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RESEARCH PAPER

Urotensin-II Induces Ear Flushing in Rats

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Background and purpose: While investigating the effects of systemic urotensin II (U-II), a potent vasoactive peptide acting at the UT receptor, we observed ear pinna flushing after systemic administration to conscious rats. In the present study, U-II-induced ear flushing was quantified in terms of ear pinna temperature change and potential mechanisms were explored.
Experimental approach: U-II-induced ear flushing was quantified by measuring lateral ear pinna temperature changes and compared to that of calcitonin gene-related peptide (CGRP), a known cutaneous vasodilator. Further, the effects of a variety of pharmacological agents on U-II-induced ear flushing were explored.
Key results: Subcutaneous injection of U-II (9 μg kg^{-1})produced localized ear pinna flushing with an onset of $\sim$15 min, a duration of $\sim$30 min and a maximal temperature change of 9°C. In contrast, CGRP caused cutaneous flushing within multiple cutaneous beds including the ear pinna with a shorter onset and greater duration than U-II. A potent UT receptor antagonist, urantide, blocked U-II-induced ear flushing but did not affect CGRP-induced ear flushing. Pretreatment with indomethacin or L-N$^{\omega}$ -nitroarginine methylester (L-NAME) abolished U-II-induced ear flushing. Mecamylamine or propranolol did not affect this response to U-II. Direct intracerebroventricular injection studies suggested that the ear flushing response to U-II was not mediated directly by the CNS.
Conclusion and implications: Our results suggest that U-II-induced ear flushing and temperature increase is mediated by peripheral activation of the UT receptor and involves prostaglandin- and nitric oxide-mediated vasodilation of small capillary beds in the rat ear pinna.
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Keywords: urotensin II; ear flush; ear pinna surface temperature; vasodilation; prostaglandin; nitric oxide; rat

Abbreviations: CGRP, calcitonin gene-related peptide; i.c.v., intracerebroventricular; L-NAME, L-*N*$^{\omega}$-nitroarginine methylester; U-II, urotensin II; URP, urotensin-II related peptide; UT receptor, urotensin II receptor

Introduction

Urotensin-II (U-II), a somatostatin-like peptide originally isolated from goby fish, has been found to be expressed widely in mammals. The U-II peptide contains a cyclic C-terminal hexapeptide sequence that is biologically active and conserved across species (Coulouarn *et al*., 1999). U-II has been identified as an endogenous ligand for the G-protein-coupled receptor, GPR14 (now named the urotensin II receptor (UT receptor)) (Ames *et al*., 1999). Initially shown to be a potent vasoconstrictor (Ames *et al*., 1999; Maguire *et al*., 2000), U-II has been subsequently found to produce a number of other functional responses affecting endothelium-dependent vasodilation (Bottrill *et al*., 2000; Zhang *et al*., 2003), cardiac remodeling (Tzanidis *et al*., 2003; Kompa *et al*., 2004), body fluid regulation (Zhang *et al*., 2003; Balment *et al*., 2005; Song *et al*., 2006) and central sympathetic activity (Lin *et al*., 2003; Watson *et al*., 2003).

The role of U-II and its receptor in normal mammalian physiology and in pathophysiological states is not fully understood. However, U-II and the UT receptor have been implicated in a number of disease states including congestive heart failure (Douglas *et al*., 2002; Ng *et al*., 2002; Russell *et al*., 2003), essential hypertension (Matsushita *et al*., 2001; Cheung *et al*., 2004) and chronic renal failure (Totsune *et al*., 2001; Langham *et al*., 2004).

U-II and its receptor are found in a variety of tissues including blood vessels, pancreas, liver, kidney, heart, skeletal muscle, lung, the adrenal gland and other organs (Elshourbagy *et al*., 2002). In addition to the periphery, U-II and the UT receptor are distributed in a variety of structures within the brain and spinal cord (Jegou *et al*., 2006). Expression of U-II and UT receptors in the central nervous system prompted us to investigate the effects of intraplantar U-II administration on thermal nociceptive thresholds in conscious Sprague–Dawley rats. In doing so, we observed

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that U-II administered in this manner produced a reproducible and pronounced increase in ear redness that was accompanied by a marked increase in ear pinna surface temperature. The present experiments were designed to characterize more fully and quantify the ear flushing response as well as to explore potential mechanisms mediating this response.

Methods

All procedures involving the use of animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996) and the Animal Care and Use Committee, Johnson and Johnson Pharmaceutical Research and Development. A total of 150 male Sprague–Dawley rats from Charles River Laboratories (Wilmington, MA, USA), weighing 250–350 g, were used in these studies. Rats were allowed free access to food and water and kept on a 12 h light–dark cycle.

Experimental protocols

General. Rats were acclimatized to a room temperature of 18°C±0.5 for at least 1 h before the experiment and maintained at this temperature throughout the course of the experiment. Doses of U-II were dissolved in sterile normal saline for a fixed dosing volume of 1 ml kg^{-1}. Where applicable, intravenously administered agents were given via the tail vein in animals restrained in standard plastic cylinders. The lateral inner ear pinna surface temperature was measured using a Raytek High Performance Infrared Thermometer (Raytek Corporation, Santa Cruz, CA, USA) in freely moving animals. Instantaneous temperature was recorded by aiming the beam at the ventrolateral surface of the ear. Following measurement of pre-dose or zero-time ear surface temperature, U-II, calcitonin gene-related peptide (CGRP) or vehicle (normal saline) was immediately injected subcutaneously in the mid-scapular region (0-min time point). Temperature measurements were subsequently made at repeated 3–5 min time intervals. In other pilot experiments, the effects of U-II on ear flush were also evaluated following other routes of administration, including intraplantar, intramuscular and intravenous.

