Urocanic Acid Isomers do not Modulate Intracellular Calcium and Cyclic AMP in Human Natural Killer Cells

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INVESTIGATIVE REPORT

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Ultraviolet (UV) light exposure on the mammalian skin induces immunosuppression and immunological tolerance. A major UV chromophore component in the stratum corneum, urocanic acid (UCA), has materialized as a candidate photoreceptor initiating the photoimmunological processes (1). UV-light irradiation influences natural killer cell function both in vitro and in vivo. The postulated ultraviolet photoreceptor in the epidermis, urocanic acid, has been reported to depress the cytotoxic activity of human natural killer cells. Therefore, this study investigated whether this would occur through specific second messengers, using a radioimmunoassay for intracellular adenosine 3',5'-cyclic monophosphate (cAMP) and Fluo-3 staining plus flow cytometry for free calcium. Both isolated lymphocytes and enriched CD16+ cells were used. A combination of the trans- and cis-isomers of urocanic acid (200 μg/ml) induced cAMP in both CD16+ and CD16− cells, but individual, stereospecific effects were not demonstrable. Urocanic acid did not induce significant changes in calcium levels in lymphocytes, or natural killer cells alone or conjugated to K562 target cells. Evidently, the biochemical evidence of urocanic acid-mediated natural killer-cell modulation is complex, and the cellular receptor(s) and specific signal transduction pathway(s) mediating the biological effects of urocanic acid remain elusive. Key words: flow cytometry; immunomagnetic separation; CD16; K562 cells.

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Ultraviolet (UV) light exposure on the mammalian skin induces immunosuppression and immunological tolerance. A major UV chromophore component in the stratum corneum, urocanic acid (UCA), has materialized as a candidate photoreceptor initiating the photoimmunological processes (1). Trans-UCA absorbs UV radiation and photoisomerizes to cis-UCA. Various studies have demonstrated that cis-UCA contributes to UV-induced suppression of cell-mediated immunity, although the mechanisms have remained uncertain (for review see Ref. 2). Cis-UCA is substantially more water soluble (2, 3), and it has been detected in the circulation of mice (4) and in the urine of human subjects (5) following UV irradiation.

Natural killer (NK) cells mediate the spontaneous lysis of certain malignant cells. The classical experimental target for human NK cells is the erythroleukaemic cell line K562, but NK cells are also capable of lysing a variety of neoplastic cells, and there is evidence for a role of NK and lymphokine-activated killer cells in immunosurveillance of cutaneous tumours in vivo, including malignant melanoma (6). UV light regulates NK cell cytotoxicity both in vitro (7, 8) and in vivo (9). Experimental evidence also suggests that UCA isomers affect the NK activity of human peripheral blood lymphocytes (10, 11). Second messengers are involved in the cytotoxic activity of NK cells, but their roles in the function of UCA in vitro are unknown. The cellular receptors for UCA have not been characterized, and the present study was undertaken to delineate the regulatory pathways at the intracellular level. Data are presented on 2 second messengers, intracellular calcium (Ca2+) and cyclic AMP (cAMP), in human peripheral blood lymphocytes and enriched NK cells, which suggest that trans- and cis-UCA do not stereospecifically modulate the levels of these mediators.

MATERIAL AND METHODS

Urocanic acid

Trans-UCA [3-(1H-imidazol-4-yl)-propenoic acid] was purchased from Sigma (St Louis, MO, USA). Cis-UCA was prepared from trans-UCA as described previously (12). The purity of both isomers was more than 98%, as detected by high-performance liquid chromatography (HPLC) (12). The isomers were dissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml, diluted in test buffers and added to cells. Corresponding amounts of DMSO were added to control tubes.

