cis-Urocanic Acid Stimulates Neuropeptide Release from Peripheral Sensory Nerves

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Previous studies using an antibody to *cis*-urocanic acid and mast-cell-depleted mice implicated both *cis*-urocanic acid and mast cells in the mechanisms by which ultraviolet B light suppresses systemic contact hypersensitivity responses in mice. In the absence of a direct stimulatory effect of *cis*-urocanic acid on connective tissue mast cells, an indirect association was investigated. A blister induced in the rat hind footpad was used to examine the effects of slowly perfused *cis*-urocanic acid on cutaneous blood flow. *cis*-Urocanic acid but not *trans*-urocanic acid increased microvascular flow by a mechanism largely dependent on the combined activity of the neuropeptides, substance P and calcitonin gene-related peptide. Perfusion of *cis*-urocanic acid over the base of blisters induced in sensory-neuropeptide-depleted rats did not have any stimulatory effect above that seen with perfusion of *cis*-urocanic acid together with neuropeptide receptor antagonists in control rats. There was a small direct effect of *cis*-urocanic acid on microvascular blood flow. As both substance P and calcitonin gene-related peptide could directly degranulate connective tissue mast cells, this study suggests that *cis*-urocanic acid indirectly activates mast cells via its effects on peripheral terminals of unmyelinated primary afferent sensory nerves. *cis*-Urocanic-acid-induced neuropeptides may also contribute to ultraviolet-B-induced cutaneous inflammation and alterations to Langerhans cell activity.* ***Key words: histamine/mast cells/rodent/skin. J Invest Dermatol 117:886–891, 2001***

Ultraviolet radiation of wavelength 280–320 nm (UVB) not only causes skin erythema and edema, but is also carcinogenic and immunoregulatory (Daynes *et al*, 1981; Kripke, 1981). There have been definitive studies in rodents showing that UV-induced tumors progress because of the adverse effects of UV radiation on immunocompetency (Daynes *et al*, 1981; Kripke, 1981). Over the past two decades, the photoreceptors in skin for UVB have been investigated and the mechanisms of this immunoregulation have been studied in rodents and, more recently, humans (Streilein *et al*, 1994; Beissert and Schwarz, 1999; Duthie *et al*, 1999). Very little UVB penetrates skin lower than the epidermis. UV-initiated responses in the epidermis that have been implicated in the mechanisms of immunomodulation include cellular DNA damage, membrane oxidation, or altered intracellular signaling leading to altered function or production of immunosuppressive molecules (Applegate *et al*, 1989; Schwarz, 1998). Another important process induced by UVB is the isomerization of *trans*-urocanic acid (UCA) to its *cis* isomer (Noonan and De Fabo, 1992). *trans*-UCA (deaminated histidine) is a molecular species located superficially in the stratum corneum of skin. On irradiation it is converted to the more soluble *cis* isomer. The action spectrum of UVB-induced suppression of contact hypersensitivity (CHS) responses in mice closely follows the absorption spectrum of UCA (De Fabo and Noonan, 1983), and mice genetically deficient in histidase, and thus in skin UCA, are not susceptible to immunosuppression by UVB irradiation (Noonan and De Fabo, 1992).

The role of *cis*-UCA in UVB immunomodulation may vary with the response examined and the experimental model adopted. An antibody to *cis*-UCA neutralizes the immunosuppressive effects of UVB in many experimental models; in particular UVB can suppress delayed type hypersensitivity responses to viral or allogeneic antigens (El-Ghorr and Norval, 1995; Moodycliffe *et al*, 1996) and CHS responses to experimental haptens (Kondo *et al*, 1995). In our studies of systemic CHS responses to trinitrochlorobenzene (TNCB) in mice, a single intraperitoneal injection of 0.1 μg *cis*-UCA antibody 4 h before UVB irradiation removed approximately 60% of the immunosuppression caused by 2 kJ per m^2 UVB in C57BL/6 mice and 12 kJ per m^2 UVB in BALB/c mice (Hart *et al*, 1997). Under these same experimental conditions, the effect of *cis*-UCA (20 μg per mouse) was negated by approximately 60% (Hart *et al*, 1997).

In vitro effects of *cis*-UCA have been described on purified keratinocytes (Mitra *et al*, 1993; Jaksic *et al*, 1995; Norval *et al*, 1995), Langerhans cells (Beissert *et al*, 1997), fibroblasts (Bouscarel *et al*, 1997), T lymphocytes (Holan *et al*, 1998), natural killer cells (Gilmour *et al*, 1993), and monocytes (Hart *et al*, 1993). The receptor for *cis*-UCA was first hypothesized to be a histamine receptor but, after different effects of these two molecules were described on monocytes (Hart *et al*, 1993), keratinocytes (Jaksic *et al*,

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Abbreviations: CGRP, calcitonin gene-related peptide$_{1–37}$; CHS, contact hypersensitivity; GABA, γ-amino-butyric acid; TNCB, 2,4,6-trinitrochlorobenzene; UCA, urocanic acid.

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1995), and fibroblasts (Bouscarel *et al*, 1997), this theory was discounted.

Recently we noted that dermal mast cells are critical to the initial mechanisms by which *cis*-UCA suppresses a ''systemic'' CHS response in mice (Hart *et al*, 1999). Mast-cell-depleted mice were not responsive to UVB or *cis*-UCA for immunomodulation unless they were reconstituted at the irradiated/injection site with cultured bone-marrow-derived mast cell precursors from mast-cell-replete mice (Hart *et al*, 1998a, 1999). We have also shown that humans with basal cell carcinomas have a higher dermal mast cell prevalence in sun-nonexposed skin, which supports the concept that mast cells are critical to UVB-induced systemic immunosuppression and further that UVB-induced immunosuppression is essential for skin cancer development (Grimbaldeston *et al*, 2000).