In vitro *selectivity profile of urantide*

The selectivity of unantide for the UT receptor was assessed by examining the interaction (binding) between urantide and a total of 51 distinct G-protein-coupled receptors and ion channels counter-screening assays using established protocols. No significant activity was observed at 1 μM. G-protein-coupled receptors screened included adenosine ($A_{1/2A/3}$), adrenergic (alpha 1 non-selective, alpha 2 non-selective, β1), dopamine ($D_{1/2S}$), muscarinic ($M_{1/2/3}$), serotonin (5-$HT_{1A/B}$, HT_{2A}, HT_3, HT_{5A}, HT_6, HT_7), histamine ($H_{1/2}$), angiotensin II (AT_1), bradykinin (B_2), melanocortin (MC_4R), melatonin (ML_1), neuropeptide Y ($Y_{1/2}$), endothelin (ET_A), neurotensin (NT_1), cholecystokinin (CCK_A), neurokinin ($NK_{2/3}$), benzodiazepine (BZD, central), galanin (GalR2), somatostatin (non-selective), vasoactive intestinal peptide ($VPAC_1$), opioid (δ, κ, μ), vasopressin (V_{1a}), GABA (non-selective), IL-8B (CXCB2), CCR1, and ORL1. Urantide was also evaluated in a variety of ion channels including sodium (site 2), calcium (L-type), potassium (SK_{Ca}, K_V) and chloride (picrotoxinin sensitive) channels and biogenic amine uptake (noradrenaline and dopamine transporters) assays.

Mechanism studies

In some experiments, pharmacological agents or their corresponding vehicles were administered to separate groups of animals before U-II administration. The U-II response in the animals receiving active pretreatment was compared to that in animals receiving vehicle pretreatment. L-N^{ω}-nitroarginine methylester (L-NAME) (100 mg kg^{-1}), dissolved in normal saline, was administered intraperitoneally, 4 h before U-II administration. Indomethacin (30 mg kg^{-1}) was administered in carboxymethylcellulose orally 2 h before U-II administration. Urantide (150–200 μg kg^{-1}), a peptide antagonist of the UT receptor, was administered in saline by intravenous bolus injection 30 min before U-II or CGRP administration. Mecamylamine (1 mg kg^{-1}) or propranolol (0.5 mg kg^{-1}) was administered by intravenous bolus injection 15 min before U-II administration (3 μg kg^{-1}). CGRP, a peptide known to cause generalized cutaneous vasodilation, was injected subcutaneously at multiple dose levels ranging from 1 to 100 μg kg^{-1}.

Core temperature was measured with a rectal probe using a thermocouple thermometer (Kent Scientific, Torrington, CT, USA). Rats were acclimatized to rectal probe insertion for 1 h daily, 5 days before the actual experiment. On the day of the experiment the probe was inserted and core temperature monitored until it stabilized. A baseline ear surface temperature was recorded and then U-II injected as described above. Core temperature and ear surface temperature were monitored at various times after U-II administration.

To determine whether the ear flush response could be evoked by direct brain injection, rats with indwelling cannulae implanted in the lateral cerebral ventricle were purchased from Charles River Laboratories. Intracerebroventricular (i.c.v.) injections were made using a 100 μl Hamilton glass syringe attached via a polyethylene catheter to a 30 G needle inserted into the indwelling cannula. U-II (0.53, 1.6, 5.3 or 53 μg kg^{-1}) or saline was infused intracerebroventricularly to conscious rats in a dosing volume of 5 μl over 15 s using a Harvard syringe pump. The injector needle was kept in place within the lateral ventricle for 15 s to help ensure diffusion into the cerebrospinal fluid, and was then withdrawn. Each rat received one i.c.v. injection at each particular dose level on 5 consecutive days, such that each animal received all dose levels and vehicle during the 5-day period. Lateral ear temperature was monitored just before each i.c.v. injection and up to 45 min following the injection. Three days after the last injection, ear flush was confirmed in all animals with a subcutaneous dose of U-II at 10 μg kg^{-1}. I.c.v. catheter placement was confirmed following the experiment by injection of 5 μl of Evans Blue dye.

In order to determine whether systemically administered U-II affected blood pressure, rat arterial pressure was

measured by a volume pressure tail cuff method (XBP1000 Series Rat Tail Blood Pressure System, Kent Scientific, Torrington, CT, USA). Rats were adapted to both restraint and warming procedures. Rats were gently warmed using a warming pad until their tail surface temperature reached 34°C. After several weeks of adaptation training, rats were injected subcutaneously with either saline or ascending U-II doses (10, 30 and 100 $\mu g\,kg^{-1}$) once every 2 weeks. Two weeks after the highest dose of U-II was administered, saline was re-administered as an additional control. Blood pressure and heart rate were measured for 20 min after saline or U-II injections.

Data analysis

All results are expressed as mean ± s.e.m. For all experiments, '*n*' refers the number of different animals evaluated per test group. For the multi-group area under the curve (AUC) results, comparisons were made between group means versus vehicle control using one-way analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test. *P*-values less than 0.05 were considered significant.

Materials

Urantide and urotensin-II-related peptide (URP) were purchased from Peptide International (Louisville, KY, USA). Rat U-II, CGRP, L-NAME, indomethacin, mecamylamine and propranolol were obtained from Sigma (St Louis, MO, USA).