Antibodies

For the identification and isolation of peripheral blood cells, the following primary anti-human monoclonal antibodies were used: anti-CD16 (Leu-11a; Becton-Dickinson, Mountain View, CA, USA), anti-human T-cell (OKT3 IgG2a; ATCC CRL-8001) and anti-human monocyte (CD14; ATCC TIB228) supernatants. Antibody labelling was performed at 4°C in phosphate-buffered saline (PBS) containing 2% fetal calf serum (Biological Industries, Kibbutz Bet Haemek, Israel).

Enrichment of lymphocytes and natural killer cells

Mononuclear cells were isolated from venous blood of apparently healthy volunteers by Ficoll-Isopaque (Pharmacal, Uppsala, Sweden) gradient centrifugation. In assays for intracellular cAMP, the isolated cells were depleted of monocytes by adherence to plastic tissue culture flasks (Costar, Cambridge, MA, USA) after incubation in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with gentamycin (50 μg/ml; Orion, Espoo, Finland) and 10% fetal calf serum, referred to as complete medium, at 37°C for 1 h. The non-adherent peripheral blood lymphocytes were collected, washed and resuspended in complete medium. The proportion of CD16+ cells within peripheral blood lymphocytes ranged between 9.7 and 11.9%.

Magnetic enrichment of cells was performed using 2 separate methods. Peripheral blood lymphocytes were depleted of T-cells by reaction with the OKT3 antibody against the CD3 epitope at 4°C for 30 min. The CD3+ cells were removed with magnetic beads (Dynabeads; Dynal, Oslo, Norway) coated with sheep anti-mouse immunoglobulin G (IgG) antibodies. The yield of CD3+ cells was 91.1% of the total number of peripheral blood lymphocytes. Alternatively, rat anti-mouse IgG2a/2b-conjugated superparamagnetic microbeads (MACS; Milteny, Bergisch Gladbach, Germany) were applied to cells labelled with both mouse anti-human T-cell and mouse anti-human monocyte antibodies. After negative selection, 23.5% of the cells were recovered, representing CD3+CD14+ double-negative cells. The enriched NK population was identified with phycoerythrin-conjugated anti-CD16 antibody. Relative fluorescence intensities were
monitored with flow cytometry (FACScan; Becton Dickinson). For selected cAMP analyses, the cells were also sorted using a FACStar plus cytometer (Becton Dickinson).

**Assay for intracellular cyclic AMP**

Peripheral blood lymphocytes or CD3-depleted peripheral blood lymphocytes were incubated with or without UCA for various periods. The concentrations of the UCA isomers did not affect the viability of the lymphocytes. No spontaneous lysis was observed. DMSO was used as a diluent control for UCA. In some experiments, 3-isobutyl-1-methylxanthine (MIX) and forskolin were used. The stock solution of MIX was heated in a boiling water bath and diluted 1:100 in complete medium to keep the agent in solution. Forskolin was dissolved in ethanol and corresponding volumes of ethanol were added to test tubes without forskolin. The incubation was stopped either in a block heater (about 95°C) for 5 min or by adding ethanol to a final concentration of 60% to lyse the cells. Total intracellular cAMP in the lymphocytes cultured with or without UCA was measured by radioimmunoassay using the acetylation method (13). Radiolabeled 2'-O-monomethyladenosine 3',5'-cyclic monophosphate tyrosyl methyl ester was used as a tracer, and it was labeled according to Brooker et al. (14). With lymphocyte incubations stopped with ethanol, the same portion of ethanol was added to the standards.

**Analysis of intracellular free calcium**

The isolated cells were loaded with the fluorescent calcium indicator as follows. Fluor-3/AM (50 μg corresponding to 44 nmol of F-1242; Molecular Probes, Leiden, The Netherlands) was reconstituted in DMSO to yield a stock solution of 4 mM. Then, 3–10 × 10⁸ cells/ml in 10 mM HEPES-buffered Hank’s balanced salt solution (HBSS, without phenol red), pH 7.4, were incubated with 1 or 2 μM Fluor-3/AM in polypropylene tubes at 37°C for approximately 30 min. Corresponding concentrations of DMSO served as unloaded controls. Ionomycin at 2 μM was added as a positive control for maximal Ca²⁺ response.