Thus, in our studies of UVB immunomodulation in mice, both *cis*-UCA and mast cells were implicated (Hart *et al*, 1997, 1999). Mast cells were also implicated in the studies of Streilein and colleagues of UVB suppression of ''local'' CHS responses when the sensitizing antigen was applied within 30 min to the irradiated site (Niizeki *et al*, 1997; Alard *et al*, 1999). A further study had concluded that *cis*-UCA stimulated mast cell degranulation, but in that study human skin organ cultures, not purified mast cells, had been used (Wille *et al*, 1999). In this study we investigated whether *cis*-UCA could activate mast cells directly or indirectly via activation of sensory nerve terminals. Sensory-neuropeptide-depleted rats and neuropeptide receptor antagonists were used to investigate the latter possibility.

MATERIALS AND METHODS

Animals For studies of microvascular flow and peritoneal mast cell isolation, outbred male Sprague-Dawley rats (3 mo of age) were used. Anesthesia was induced with sodium pentobarbitone (60 mg per kg, intraperitoneally) and maintained by supplementary injections. Body temperature was maintained at 37°C. Animals were sacrificed by barbiturate overdose at the completion of experiments.

Female BALB/c mice (4–8 wk of age) were obtained from the Animal Resource Center of the South Australian Department of Agriculture. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia.

UCA and UV irradiation The *trans* isomer of UCA was purchased from Sigma (St. Louis, MO) and UV irradiated using a bank of FS40 sunlamps (Westinghouse, Pittsburgh, PA) emitting a broad band of UV, 250–360 nm, with 65% of the output in the UVB range (280–320 nm). *cis*-UCA was purified from irradiated *trans*-UCA by ion-exchange chromatography (Hart *et al*, 1993). Both UCA isomers (200 μg per mouse dissolved in 200 μl phosphate-buffered saline) were injected subcutaneously into the upper backs of mice. Using the same bank of FS40 sunlamps, mice were irradiated on clean-shaven 8 cm^2 dorsal skin as previously described using a PVC plastic film to screen out wavelengths less than 290 nm (Jaksic *et al*, 1995; Hart *et al*, 1998a; 1999). The ears of the mice were protected with black adhesive insulation tape and the mice were irradiated in individual compartments of Perspex cages. The sunlamps were held 20 cm above the cages.

Isolation of rat peritoneal mast cells Rat peritoneal cells were obtained by lavage with Tyrodes buffer with 0.1% gelatin (pH 6.7). Washouts were centrifuged at 150 × *g* for 10 min, and cell pellets were resuspended in the same buffer and layered upon 22% metrizamide (Sigma) gradients. Gradients were centrifuged at 280 × *g* for 15 min; cell pellets were washed and layered on a second 22% metrizamide gradient. After centrifugation at 25 × *g* for 20 min, the gradients were harvested with the bottom third containing 99% pure mast cells as judged by their size and granular content. These cells were mature connective tissue mast cells as judged by their red color after staining with alcian blue-safranin (Csaba's mast cell stain) (Hart *et al*, 1998a).

Quantification of histamine release The histamine released from rat peritoneal mast cells in response to mediators was determined by fluorometric analysis as previously described (Skov *et al*, 1985). Briefly, 2000 cells in a final volume of 200 μl Tyrodes buffer with 0.1% gelatin were incubated with mediators for 15 min in a 37°C water bath. The mediators dissolved in Tyrodes buffer with 0.1% gelatin included substance P (1–100 μM, Sigma), calcitonin gene-related peptide$_{1-37}$ (CGRP$_{1-37}$; 0.1–50 μM, CGRP, Peninsula Laboratories Europe, St. Helens, Merseyside, U.K.), and 48/80 (1–10 μg per ml, Sigma). Reactions were terminated by centrifugation at 300 × *g*, 4°C for 5 min and 25 μl supernatant aliquots were added to glass microfibre-coated 96-well assay plates for analysis (Skov *et al*, 1985). The total histamine content was calculated as that measured in an aliquot of boiled cells. Histamine release was expressed as a percentage of the total cellular histamine content corrected for spontaneous release (i.e., that occurring in the absence of any stimulus).

Sensory neuropeptide depletion in rats and mice Neonatal rats were pretreated on the second day of life with a single subcutaneous injection of 50 mg per kg capsaicin. This treatment has been shown to permanently destroy the majority of sensory nerve fibres (Lembeck and Holzer, 1979) allowing the assessment of the role of sensory fibres in sensory-mediated physiologic responses. Blisters were induced and peptides perfused in these rats at the age of 3 mo. Efficacy of this treatment was confirmed as previously described (Khalil *et al*, 1994; Khalil, 1999).

Mice were depleted of sensory neuropeptides as previously described (Garssen *et al*, 1998). When mice were 4 wk of age, they were anesthetized (ketamine 75 mg per kg and medetomidine 1 mg per kg intraperitoneally) and injected subcutaneously (50 μl) in the neck region with capsaicin (25 mg per kg; Sigma) or vehicle control (alcohol:Tween-80:saline, 2:1:7) on two consecutive days. Mice were not UV irradiated or administered UCA until they were 8–9 wk of age. After completion of the CHS assays, sensory neuropeptide depletion was confirmed as previously described (Garssen *et al*, 1998).