Results

U-II-induced ear flush

Cutaneous flushing of the rat ear pinna was first observed when we administered U-II by intraplantar administration. A similar response was observed with multiple routes of administration including subcutaneous, intramuscular and intravenous in conscious rats. We adopted the subcutaneous route for U-II administration in all subsequent studies. Photographs of the cutaneous ear pinna flushing following subcutaneous administration of either U-II or CGRP (both 10 $\mu g\,kg^{-1}$) are shown in Figure 1.

The present study establishes that the U-II-induced ear flush response was accompanied by graded and marked increases in ear pinna surface temperature, as assessed with a remote non-contact laser thermometer. Therefore, ear surface temperature was employed as a sensitive means of quantifying the response. Figure 2a shows the time course of the ear temperature changes in response to increasing doses of U-II. In general, ear temperature began to rise within approximately 6–10 min of U-II administration, reaching a peak response within 15–20 min. The dose–response relationship as illustrated in Figure 2b by the integrated area under the temperature–time curve was bell-shaped, with a maximal response at about 9 $\mu g\,kg^{-1}$, followed by a gradually declining response that plateaued at about 50% of the maximum response at the highest dose tested (Figure 2b). The area under the temperature–time curve as a result of U-II administration was significantly different from vehicle

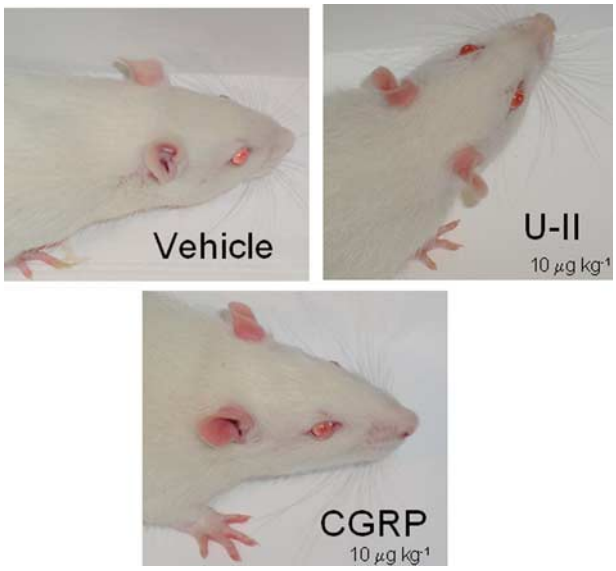

Figure 1 Photographs of U-II-induced ear flush response in rats. Left panel: rat injected with saline subcutaneously; Right panel: rat injected with 10 $\mu g\,kg^{-1}$ U-II, subcutaneously. Bottom panel: rat injected with 10 $\mu g\,kg^{-1}$ CGRP. Photographs were taken 15 min after vehicle or peptide administration.

controls over most of the doses used (Figure 2b). We have previously reported that the conserved cyclic peptide sequence of U-II, CFWKYC, is critical for U-II functional activity and binding of U-II to its receptor. Replacement of K (lysine) with A (alanine) dramatically reduced affinity for the recombinant rat UT receptor (Kinney *et al*., 2002). Administration of the alanine substituted and truncated goby U-II peptide, ADCFWAYCV, failed to induce an ear flush response at 100 $\mu g\,kg^{-1}$ (data not shown).

In order to confirm that the ear flush response to U-II was mediated by UT receptor stimulation, the effects of pretreatment with a UT-selective antagonist on U-II-induced ear temperature changes were examined. Urantide is a recently identified peptide that has been shown to be a potent and selective antagonist of the UT receptor based on inhibition of U-II-induced vasoconstriction of rat aortic rings (Patacchini *et al*., 2003). It is inactive when cross-screened against a diverse range of G-protein-coupled receptors, ion channels, and transporters (see the methods section). Urantide, administered intravenously at 150 $\mu g\,kg^{-1}$, had no effect on ear temperature when administered alone (Figure 3). However, the same dose of urantide effectively antagonized U-II (3 $\mu g\,kg^{-1}$, s.c.)-induced ear pinna temperature elevation and flush (Figure 3) when it was administered 30 min before challenge with U-II.

U-II-induced ear flush mechanisms

To explore the possibility that U-II-mediated ear flush is related to global thermoregulation, core temperature was monitored following U-II administration in a separate group of animals. U-II administered at a dose that significantly increased ear temperature (140 $\mu g\,kg^{-1}$), had no effect on core body temperature (Figure 4). Further, the U-II-mediated