**Conjugation of lymphocytes or enriched natural killer cells with K562 targets**

Conjugation studies were constructed according to the guidelines provided by Van Graft et al. (15) and Callewaert et al. (16). K562 cells were maintained in exponential growth phase in complete medium. After enrichment, antibody labelling or calcium indicator loading, the effector lymphocytes were washed with HBSS/HEPES and resuspended to 2.5–10 × 10⁶ cells/ml. UCA isoforms, freshly dissolved in the same medium, were added. Keeping all cells at 0°C, the effecter lymphocytes and K562 targets (ratio 1:1) were brought together and briefly centrifuged at 4°C. The supernatant was aspirated, and the cell suspension was gently shaken without vortexing and kept on ice until analysis with flow cytometry. PBS containing the appropriate concentrations of UCA was added, and the cells were incubated with occasional shaking in a water bath at about 37°C for various periods and analysed immediately with a FACScan flow cytometer.

**Statistics**

Measured concentrations of cAMP and Ca²⁺ were compared between the UCA-treated and control cells using a 2-tailed Student’s t-test after preanalysis with the F-test to determine whether the samples had different variances.

**RESULTS**

**Cyclic AMP**

Immunostaining and flow-cytometric cell sorting were performed to enrich CD16⁺ cells. CD16⁻ cells served as a reference. The cAMP levels measured after incubation of triplicate samples with high concentrations (200 μg/ml) of cis- and trans-UCA for 2 h were always above those of control cells (Fig. 1). This was true especially for trans-UCA in the CD16⁺ cells and for cis-UCA in CD16⁻ lymphocytes. In addition, cis-UCA inhibited cAMP in CD16⁺ cells at 10 μg/ml, although these effects lacked statistical significance. The isomers additively stimulated both cell populations at 200 μg/ml (total UCA concentrations 400 μg/ml). The elevation in cAMP was proportionally larger in CD16⁺ cells, where the basal concentrations were lower than in CD16⁻ cells (p = 0.014 and p = 0.015, respectively) (Fig. 1).

Attempts were made to elevate the basal steady-state levels of cAMP by inhibiting its further metabolism in peripheral blood lymphocytes by adding the phosphodiesterase inhibitor MIX (0.1 mM). The cis- and trans-UCA-incubated peripheral blood lymphocytes showed equally slight increases in cAMP levels after 20 min, but the levels were reduced close to control values after 2 h (data not shown). No increase could be observed without MIX. The isomers acted additively and stimulated cAMP during the 20-min incubation, decreasing after 2 h (Table I).

When NK cells were enriched by depletion of CD3⁺ (T) cells and incubated with UCA and MIX for 20 min, no effect of either the UCA isoforms or MIX could be seen. The cAMP-generating adenylyl cyclase stimulator forskolin (50 μM) was then combined with MIX to induce the synthesis of cAMP in peripheral blood lymphocytes. Cyclic AMP levels increased up to a plateau of 3–4 times the initial concentration with no effect of UCA (data not shown).

![Fig. 1. Concentrations of intracellular cyclic AMP (mean, SEM) in (A) CD16-positive and (B) CD16-negative cells after incubation with urocanic acid (UCA) isomers. The sorted cells (90,000 / tube) were incubated in triplicate tubes for 2 h.](image-url)
Table I. Levels of cyclic AMP in peripheral blood lymphocytes, 250,000 cells/0.5 ml, incubated in duplicate tubes with equal amounts (1:1) of the urocanic acid (UCA) isomers and 0.1 mM 3-isobutyl-1-methylxanthine (MIX).

| Incubation time (min) | MIX | Concentration of total UCA (µg/ml) |
|-----------------------|-----|---------------------------------|
|                       |     | 0                              | 20               | 400              |
| 20                    | –   | 42.9 ± 2.7                     | 50.7 ± 5.0       |
|                       | +   | 81.0 ± 4.8                     | 94.5 ± 11.2      |
| 120                   | –   | 17.4 ± 3.3                     | 21.0 ± 4.5       |
|                       | +   | 22.7 ± 6.4                     | 37.5 ± 1.0       |

The figures are in fmol/25,000 cells (mean ± SEM). Dimethyl sulphoxide was added into control tubes in place of UCA.