Blister induction and peptide perfusion A blister of 0.25 cm^2 was induced on the hind footpad of the anesthetized rat by applying a vacuum pressure of –40 kPa to the glabrous skin for approximately 30 min, using a metal suction cap heated to 40°C by an attached heating element (Khalil *et al*, 1994; Khalil, 1999). This induction period lasted 30 min in both control and capsaicin-pretreated rats. When a blister was established, the surface epithelium was removed and a Perspex chamber with inlet and outlet ports was fixed over the blister base. Perfusion of the drugs over the blister was maintained at 4 ml per h by a peristaltic pump (Microperpex S, LKB, Sweden). Both perfusion temperature and body temperature were kept at 37°C. The experimental protocol consisted of an initial 20 min equilibration with Ringer's solution to establish a stable baseline. Sodium nitroprusside, a direct smooth muscle vasodilator, was perfused at 100 μM for 10 min. The latter is used to control for the variability in smooth muscle reactivity between rats. This was followed by perfusion of Ringer's solution to re-stabilize the baseline. *trans*-UCA or *cis*-UCA at different concentrations in Ringer's solution was then perfused for 20 min. Finally there was a 10 min post-stimulation period with perfusion of Ringer's solution followed by a second perfusion of sodium nitroprusside to test for the indirect effect of UCA on smooth muscle reactivity. In some experiments, the substance P receptor antagonist RP-67580 (10 μM, gift of Rhone Poulenc Rorer Chemicals, France) and the CGRP receptor antagonist CGRP$_{8-37}$ (10 μM, Sigma) were perfused for 10 min prior to, and concomitant with, perfusion of *cis*-UCA or *trans*-UCA.

Measurement of cutaneous blood flow A laser Doppler flowmeter probe (Periflux, PF2B, Perimed, Sweden) was positioned vertically over the exposed blister in the hindpaw via the Perspex chamber. The flux output of the laser Doppler monitor is a function of the concentration and the velocity of the red blood cells moving in the tissue penetrated by the laser light. The changes in relative blood flow (as determined by changes in red cell flux) were continuously displayed on a chart recorder. Raw data were evaluated by calculating the area under the response curve (cm^2). All measurements were made relative to a stable baseline obtained prior to drug perfusion. The baselines did not differ between control and capsaicin-pretreated rats or control and any other acute treatment groups.

Assay of CHS Five days after UV irradiation or UCA administration, mice (generally five per group) were sensitized on the shaved ventral skin with 100 μl freshly prepared 5% (wt/vol) TNCB (Tokyo Kasei Kogyo, Tokyo, Japan) in acetone. Five days later and after coding the identities of the mouse groups, a CHS response was elicited by applying 10 μl freshly prepared 1% TNCB in acetone to each of the ventral and dorsal surfaces of both ears. Twenty-four hours after challenge, the ear thickness was measured with a micrometer (Mitutoyo, Tokyo, Japan) and the extent of ear swelling for each mouse was calculated by subtracting the ear thickness before challenge. From this value was

Table I. *cis*-UCA does not degranulate rat peritoneal mast cells

Mediator	*n*	% histamine release[a] (mean + SEM)[b]
trans-UCA 700 μM	21	1 + 2
cis-UCA 700 μμ	26	1 + 1
substance P 1 μμ	8	3 + 2
substance P 10 μμ	8	5 + 2
substance P 100 μμ	8	30 + 8[c]
CGRP 0.1 μM	3	1 + 1
CGRP 2.0 μM	3	1 + 1
CGRP 25 μM	3	7 + 1
CGRP 50 μM	3	30 + 1[c]
48/80 1 μg per ml	16	35 + 7[c]
48/80 5 μg per ml	16	45 + 7[c]
48/80 10 μg per ml	16	57 + 7[c]

[a]Histamine release has been expressed as a percentage of the total cellular histamine content corrected for spontaneous release.
[b]The mean value for replicates from each of *n* experiments has been used to calculate the mean + SEM for *n* experiments.
[c]$p < 0.05$.

subtracted the mean swelling measured in mice that were challenged, but not sensitized, with TNCB (approximately 0.03 mm).

Expression of data and statistical analysis Vasodilator responses were measured as the area under the response curve (cm^2) using a digital planimeter (Tamaya, Japan). Results are expressed as mean ± SEM. Statistical analyses were performed using one-way analysis of variance followed by a Student–Newman–Keuls *post hoc* test. SNP responses were used as a covariate in the analysis. For measurements of histamine release from mast cells, the mean results from each of multiple experiments were used to calculate the mean value ± SEM. In the CHS assays, a multiple comparison procedure using a one-way analysis of variance and Fisher's least significance difference test was used to determine the statistical significance. Significance was set at $p < 0.05$.

RESULTS

***cis*-UCA does not degranulate rat peritoneal mast cells** Neither *cis*- nor *trans*-UCA (700 μM) significantly increased histamine release from rat peritoneal mast cells (**Table I**). Lower concentrations of UCA (7–70 μM) were also ineffective (data not shown). In contrast, the neuropeptides substance P and CGRP both stimulated histamine release (**Table I**). Increasing concentrations of 48/80 were tested as a positive mast cell degranulant.