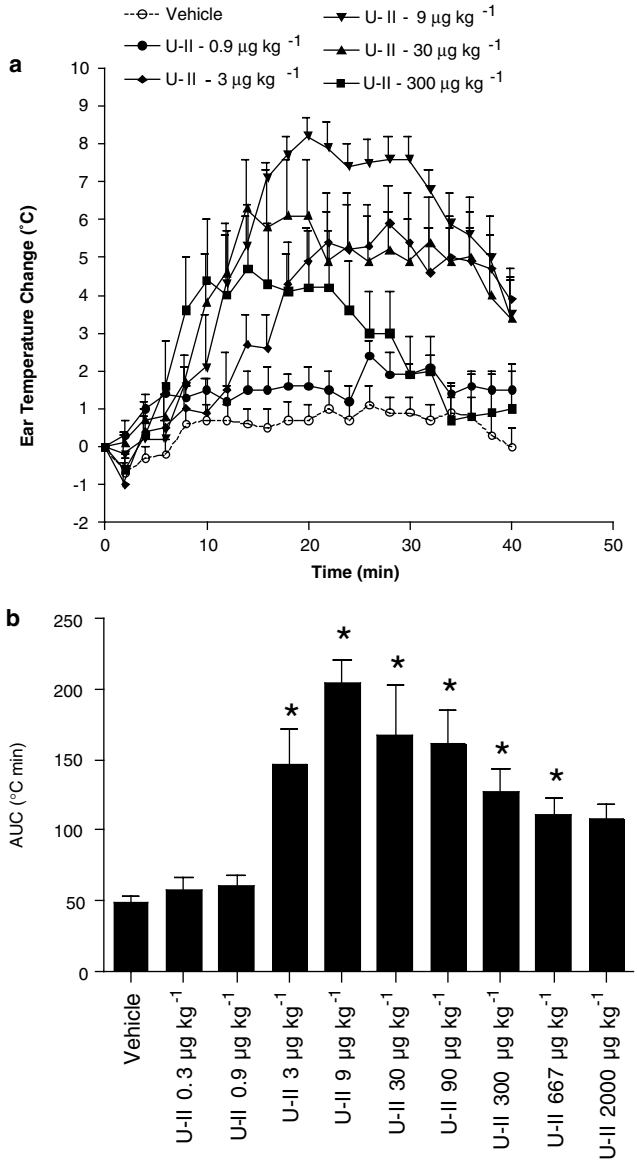

Figure 2 Ear surface temperature increase at various. U-II dose levels (0.3–2000 μg kg^{-1}; s.c.). (**a**) Time course of U-II-induced ear temperature increase (select dose levels shown for clarity). (**b**) Dose–response of U-II-induced ear flush as quantified by area under the temperature change–time curve (AUC; includes additional dose levels not shown in **a**). Data are shown as mean temperature change from baseline or AUC$\pm$s.e.m. *$P<0.05$ compared to vehicle response using one-way ANOVA ($n=6$ per treatment group).

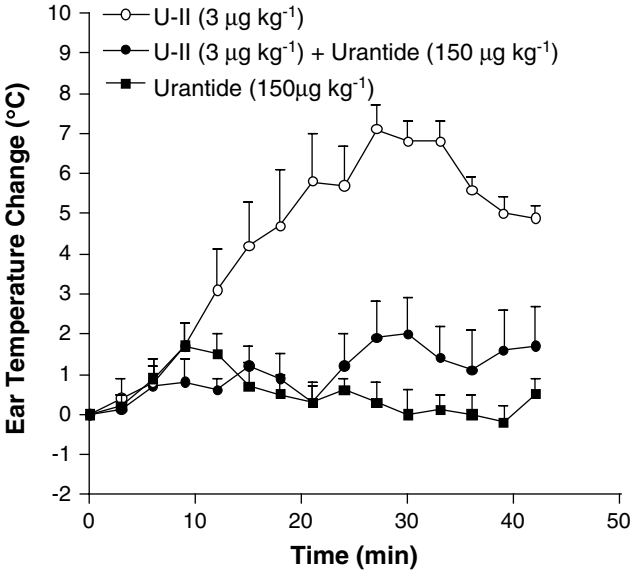

Figure 3 Effects of urantide on U-II-induced ear temperature changes. Data shown are mean temperature change from baseline$\pm$s.e.m. ($n=4$ per treatment group).

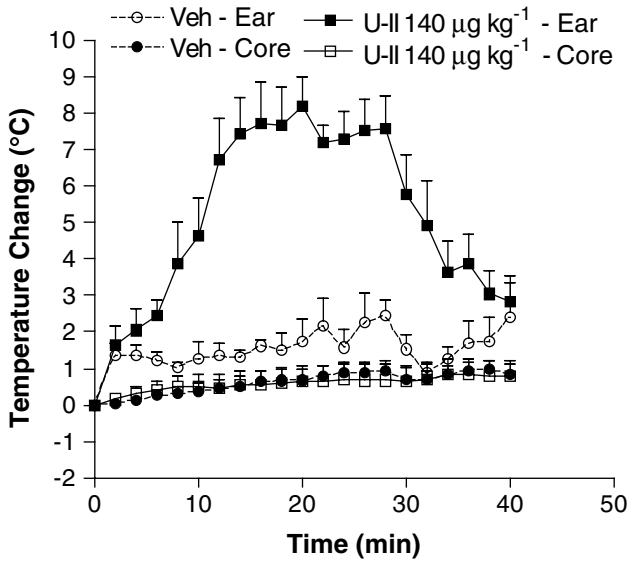

Figure 4 Assessment of core temperature in comparison to ear surface temperature in response to U-II. Effect of vehicle or U-II (s.c.) on ear pinna and core body temperature. Data shown are mean temperature change from baseline$\pm$s.e.m. ($n=4$ per treatment group).

ear flush response, with an onset of approximately 6–10 min, was remarkably restricted to the ear pinna as no erythema of the eyelids, oral mucosa, snout, fore- or hindlimbs, or tail was observed.

Because of its ability to affect cutaneous blood flow, the effects of CGRP on ear pinna temperature change were also assessed. A photograph of CGRP-induced ear flush is shown in Figure 1. Grossly, CGRP produced a relatively rapid onset and persistent erythema response of the ear pinna within 3–5 min of subcutaneous administration, lasting up to 90 min. In addition, cutaneous flushing was also noted around the eyes, snout, fore- and hindlimbs and tail (forelimb erythema visible in Figure 1), the onset of which was within 20–30 min following administration. The effects of subcutaneously administered CGRP on ear pinna temperature change are shown in Figure 5a and b. In contrast to the longer onset and shorter-lived U-II-induced ear temperature changes, CGRP-induced ear pinna temperature changes occurred rapidly (within 3 min of s.c. administration) and persisted longer than 1 h. At dose levels of 30 and 60 μg kg^{-1}, animals exhibited elevated ear pinna temperatures for at