Intracellular calcium

Freshly isolated peripheral blood lymphocytes were labelled with anti-CD16, loaded with Fluo-3 and incubated with UCA isomers (200 µg/ml). After 45 min, Ca\(^{2+}\) levels were somewhat higher in both cis- and trans-UCA-treated cells. This could be seen in CD16\(^+\) and CD16\(^-\) cells as well as in lymphocytes gated by size (forward scatter) and granularity (side scatter) in flow cytometry (Fig. 2). However, none of these changes reached statistical significance. Anti-CD16 antibody alone did not change the fluorescence in Fluo-3-loaded control cells at 1, 15 or 45 min (Fig. 2 and additional data not shown). Ca\(^{2+}\) fluorescence levels were on average 90% of those measured in the ionomycin-treated control cells.

Conjugate formation

In order to study how NK cells bind to their target cells and how they become activated in the presence of UCA isomers, magnetically (MACS) enriched NK cells (CD3\(^-\)/CD14\(^-\)) were allowed to form conjugates with K562 targets and were identified by analysing the cells in Fluo-3 versus forward scatter gates by FACS (Fig. 3A). The number of conjugates localized in flow cytometry, and Ca\(^{2+}\) levels were measured by Fluo-3 fluorescence (B). The Fluo-3 intensity of the gated unconjugated lymphocytes in the same samples is shown for reference (open symbols). A total of 10,000 cells was analysed at the indicated incubation time points within 29–31 s.

Fig. 3. Ca\(^{2+}\) response of natural killer (NK)/K562 target cell conjugates treated with urocanic acid (UCA) isomers. Human peripheral blood lymphocytes were magnetically (MACS) depleted of T cells (CD3\(^-\)) and monocytes (CD14\(^-\)), and they were allowed to conjugate with K562 cells (cell ratio 1:1) at 0°C in the presence of UCA isomers (200 µg/ml). The cells were then incubated at 37°C. The conjugates of NK effector lymphocytes and K562 targets were electronically localized in flow cytometry (A), and Ca\(^{2+}\) levels were measured by Fluo-3 fluorescence (B). The Fluo-3 intensity of the gated unconjugated lymphocytes in the same samples is shown for reference (open symbols). A total of 10,000 cells was analysed at the indicated incubation time points within 29–31 s.
remained the same in the presence of trans-UCA (200 μg/ml; mean number of conjugates 6.7%, range 5.7–9.8%) and cis-UCA (mean 5.9%, range 5.3–7.3%) in comparison with control cells (5.7%, range 5.4–5.9%). In peripheral blood lymphocytes without enrichment, the proportion of conjugated cells ranged between 4.1 and 4.4%. The total number of analysed cells in FACS was 10,000, including effector cells. K562 target cells and their conjugates. The Fluo-3-loaded enriched NK cells had higher fluorescence in the conjugate gate with K562 targets than the NK cells alone. UCA had no effect on Ca\(^{2+}\) in the NK cells conjugated to targets (Fig. 3B).

**DISCUSSION**

Although the immunological functions of UCA have been widely investigated, little is known about the cellular receptor(s) or signal transduction system(s) mediating these effects (2). Based on both the close structural relationship with histamine and some indirect functional evidence, it has been postulated that histamine-like receptors would mediate the biological signal of cis-UCA at the cellular level. Recent reports have suggested that cis-UCA attenuates cAMP signaling induced by histamine in cultured fibroblasts (17, 18) and keratinocytes from the human skin (19), and epidermal cells from the pig skin (20), but not in human monocytes (21), hepatocytes isolated from the hamsters or an adenocarcinoma cell line (18). In all but one (17) of these reports UCA isomers alone failed to stimulate cAMP synthesis. It is presently widely accepted that although histaminergic signalling may take place in the whole process of UV-induced immunosuppression, histamine receptors are not the actual receptors for cis-UCA (3, 21–23). Other candidate receptors such as the imidazoline (24) and α\(_2\)-adrenergic receptors (18, 24) have also been proven non-reactive to UCA.