***cis*-UCA increases microvascular blood flow** *cis*-UCA dose dependently increased microvascular blood flow in a rat hind paw blister base (**Fig 1A**). The area under the response curve reached 12.8 ± 1.2 cm^2 and 26.2 ± 4.0 cm^2 in response to 1 and 10 mM perfusion, respectively. *trans*-UCA was tested at both 1 and 10 mM but was without effect (**Fig 1B**). To determine if *trans*-UCA (10 mM) competed with cis-UCA (10 mM) for receptor occupancy, *trans*-UCA was perfused simultaneously with *cis*-UCA over the blister base but no antagonistic effect was observed (**Fig 1B**).

***cis*-UCA effects on microvascular flow due to neuropeptide release** To investigate the mechanism by which *cis*-UCA increased microvascular flow, the blister base was perfused with RP-67580 and $CGRP_{8-37}$, each at 10 μM, prior to *cis*-UCA. RP-67580 significantly inhibited the vascular response by 56% with the area under the response curve reaching 11.2 ± 0.9 cm^2. $CGRP_{8-37}$, on the other hand, reduced the response by 32% to 17.8 ± 2.4 cm^2. This reduction, however, did not reach statistical significance. **Figure 2** also demonstrates that substance P and CGRP were responsible in a cumulative fashion for 76% of the effect seen in response to *cis*-UCA. The vascular response to

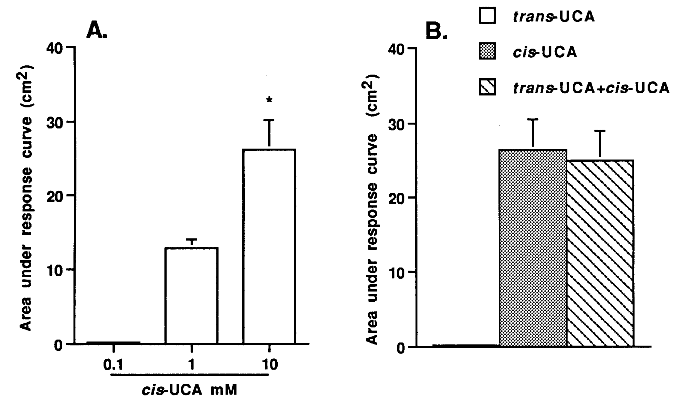

Figure 1. The effect of *cis*-UCA on microvascular blood flow. (*A*) Dose-dependent increase in microvascular blood flow in response to *cis*-UCA perfused for 20 min over the base of a blister raised on the hind footpad of naïve rats. *cis*-UCA was perfused at concentrations of 100 μM, 1 mM, and 10 mM for six to eight rats per group. An asterisk denotes significantly different from *cis*-UCA at 1 mM. (*B*) The effect of *trans*-UCA and *cis*-UCA (each at 10 mM) perfused for 20 min in isolation or together on microvascular blood flow recorded from the base of blisters raised on the hind footpad of naïve rats. For six to eight rats per group, *trans*-UCA did not induce a change in baseline blood flow. *cis*-UCA alone induced an increase in microvascular blood flow that was not significantly different from the response obtained to combined perfusion of both *cis*-UCA and *trans*-UCA.

cis-UCA in the presence of the combined antagonists (6.2 ± 1.4 cm^2) was significantly different from the control response as well as from the response in the presence of the substance P antagonist alone, indicating a significant contribution of CGRP.

***cis*-UCA has residual effect on microvascular blood flow in sensory-neuropeptide-depleted rats** To confirm that the effect of *cis*-UCA on microvascular flow was due, in large part, to its effects on sensory nerves, rats were depleted of sensory neuropeptides with capsaicin early in life. As shown in **Fig 3**, even in the sensory-neuropeptide-depleted rats, *cis*-UCA had a small stimulatory effect on microvascular flow that was not reduced by antagonists to substance P or CGRP, tested in isolation or together. Thus, *cis*-UCA has a small direct microvascular effect.

***cis*-UCA, like UVB, does not suppress systemic CHS responses in sensory-neuropeptide-depleted mice** To determine the importance of *cis*-UCA on sensory nerves for neuropeptide release to suppression of systemic CHS responses by UVB and *cis*-UCA in rodents, the immunomodulatory effects of UVB (8 kJ per m^2) and *cis*-UCA (200 μg per mouse subcutaneously) on CHS responses were examined in capsaicin-treated BALB/c mice. As previously published by Garssen and coworkers for UVB suppression of CHS responses in mice (Garssen *et al*, 1998), the immunomodulatory effects of UVB and *cis*-UCA as detected in control mice were not detected in the capsaicin-pretreated mice (**Fig 4**).

DISCUSSION

Our previous studies demonstrated that both *cis*-UCA and dermal mast cells by their release of histamine were critical to the ability of UVB to suppress "systemic" CHS responses in mice (Hart *et al*, 1997, 1998a,b, 1999). *cis*-UCA could not directly degranulate peritoneal mast cells, however, which are connective tissue mast cells like those in skin. In contrast, both substance P and CGRP dose-dependently stimulated histamine release. *cis*-UCA was also unable to stimulate histamine release from less mature, bone-marrow-derived mast cells (data not shown). As mast cells are located adjacent to nerves in skin (Egan *et al*, 1998), we investigated