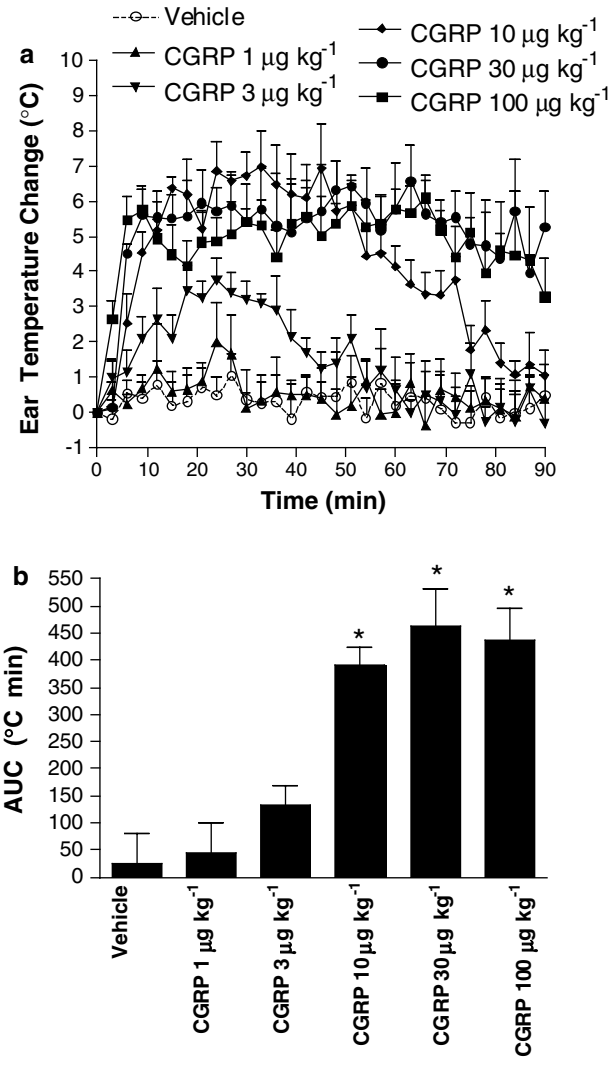

Figure 5 Effects of CGRP, injected s.c., on ear pinna temperature. (**a**) Time course of CGRP-induced ear pinna temperature change, shown as mean temperature change from baseline (±s.e.m.). (**b**) Effects of CGRP on area under the temperature change–time curve reported as mean AUC ±s.e.m. at various CGRP dose levels. $n=5$ per treatment group, *$P<0.01$ using one-way ANOVA.

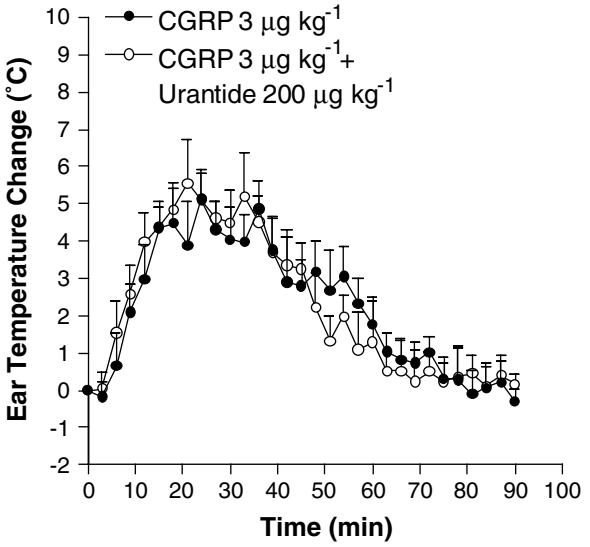

Figure 6 Effects of urantide on CGRP-induced ear flush. The effects of CGRP or CGRP + urantide on ear pinna temperature change are shown as mean temperature change ±s.e.m. ($n=5$ per treatment group).

least 90 min. The calculated area under the temperature–time curve exhibited a dose-dependent increase with a maximal effect being observed at a dose of approximately 10 $\mu g\,kg^{-1}$ (Figure 5b). The effects of subcutaneously administered CGRP at a dose of 3 $\mu g\,kg^{-1}$ on ear pinna temperature were not affected by intravenously administered urantide at a dose that antagonized U-II-induced ear flush (Figure 6).

As U-II-induced vasodilation has been shown to be mediated by both nitric oxide (NO) and prostacyclin (Katano *et al*., 2000; Gray *et al*., 2001; Zhang *et al*., 2003; Gardiner *et al*., 2004), the effects of pretreatment with L-NAME, an inhibitor of nitric oxide synthase (NOS), and indomethacin, a cyclooxygenase inhibitor, on U-II-induced ear temperature changes were evaluated. Pretreatment with intraperitoneally administered L-NAME markedly inhibited the U-II-induced elevation in ear pinna temperature (Figure 7a). Pretreatment with indomethacin (30 $mg\,kg^{-1}$, p.o.) completely inhibited the U-II-induced ear flush response (Figure 7b). Before U-II challenge, ear flush (assessed visually) was not observed during the 4-h pretreatment period following L-NAME or during the 2-h pretreatment period following indomethacin. In addition, the baseline ear pinna temperature immediately before U-II administration did not differ among the pretreatment groups (see Figure 7 legend). In a separate set of animals, pretreatment with the anti-inflammatory glucocorticoid dexamethasone (8 $mg\,kg^{-1}$, i.p. 2 h before U-II challenge), had no effect on the U-II-induced ear flush response (data not shown).

