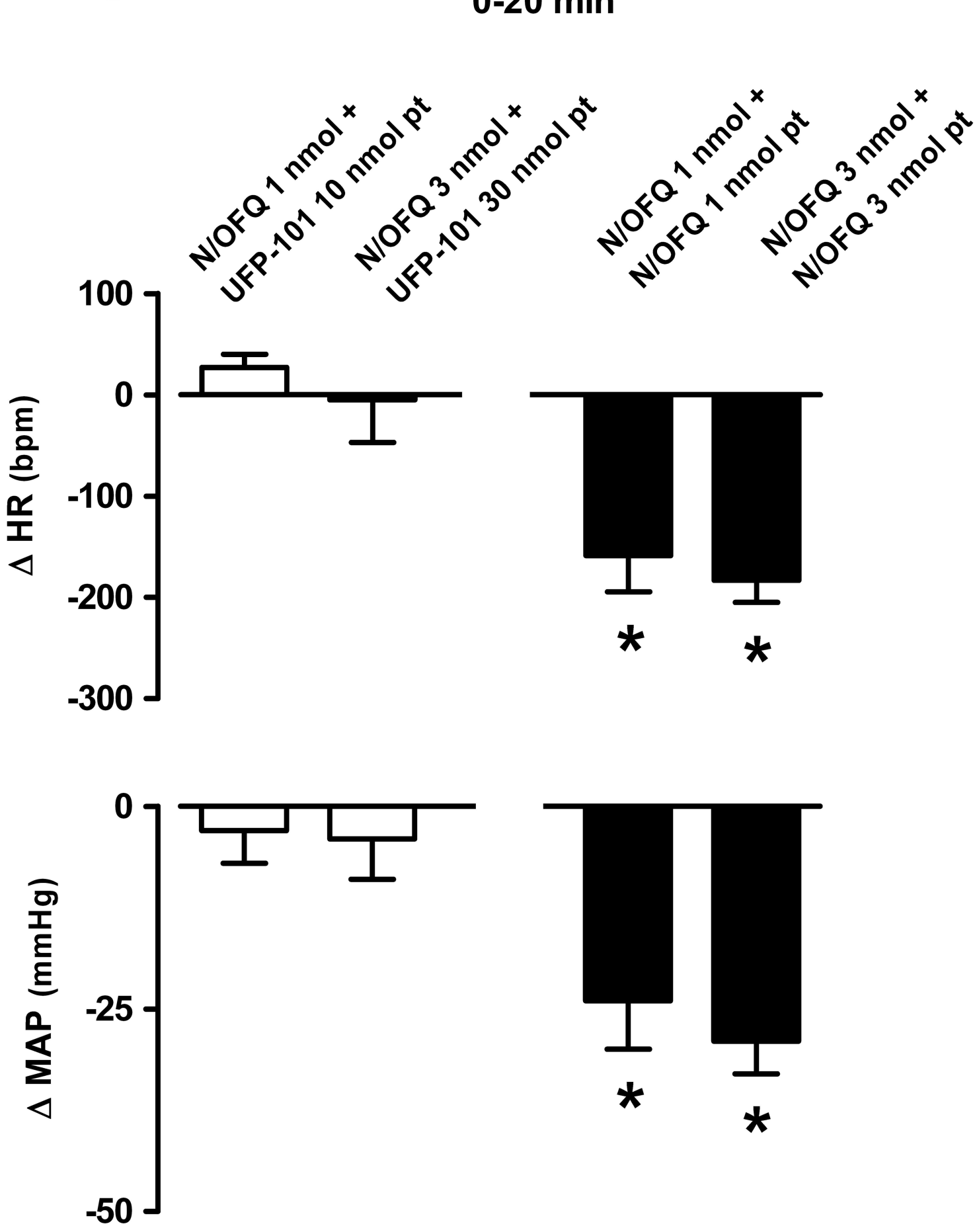
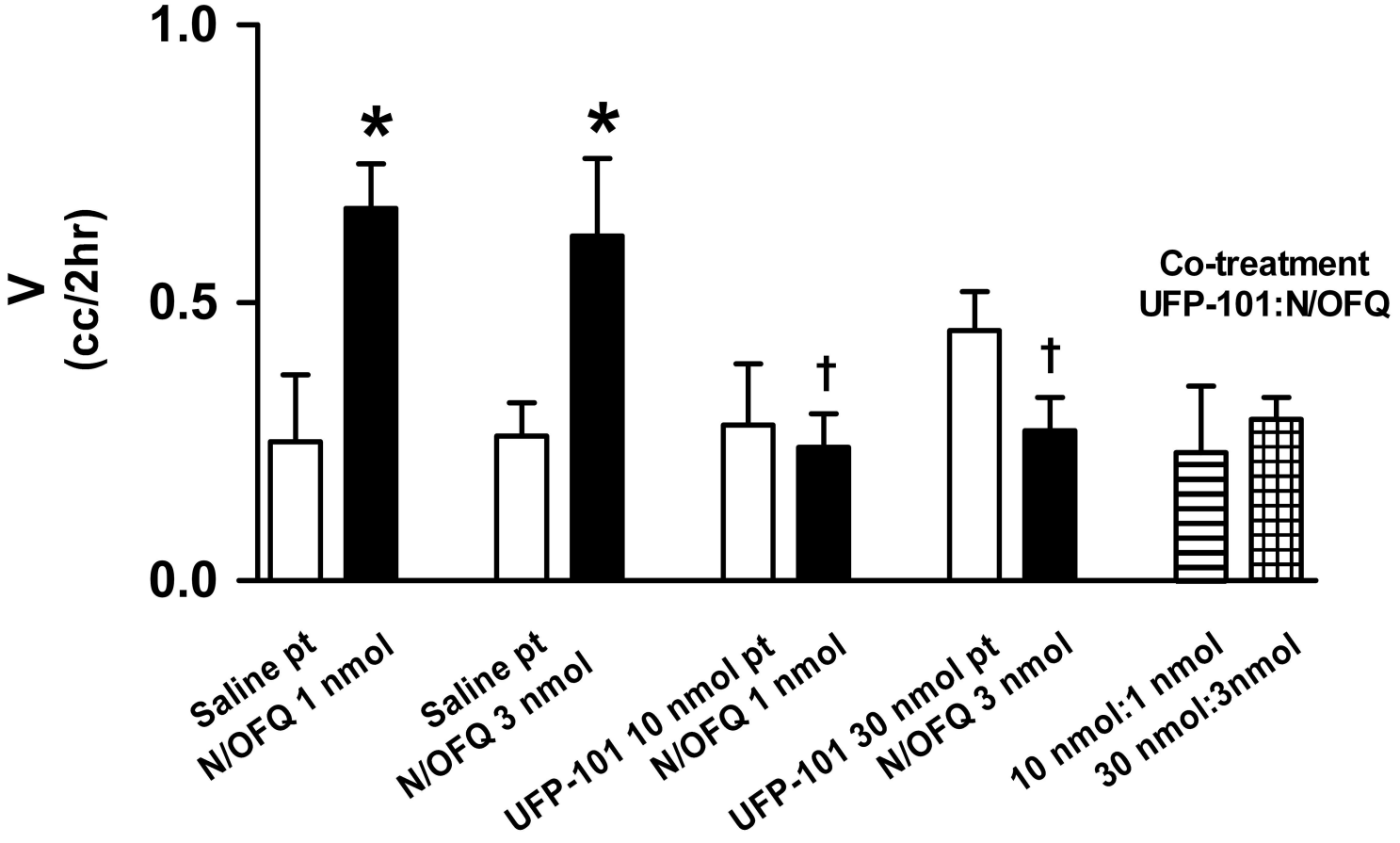


Figure 4


1.0
0.5
0.0
V
(cc/2hr)
*
*
†
†
Co-treatment
UFP-101:N/OFQ
Saline pt
N/OFQ 1 nmol
Saline pt
N/OFQ 3 nmol
UFP-101 10 nmol pt
N/OFQ 1 nmol
UFP-101 30 nmol pt
N/OFQ 3 nmol
10 nmol:1 nmol
30 nmol:3nmol