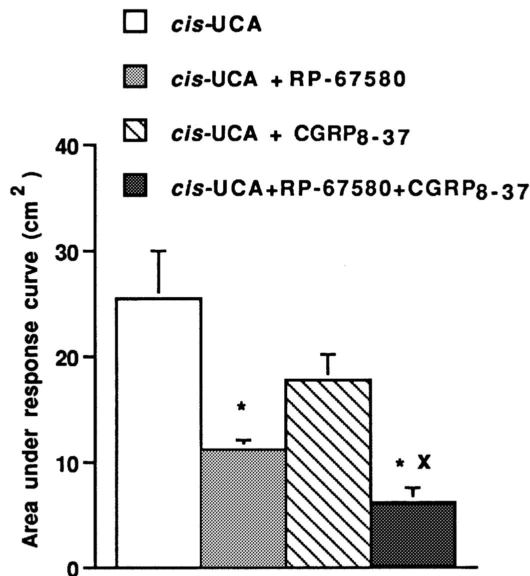

Figure 2. The effect of receptor antagonists to substance P and CGRP (RP-67580 and $CGRP_{8-37}$, respectively, each at 10 μM) on the vascular response to *cis*-UCA (10 mM). For six to eight rats per group, vascular responses were recorded from the base of blisters raised on the hind footpads of naïve rats. The antagonists (alone or in combination) were perfused for 10 min prior to and concomitant with *cis*-UCA. An asterisk denotes a significant difference from *cis*-UCA (10 mM). A cross denotes a significant difference from *cis*-UCA (10 mM) in the presence of RP-67580 alone.

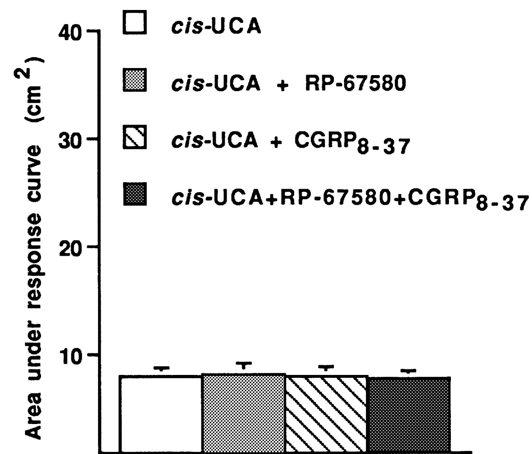

Figure 3. The effect of substance P and CGRP receptor antagonists (RP-67580 and $CGRP_{8-37}$, respectively, each at 10 μM) on the vascular response to *cis*-UCA (10 mM) in sensory-neuropeptide-depleted rats. For six to eight rats per group, vascular responses were recorded from the base of blisters raised on the hind footpads of capsaicin-pretreated rats. The receptor antagonists (alone or in combination) were perfused for 10 min prior to and concomitant with *cis*-UCA. No significant differences were observed between groups.

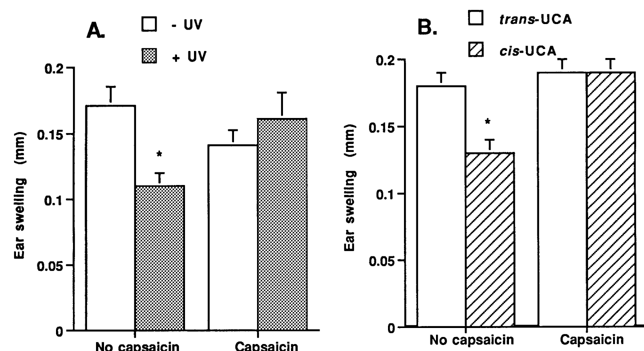

Figure 4. The effect of UVB and *cis*-UCA on the CHS responses of sensory-neuropeptide-depleted and control BALB/c mice to the hapten, TNCB. Four-week-old mice were injected subcutaneously in the neck region with capsaicin (25 mg per kg) on two consecutive days. After a further 4 wk, in (*A*) mice were untreated or irradiated with 8 kJ per m^2 UVB. In (*B*) mice were injected subcutaneously on their backs with *cis*-UCA (200 μg per mouse) or, as a control, *trans*-UCA (200 μg per mouse). The mean change in ear swelling (+ SEM) for five mice per group is shown; the groups of mice were always coded before challenge with hapten and ear thickness measurements. An asterisk denotes a result significantly different ($p < 0.05$) from that of nonirradiated mice in (*A*) or mice treated with *trans*-UCA in (*B*).

whether *cis*-UCA could indirectly stimulate mast cell degranulation by activating sensory nerves for neuropeptide release. A well-documented read-out system was adopted that allowed the dissection of neuropeptide-dependent changes in microvascular flow over a rat hind-paw blister base (Khalil *et al*, 1994; Khalil, 1999). *cis*-UCA, but not *trans*-UCA, increased microvascular flow in a predominantly substance P- and CGRP-dependent manner. This is the first report of a functional effect of *cis*-UCA on the peripheral terminals of primary afferent sensory nerves. That neuropeptides were important in *cis*-UCA-induced immunomodulation was confirmed in sensory-neuropeptide-depleted mice. *cis*-UCA and γ-amino-butyric acid (GABA) are analogs and it was reported that *cis*-UCA bound competitively to GABA receptors in rat cortex membranes (Laihia *et al*, 1998). The functional relevance of this binding was challenged, however, because GABA receptors are not generally found on peripheral nerves. GABA (10 mM) did not alter microvascular flow in our blister model (data not shown). In this study, *cis*-UCA stimulated neuropeptide release, which linked *cis*-UCA with control of mast cell degranulation. The contribution of microvascular changes induced by *cis*-UCA to UV-induced immunomodulation remains unknown. It has been published that sensory nerves in the skin terminate near Langerhans cells in the epidermis (Hosoi *et al*, 1993) and are therefore close to the site of *cis*-UCA formation and in the diffusion path of the more soluble UCA isomer. Dermal mast cells are generally located in skin near nerves and blood vessels. Thus, a functional link between *cis*-UCA, sensory nerves, mast cells, and, in our blister model, blood vessels, is anatomically defensible.