We investigated the effects of the β-adrenergic antagonist, propranolol, and the ganglionic blocker, mecamylamine, on the U-II-induced ear flush response. Pretreatment with propranolol (0.5 $mg\,kg^{-1}$, i.v.) or mecamylamine (1 $mg\,kg^{-1}$, i.v.) had no effect on the U-II-induced ear flush response (Figure 8a and b). The dose of mecamylamine used was double that which was shown to abolish ganglionic neurotransmission for more than 2 h (Wang and Pang, 1991).

Finally, to determine whether the ear flush response was centrally mediated, ear temperature changes were monitored following i.c.v. infusion of U-II. Centrally administered U-II failed to induce any change in ear temperature over the 45 min observation period and up to a dose of 5.3 $\mu g\,kg^{-1}$ (Figure 9). If this dose were administered subcutaneously, a robust change in ear pinna temperature would have been observed. I.c.v. infusion of a higher dose of 53 $\mu g\,kg^{-1}$ U-II did induce a delayed (>20 min) but demonstrable ear flush response.

Discussion

The present studies have demonstrated a unique, dose-dependent response to parenterally administered U-II in

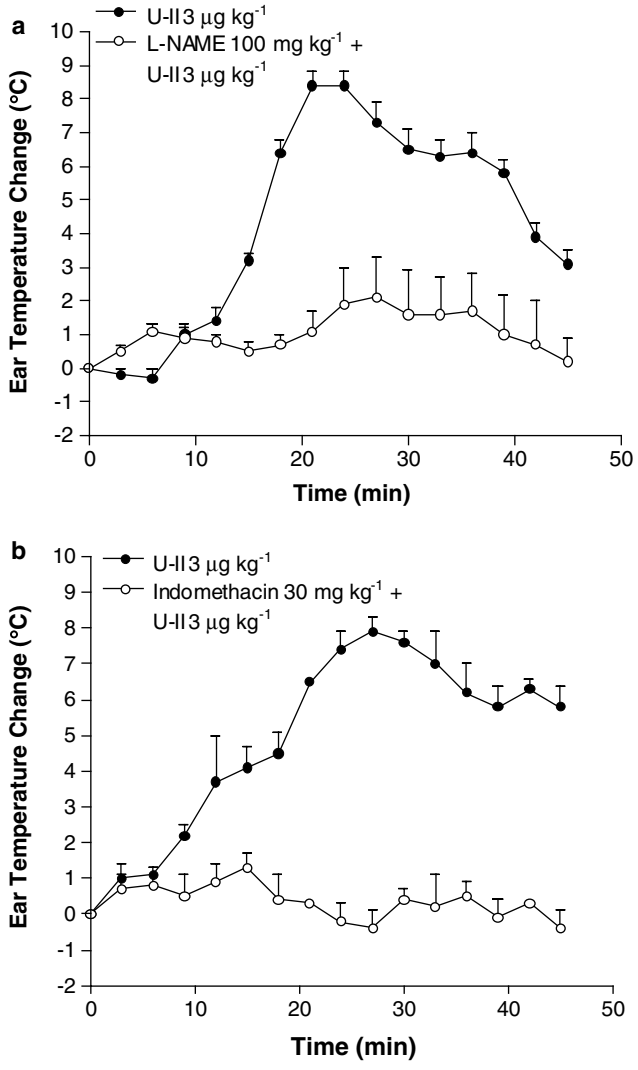

Figure 7 Effects of L-NAME or indomethacin on U-II-induced ear flush response. Shown are the effects of U-II on ear temperature change (**a**) in vehicle-pretreated animals (baseline pinna temperature, 25.1±0.1°C) or in L-NAME-pretreated animals (baseline pinna temperature, 24.9±0.1°C); (**b**) in vehicle-pretreated animals (baseline pinna temperature, 25.4±0.2°C) or in indomethacin-pretreated animals (baseline pinna temperature, 25.3±0.3°C). Temperature changes are reported as mean temperature change ±s.e.m. ($n=4$–5 per treatment group).

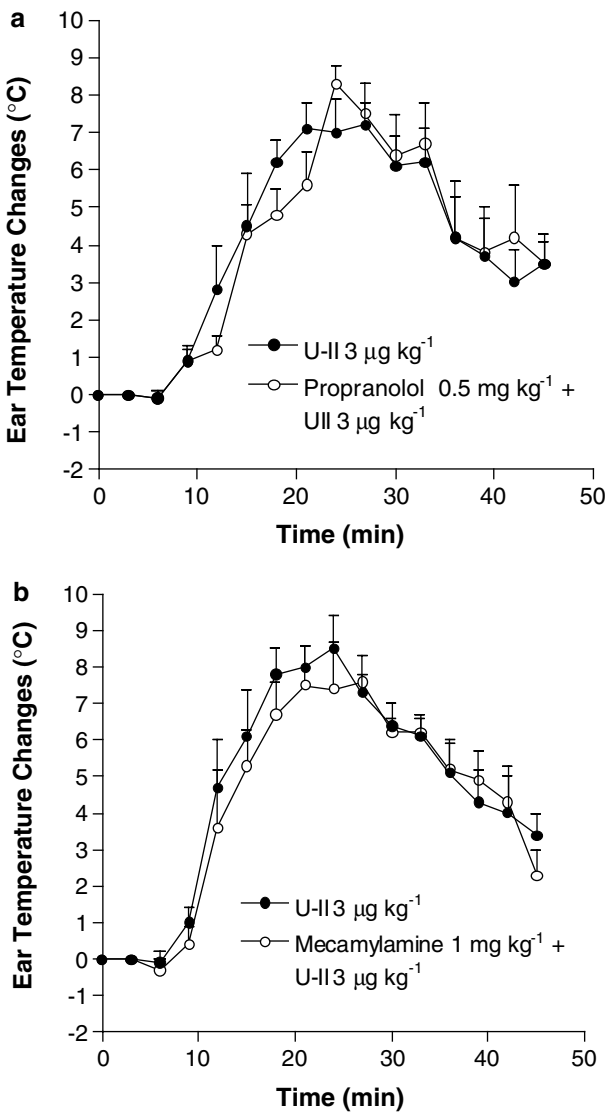

Figure 8 Effects of propranolol or mecamylamine on U-II-induced ear flush response. In (**a**), the effects of U-II or U-II + propranolol treatment are shown as mean temperature change ±s.e.m.). In (**b**) responses to U-II or U-II + mecamylamine treatment are shown as mean temperature change ±s.e.m. ($n=4$–5 per treatment group).