The cytotoxic activity of NK cells is regulated by both activating and inhibitory signals. Two groups of receptors transmitting signals that inhibit human NK cell function have recently been identified (for review see Ref. 25). Both of these receptor classes, the Ig superfamily-like and the lectin-like receptors, carry the immune tyrosine-based inhibitory motifs and, when bound to major histocompatibility complex I molecules on the target cell, abrogate the signal transduction cascade initiated by the activating receptors. Measurement of specific second messengers provides indirect information on the receptor types involved in cell signalling. For example, adenylyl cyclase-coupled receptors produce cAMP as a second messenger, whereas phospholipase C-mediated effects typically involve a Ca\(^{2+}\) increase in the cell. Taking advantage of the effect of UCA isomers on human NK cell function (10, 11) and the fact that second messengers, such as Ca\(^{2+}\) (26) and cAMP (27, 28), are critically involved in NK cell activation, human NK cells were used in this study as the model system for investigating the signal transduction pathways in UCA action. Most biological effects of UCA have been attributed to the cis isomer, but Palaszynski et al. showed that trans-UCA induces the formation of cAMP in a human dermal fibroblast lineage and that cis-UCA reverses this effect (17). The cytotoxic activity of human NK cells is strongly suppressed in the presence of trans-UCA in vitro (10). In the present signal-transduction experiments neither cis- nor trans-UCA showed activity alone, but a combination of the isomers elicited a significant stimulation of cAMP production in both NK cells and non-NK cells (Fig. 1, Table 1). Findings indicating inhibition of human polymorphonuclear leukocyte function *in vitro* without stereospecific effects by UCA isomers have been published (29). In corroboration with the present results, UCA did not affect cAMP signalling in those cells (29).

In the experiments on target cell binding of NK cells, the number of NK–K562 conjugates was only slightly increased by both trans- and cis-UCA. The Ca\(^{2+}\) levels in NK cells conjugated to targets were clearly higher than in unconjugated NK cells, but the values were not affected by UCA isomers either (Fig. 3B). Thus, the isomers do not regulate NK cell activity at a signal-transduction point characteristic of target cell recognition (26). This does not exclude the possibility that the lytic process may be inhibited at the post-binding level (30). According to recent data, conjugation of NK effectors and K562 targets initializes a process of lipid raft polarization to the NK-cell membrane site contacting with the target cell (31). The functional and temporal correlation of raft formation and intracellular signalling is not fully clear (31), and it may be that exogenous substances capable of directly modifying membrane raft polarization are not distinguishable at the second messenger level such as cAMP and Ca\(^{2+}\) signalling. If it were hypothesized that UCA would affect NK cell function through lipid raft formation, the observed stereospecific effects (10, 11) could partly reflect the differential solubility (lipophilicity) of cis- and trans-UCA.

Evidently, the biochemistry of UCA-mediated effects is complex. The present data do not clarify the general nature of the putative cellular UCA receptor(s) or the more specific question of the cellular signalling pathways in UCA-mediated NK modulation. There may be different receptors and signal-transduction pathways in different cell types. In addition, accessory effector cells may be involved; for example, the presence of monocytes has been found to regulate NK cell activity *in vitro*. The contradictory results of two recent investigations (10, 11) may be derived from the influence of monocytes and dendritic cells on NK cells in the latter study, as pointed out by Norval et al. (32). Elucidation of the cell-binding and signal-transduction mechanisms of UCA will need further research.

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