Our conclusions contrast with those of Wille and colleagues who used human skin organ cultures (Wille *et al*, 1999). It has been shown that, although the axons of sensory nerves are cut in the preparation of small skin explants, the nerve endings seal up at the point of severance and remain susceptible for at least 24 h to activation *in vitro* (Kilo *et al*, 1997). We hypothesize that in the skin organ cultures *cis*-UCA was activating dermal mast cells indirectly via its effect on the sensory nerves. The microvascular changes detected could not have been due to *cis*-UCA degranulating mast cells directly. Perfusion of the mast cell product, histamine, at 100 mM was without effect on cutaneous blood flow (data not shown). At 100 mM, however, histamine could potentiate the vascular response to substance P (data not shown).

A role for neuropeptides in UVB-induced "systemic" immunosuppression was first proposed by Garssen *et al* (1998). CGRP had been previously implicated in stimulating degranulation of mast cells in UV-irradiated skin (Niizeki *et al*, 1997; Alard *et al*, 1999). Streilein and colleagues previously reported that mast cell degranulation was critical for UV-induced "local" immunosuppression in mice (hapten applied within 30 min to the irradiated site) and that this effect was reduced by intradermal administration of a CGRP receptor antagonist (Niizeki *et al*, 1997). By use of

CGRP and substance P receptor antagonists administered systemically, Garssen and coworkers also found a role for CGRP, but not substance P, in UVB-induced systemic immunosuppression (Garssen *et al*, 1998). Garssen and colleagues used RP-67580, the same substance P receptor antagonist that was used in this study, to inhibit substance P binding to its receptor on sensory nerves. We hypothesize that substance P was not implicated in that study because substance P does not act on mast cells by a receptor-dependent mechanism (which RP-67580 will affect) but by a unique pertussis toxin-sensitive, cation-dependent, receptor-independent process (Mousli *et al*, 1989; Cocchiara *et al*, 1999). These previous studies did not implicate *cis*-UCA in these neuropeptide-dependent effects of UVB. Attempts to cause an immunosuppression by administration of CGRP and substance P 5 d prior to hapten sensitization were unsuccessful (data not shown), presumably because of the short half-life of the neuropeptides.

Although a well-known mast cell degranulating agent, substance P has not been previously recognized as an immunosuppressive molecule. In fact, it is generally recognized to stimulate certain parameters of the immune response (Ansel *et al*, 1996) and a substance P agonist administered within 30 min of the sensitizing antigen could act as an adjuvant to promote hapten-specific skin immunity (Niizeki *et al*, 1999). There is evidence that substance P can cause immune enhancement or immune suppression depending on the other factors involved (Khalil, 1999; Khalil *et al*, 2000).

The concentration of *cis*-UCA required for neuropeptide release was 1 mM, i.e., 184 μg during the 20 min perfusion of 1.3 ml. If *cis*-UCA acts by a receptor-driven system, this may appear high particularly as the concentration of UCA in rat dorsal or ear skin is 4–8 μg per cm^2 (Garssen *et al*, 1999). Due to the extravascular route of administration used in this study, however, a 100-fold higher concentration than would be required if administered intravascularly was required to overcome the diffusion barrier. A concentration of 1 mM *cis*-UCA was previously found to block 50% of specific binding of GABA to rat cortex membranes whereas 1 mM *trans*-UCA inhibited 23% of binding of GABA to its receptor (Laihia *et al*, 1998). In our studies of *cis*-UCA on monocyte activity, *cis*-UCA was active at a similar range of concentrations, i.e., *cis*-UCA at concentrations greater than or equal to 0.1 mM (Hart *et al*, 1993). For human skin, a concentration of UCA of 0.3–8.9 mM within the estimated 70 μm thick epidermis has been calculated (Bruls *et al*, 1984; Laihia *et al*, 1998), of which 50% would be *cis*-UCA after UVB irradiation. In human skin organ cultures (Wille *et al*, 1999), a concentration of *cis*-UCA of 75 μg per ml (0.5 mM) was required for maximal induction of E-selectin on microvessels by a pathway attributed to mast-cell-derived tumor necrosis factor α.

There have been several reports that CGRP can downregulate the activity of Langerhans cells, the antigen-presenting cells of epidermal skin (Hosoi *et al*, 1993; Lambert and Granstein, 1998). Activation of the CGRP receptor on Langerhans cells increases production of cAMP by adenylate cyclase and augmentation of lipopolysaccharide- and granulocyte–macrophage colony stimulating factor-induced interleukin 10 (IL-10) production (Asahina *et al*, 1995). IL-10 downregulates B7-2 and inhibits antigen presentation by Langerhans cells (Lambert and Granstein, 1998). In models of local UVB-induced immunomodulation, *cis*-UCA-induced production of CGRP may be important in regulation of Langerhans cell activity. *cis*-UCA-induced neuropeptides may also regulate other cells of irradiated skin, including keratinocytes. Substance P can bind normal human keratinocytes and stimulate IL-1 secretion (Kemeny *et al*, 1994; Lambert and Granstein, 1998).

In summary, this study has identified unmyelinated capsaicin-sensitive sensory nerves as an important responder cell to *cis*-UCA produced by UV isomerization of *trans*-UCA. In turn, neuropeptides are released that may activate neighboring mast cells. These *cis*-UCA-induced neuropeptides may also play an important role in regulation of other immune and inflammatory cells involved in cutaneous inflammation following UV exposure.