conscious rats consisting of pronounced ear pinna cutaneous flushing that is accompanied by readily quantifiable increases in ear surface temperature. We further explored potential physiological mechanisms involved in the ear flush response to U-II and propose that it might serve as a useful model for pharmacological evaluation of agents that interact with the UT receptor, in particular UT receptor antagonists.

Vasodilation in the rat ear pinna is physiologically complex. The external ears of the rat are relatively thin, flat organs with blood vessels lying throughout the plane of the ear pinna. Although it is tempting to regard the ear pinna as a major thermoregulatory organ in rats as it is in other species (e.g. guinea-pig and rabbit) (Folkow, 1955), Grant (1963) showed that unlike the response to body warming in the rat tail, the lateral ear pinna temperature and corresponding arterial caliber do not increase as the body is warmed. However, rat ear vessels do react to sensory stimulation and are innervated by peptidergic neurons expressing CGRP and substance P (Kiernan, 1976; Benrath *et al*., 1995; Blacklock *et al*., 2004). The vasodilatory effects of ultraviolet light exposure to the rat ear surface have been shown by Benrath *et al*. (1995) to be dependent upon CGRP and substance P, the former response having an NO-independent component. Further, mast cells in the ear pinna release vasoactive autacoids in response to noxious stimulation. Substance P injection in the ear pinna causes mast cell degranulation (Aloe *et al*., 1993). Histamine release from pinna mast cells promotes vasodilation (Kiernan, 1972) through H1 and H2 receptor-dependent mechanisms (Belcheva and Zhelyazkova, 1988). Related to the role of

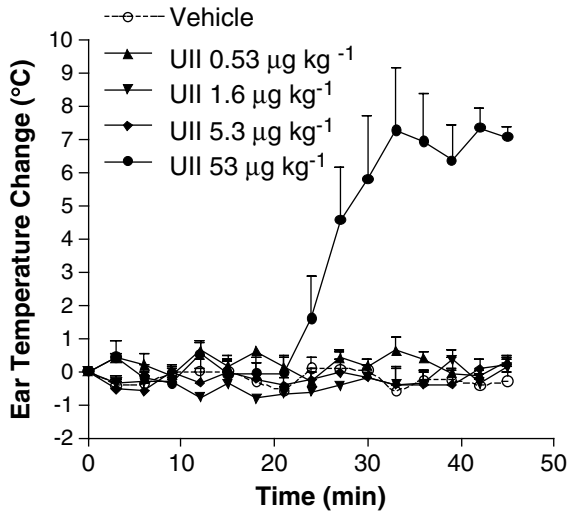

Figure 9 Effects of i.c.v. administration of U-II on ear temperature in conscious rats. The effects of different i.c.v. administered doses of U-II on ear pinna temperature change are shown in the figure as mean temperature change ±s.e.m. ($n=5$ per treatment group). One rat was excluded from the analysis owing to a misplaced i.c.v. cannula determined following the experiment using Evans Blue dye.

autacoids in rat ear flush, systemic nicotinic acid administration has been shown to cause a mild (less than 1.5°C) increase in rat ear surface temperature (Turenne *et al*., 2001).

In general, the role of U-II in vasomotor tone in animal models varies with organ, region, species and preparation. Systemic administration of human U-II to anesthetized monkeys has been shown to increase cardiac output and regional vasodilation at low U-II doses but, at higher doses, caused large increases in vascular resistance, decreases in cardiac output, and subsequent fatal circulatory collapse (Ames *et al*., 1999). In the cat, U-II causes pronounced vasoconstriction of a variety of isolated vessels as well as a dose-dependent elevation in mean arterial pressure and vascular resistance (Behm *et al*., 2004). Although U-II was initially characterized as an extremely potent vasoconstrictor in some isolated vascular preparations (Ames *et al*., 1999; Maguire *et al*., 2000), several studies show that U-II can also induce vasodilation (Bottrill *et al*., 2000; Zhang *et al*., 2003). Intravenous bolus injection of U-II in anesthetized rats produced a decrease in mean arterial pressure, left ventricular systolic pressure and cardiac contractility (Hassan *et al*., 2003). Studies in conscious rats showed a predominant systemic vasodilatation in response to U-II with accompanying dose-dependent tachycardia (Gardiner *et al*., 2001; Lin *et al*., 2003). Subsequent studies have shown that bolus injection of U-II to conscious rats evokes an initial response consisting of tachycardia and hypotension that is followed by a later phase (30–120 min post-injection) of tachycardia and hypertension (Gardiner *et al*., 2004). We have observed no or minor reductions (<10%) in mean arterial pressure 5–20 min post-U-II administration at dose levels of 10, 30 or 100 $\mu g\,kg^{-1}$ as measured by a tail cuff method in conscious rats (data not shown).