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REFERENCES

Alard P, Niizeki H, Hanninen L, Streilein JW: Local ultraviolet B irradiation impairs contact hypersensitivity induction by triggering release of tumor necrosis factor-α from mast cells. Involvement of mast cells and Langerhans cells in susceptibility to ultraviolet B. *J Invest Dermatol* 113:983–990, 1999

Ansel JC, Kaynard AH, Armstrong CA, Olerud J, Bunnett N, Payan D: Skin–nervous system interactions. *J Invest Dermatol* 106:198–204, 1996

Applegate LA, Ley RD, Alcalay J, Kripke ML: Identification of the molecular target for the suppression of contact hypersensitivity by ultraviolet radiation. *J Exp Med* 170:1117–1131, 1989

Asahina A, Moro O, Hosoi J, Lerner EA, Xu S, Takashima A, Granstein RD: Specific induction of cAMP in Langerhans cells by calcitonin gene-related peptide: relevance to functional effects. *Proc Natl Acad Sci USA* 92:8323–8327, 1995

Beissert S, Schwarz T: Mechanisms involved in ultraviolet light-induced immunosuppression. *J Invest Dermatol Symp Proc* 4:61–64, 1999

Beissert S, Mohammad T, Torri H, Lonati A, Yan Z, Morrison H, Granstein RD: Regulation of tumor antigen presentation by urocanic acid. *J Immunol* 159:92–96, 1997

Bouscarel B, Noonan F, Ceryak S, Gettys TW, Phillips TM, De Fabo EC: Regulation of stimulated cyclic AMP synthesis by urocanic acid. *Photochem Photobiol* 67:324–331, 1997

Bruls WAG, Slaper H, van der Leun JC, Berrens L: Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelengths. *Photochem Photobiol* 40:485–494, 1984

Cocchiara R, Lampiasi N, Albeggiani G, Bongiovanni A, Azzolina A, Geraci D: Mast cell production of TNF-α induced by substance P evidence for a modulatory role of substance P-antagonists. *J Neuroimmunol* 101:128–136, 1999

Daynes RA, Bernhard EJ, Gurish MF, Lynch DH: Experimental photoimmunology. Immunologic ramifications of UV-induced carcinogenesis. *J Invest Dermatol* 77:77–85, 1981

De Fabo EC, Noonan FP: Mechanism of immune suppression by ultraviolet irradiation *in vivo*. I. Evidence for the existence of a unique photoreceptor in skin and its role in photoimmunology. *J Exp Med* 157:84–98, 1983

Duthie MS, Kimber I, Norval M: The effects of ultraviolet radiation on the human immune system. *Br J Dermatol* 140:995–1009, 1999

Egan CL, Viglione-Schneck MJ, Walsh LJ, Green B, Trojanowski JQ, Whitaker-Menezes D, Murphy GF: Characterization of unmyelinated axons uniting epidermal and dermal immune cells in primate and murine skin. *J Cutan Pathol* 25:20–29, 1998

El-Ghorr AA, Norval M: A monoclonal antibody to *cis*-urocanic acid prevents the ultraviolet-induced changes in Langerhans cells and delayed hypersensitivity responses in mice, although not preventing dendritic cell accumulation in lymph nodes draining the site of irradiation and contact hypersensitivity responses. *J Invest Dermatol* 105:264–268, 1995

Garssen J, Buckley TL, Van Loveren H: A role for neuropeptides in UVB-induced systemic immunosuppression. *Photochem Photobiol* 68:205–210, 1998

Garssen J, Norval M, Crosby J, Dortant P, Van Loveren H: The role of urocanic acid in UVB-induced suppression of immunity to *Trichinella spiralis* infection in the rat. *Immunology* 96:298–306, 1999

Gilmour JW, Vestey JP, George S, Norval M: The effect of phototherapy and urocanic acid on natural killer cell function. *J Invest Dermatol* 101:169–174, 1993

Grimbaldeston MA, Skov L, Baadsgaard O, Skov BG, Marshman G, Finlay-Jones JJ, Hart PH: High dermal mast cell prevalence is a predisposing factor for basal cell carcinoma in humans. *J Invest Dermatol* 115:317–320, 2000

Hart PH, Jones CA, Jones KL, Watson CJ, Santucci I, Spencer LK, Finlay-Jones JJ: *Cis*-urocanic acid stimulates human peripheral blood monocyte prostaglandin E_2 production and suppresses indirectly tumor necrosis factor-α levels. *J Immunol* 150:4514–4523, 1993

Hart PH, Jaksic A, Swift G, Norval M, El-Ghorr AA, Finlay-Jones JJ: Histamine involvement in UVB- and *cis*-urocanic acid-induced systemic suppression of contact hypersensitivity responses. *Immunology* 91:601–608, 1997

Hart PH, Grimbaldeston MA, Swift GJ, Jaksic A, Noonan FP, Finlay-Jones JJ: Dermal mast cells determine susceptibility to ultraviolet B-induced systemic suppression of contact hypersensitivity responses in mice. *J Exp Med* 187:2045–2053, 1998a

Hart PH, Grimbaldeston MA, Swift GJ, Sedgwick JD, Korner H, Finlay-Jones JJ: TNF modulates susceptibility to UVB-induced systemic immunomodulation in mice by effects on dermal mast cell prevalence. *Eur J Immunol* 28:2893–2901, 1998b