There is some recent evidence that U-II's vasodilatory actions *in vivo* are region specific. Although ear blood flow was not measured, U-II has been shown to cause preferential mesenteric and hindquarter vasodilatation, and less pronounced effects in renal vascular conductance in conscious Sprague–Dawley rats (Gardiner *et al*., 2004). The effects of U-II in rat ear pinna vasodilation and subsequent ear temperature changes have not been described previously. Our results clearly indicate that parenteral administration of U-II produces dose-dependent ear pinna vasodilation. Further, U-II-induced ear pinna vasodilation does not appear to be the result of a general thermoregulatory response as core temperature remained unchanged throughout the observation period following U-II administration. Unlike CGRP, U-II-induced cutaneous vasodilation appears to be limited to the ear pinna as U-II failed to induce observable flushing in mucous membranes (e.g. conjunctiva and oral mucosa) as well as in a number of other cutaneous tissues including the skin of the nares, the scrotum or tail (the latter assessed by cutaneous temperature measurement).

It appears that U-II-induced ear flush is the result of direct UT receptor stimulation. Urantide is a recently identified peptide that has been shown to be a potent and selective antagonist of the UT receptor based on inhibition of U-II-induced vasoconstriction of rat aortic rings (Patacchini *et al*., 2003). We showed that administration of urantide effectively antagonizes the U-II-induced ear flush response. A more recent report showed that urantide has agonist activity in Chinese Hamster ovary cells overexpressing the human UT receptor (Camarda *et al*., 2004). However, in our studies, urantide alone did not elicit the ear flush response. We also found that a newly identified agonist of the UT receptor, URP (Sugo *et al*., 2003), also elicited an ear flush response (data not shown). In addition, truncated goby U-II peptide with an alanine substitution, a peptide that has a dramatically reduced affinity for the rat UT receptor, did not induce the ear flush response. These findings support that ear flushing in response to U-II is mediated by specific activation of the UT receptor.

Mechanistically, U-II-mediated ear flush could be either the result of central or peripheral U-II action. However, U-II-induced vasodilation does not appear to be centrally mediated as direct i.c.v. injection failed to induce ear flush at dose levels that would have done so if administered systemically. When administered intracerebroventricularly at a relatively high dose ($>50\,\mu g\,kg^{-1}$), the response was delayed compared to the response evoked by subcutaneous administration. The high dose finding is consistent with the appearance of U-II in the systemic circulation by virtue of elimination from the cerebrospinal fluid compartment. Thus, the U-II-induced ear flush response is likely to be peripherally mediated.

The mechanism by which U-II produces ear flush is complex. Unlike CGRP, which induces ear flush within approximately 3 min of subcutaneous injection, U-II-mediated ear flush following subcutaneous administration is relatively delayed, beginning at approximately 6–10 min despite the route of administration. The delay in ear flush induced by U-II compared to CGRP suggests that U-II interacts through a mechanism that requires other mediators. Several studies have shown that U-II-induced vasodilation is endothelium dependent and involves the release of

both NO and prostacyclin (Katano *et al*., 2000; Gray *et al*., 2001; Zhang *et al*., 2003; Gardiner *et al*., 2004). In rat isolated heart preparations, U-II has been shown to produce a concentration-dependent increase in coronary resistance that was increased threefold in the presence of L-NAME and the cyclooxygenase inhibitor indomethacin (Gray *et al*., 2001). In another isolated heart study, higher concentrations of U-II induced a sustained vasodilation that was significantly inhibited by a cyclooxygenase inhibitor (Katano *et al*., 2000). When directly administrated into the renal artery, U-II increased renal blood flow, consistent with renal vasodilation. This response was abolished by L-NAME (Zhang *et al*., 2003). In our studies, either indomethacin alone or L-NAME alone was sufficient to block the ear flush response whereas the anti-inflammatory steroid, dexamethasone, had no effect. This result was unexpected as NO and prostacyclin are hypothesized to contribute additively to induce vasodilation. However, two previous studies with U-II have reported findings consistent with our results. In anesthetized rats, indomethacin abolished the hypotensive response to U-II (Hasegawa *et al*., 1992). In addition, indomethacin alone was shown to abolish early and late hindquarter vasodilatation in conscious rats (Gardiner *et al*., 2004). One possible interpretation of the findings is that UT receptor activation induces prostanoid production in the ears, which, in turn, stimulates inducible nitric oxide synthase and NO release (Borda *et al*., 2002; Madrigal *et al*., 2003). Alternatively, U-II may directly stimulate NOS expression, and indomethacin may inhibit this stimulation (Di Girolamo *et al*., 2003).

We attempted to determine whether peripheral neuronal pathways mediate U-II-induced ear flush. However, ganglionic blockade with mecamylamine or β-adrenergic blockade with propranolol had no effect on the ear flush response. Furthermore, preliminary experiments involving local infiltration of lidocaine into the base of the ear pinna, which should inhibit nerve conduction in local sensory fibers, had no impact on the ear flush response (data not show) suggesting that ear flush is not mediated via peripheral neuronal pathways.

In conclusion, our studies have described and characterized a unique response to U-II consisting of pronounced ear flush that can be readily quantified using serial measurements of ear surface temperature. The response appears to be mediated by specific activation of peripheral UT receptors that results in dilation of the vasculature of the ear pinna. Our results suggest that the vasodilatory response involves the generation of products of cyclooxygenase and the release of NO. Further studies will be required to understand fully the mechanisms for this response. However, the model may be useful to evaluate the efficacy and potency of potential UT receptor antagonists as well as agonists *in vivo*.

Conflict of interest

All authors are or were employees of Johnson and Johnson Pharmaceutical Research and Development who funded this research.

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