Hart PH, Grimbaldeston MA, Swift GJ, Hosszu EK, Finlay-Jones JJ: A critical role for dermal mast cells in *cis*-urocanic acid-induced systemic suppression of contact hypersensitivity responses in mice. *Photochem Photobiol* 70:807–812, 1999

Holan V, Kuffova L, Zajicova A, Krulova M, Filipec M, Holler P, Jancarek A:

Urocanic acid enhances IL-10 production in activated CD4+ T cells. *J Immunol* 161:3237–3241, 1998

Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A, Granstein RD: Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature* 363:159–162, 1993

Jaksic A, Finlay-Jones JJ, Watson CJ, Spencer LK, Santucci I, Hart PH: *Cis*-urocanic acid synergizes with histamine for increased PGE_2 production by human keratinocytes: link to indomethacin-inhibitable UVB-induced immunosuppression. *Photochem Photobiol* 61:303–309, 1995

Kemeny L, von Restorff B, Michel G, Ruzicka T: Specific binding and lack of growth-promoting activity of substance P in cultured human keratinocytes. *J Invest Dermatol* 103:605–606, 1994

Khalil Z: Sensory peptides. Effects on ageing and wound healing. In: Brain SD, Moore PK, eds. *Pain and Neurogenic Inflammation*. Basel: Life Science, Birkhauser-Verlag, 1999:p 275

Khalil Z, Ralevic V, Bassirat M, Dusting GJ, Helme RD: Effects of ageing on sensory nerve function in rat skin. *Brain Res* 641:265–272, 1994

Khalil Z, Georgiou GM, Ogedegbe H, Cone RE, Simpson F, Little CH: Immunological and *in-vivo* neurological studies on a benzoic acid-specific T-cell-derived antigen-binding molecule from the serum of a toluene-sensitive patient. *Arch Environ Health* 55:304–318, 2000

Kilo S, Harding-Rose C, Hargreaves KM, Flores CM: Peripheral CGRP release as a marker for neurogenic inflammation: a model system for the study of neuropeptide secretion in rat paw skin. *Pain* 73:201–207, 1997

Kondo S, Sauder DN, McKenzie RC, Fujisawa H, Shivji GM, El-Ghorr A, Norval M: The role of *cis*-urocanic acid in UVB-induced suppression of contact hypersensitivity. *Immunol Lett* 48:181–186, 1995

Kripke ML: Immunologic mechanisms in UV-radiation carcinogenesis. *Adv Cancer Res* 34:69–106, 1981

Laihia JK, Attila M, Neuvonen K, Pasanen P, Tuomisto L, Jansen CT: Urocanic acid binds to GABA but not to histamine (H_1, H_2, or H_3) receptors. *J Invest Dermatol* 111:705–706, 1998

Lambert RW, Granstein RD: Neuropeptides and Langerhans cells. *Exp Dermatol* 7:73–80, 1998

Lembeck F, Holzer P: Substance P as a neurogenic mediator of antidromic vasodilatation and neurogenic plasma extravasation. *Naunyn-Schmiedeberg's Arch Pharmacol* 310:175–183, 1979

Mitra RS, Shimizu Y, Nickoloff BJ: Histamine and *cis*-urocanic acid augment tumour necrosis factor-alpha mediated induction of keratinocyte intercellular adhesion molecule-1 expression. *J Cell Pathol* 156:348–357, 1993

Moodycliffe AM, Bucana CD, Kripke ML, Norval M, Ullrich SE: Differential effects of a monoclonal antibody to *cis*-urocanic acid on the suppression of delayed and contact hypersensitivity following ultraviolet irradiation. *J Immunol* 157:2891–2899, 1996

Mousli M, Bronnert C, Beub JL, Tschirhart E, Gies JP, Landry Y: Activation of rat peritoneal mast cells by substance P and mastoparan. *J Pharm Exp Ther* 250:329–335, 1989

Niizeki H, Alard P, Streilein JW: Calcitonin gene-related peptide is necessary for ultraviolet B-impaired induction of contact hypersensitivity. *J Immunol* 59:5183–5186, 1997

Niizeki H, Kurimoto I, Streilein JW: A substance P agonist acts as an adjuvant to promote hapten-specific skin immunity. *J Invest Dermatol* 112:437–442, 1999

Noonan FP, De Fabo EC: Immunosuppression by ultraviolet B radiation: initiation by urocanic acid. *Immunol Today* 13:250–254, 1992

Norval M, Gibbs NK, Gilmour J: The role of urocanic acid in UV-induced immunosuppression: recent advances (1992–94). *Photochem Photobiol* 62:209–217, 1995

Schwarz T: UV light affects cell membrane and cytoplasmic targets. *J Photochem Photobiol B Biol* 44:91–96, 1998

Skov PS, Mosbech H, Norn S, Weeke B: Sensitive glass microfibre-based histamine analysis for allergy testing in washed blood cells. *Allergy* 40:213–218, 1985

Streilein JW, Taylor JR, Vincek V, Kurimoto I, Shimizu T, Tie C, Golomb C: Immune surveillance and sunlight-induced skin cancer. *Immunol Today* 15:174–179, 1994

Wille JJ, Kydonieus AF, Murphy GF: *cis*-Urocanic acid induced mast cell degranulation and release of preformed TNF-α: a possible mechanism linking UVB and *cis*-urocanic acid to immunosuppression of contact hypersensitivity. *Skin Pharmacol Appl Skin Physiol* 12:18–27, 1999