Urocanic acid in photodermatology
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CHAPTER 8

Suppression of contact hypersensitivity response by urocanic acid oxidation products

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Abstract

Urocanic acid (UCA) can \textit{in vitro} be photooxidized by UV-B irradiation into UCA oxidation products; three of these compounds, the imidazolic UCA oxidation products (the \textquoteleft imidazoles\textquoteright), could be detected \textit{in vivo} in corneal layers of the epidermis as well. We hypothesized that the imidazoles participate in the UV-induced systemic immunosuppression and tested this in a murine model for contact hypersensitivity (CHS). A crude mixture of photo-oxidized products of UCA (2.0 and 0.2 g/l) significantly inhibited the CHS responses. Two imidazoles, i.e. imidazole-4-carboxylic acid (ImCOOH) at 1.0 g/l and imidazole-4-acetic acid (ImAC) at 1.0 and 0.2 g/l significantly reduced the CHS response with a potency comparable to that of the well-known inhibitor \textit{cis}-UCA. A stronger reduction was observed with the third imidazole, imidazole-4-carboxaldehyde (ImCHO), either at 1.0 and 0.2 g/l. When added in combination, the three imidazoles (each 0.33 g/l) showed strong, very significant inhibition of ear swelling, which effect was more pronounced than the suppression induced by \textit{cis}-UCA. Paired combinations of the three imidazoles showed intermediate, but very significant, reductions in ear swelling. These results indicate that, in addition to \textit{cis}-UCA, the imidazolic UCA oxidation products play a role in UV-induced immunosuppression as well, possibly in concert with each other.
1. Introduction

In the last decade extensive immunological research has been carried out to characterize the immunomodulatory effect of urocanic acid (UCA), predominantly that of the cis-UCA isomer. This stereoisomer of UCA is formed upon photoisomerization of trans-UCA by UV irradiation of the skin up to a wavelength of approximately 360 nm [1]. It was shown that cis-UCA can mimic several effects of UV on the immune system such as local and systemic immunosuppression or effects that fit in UV-induced immunosuppressive reaction cascades [2,3]. Most convincing evidence for trans-UCA, as the photoreceptor, and cis-UCA, as the immunosuppressant, was obtained from the mouse model for delayed type hypersensitivity (DTH) and in contact hypersensitivity (CHS) [3]. However, in various assays in vitro no inhibitory effect of cis-UCA could be demonstrated [4-7]. The mechanism of cis-UCA induced immunosuppression is still a puzzling issue.

In a previous report [8], it was demonstrated that trans-UCA and cis-UCA are converted to oxidation products in oxidative stress circumstances. There is strong evidence that the epidermis undergoes a relatively high level of oxidative stress during UV-irradiation leading to the formation of reactive hydroxyl radicals [9-11]. Trans-UCA and UV-induced cis-UCA are present at relatively high concentrations in the epidermis and may form potential targets for hydroxyl radicals. UCA isomers are efficient hydroxyl radical scavengers [12] and several oxidation products are formed during scavenging in vitro as well as in UV-B exposed corneal skin samples [8].

We suggest that oxidation products of UCA isomers may be involved in the process of UV-induced immunosuppression. This is supported by the following observations. First, similar levels of cis-UCA can be induced by UV-A and UV-B, whereas UV-B, but not UV-A, is the principal inducer of immunosuppression [13] and UCA oxidation products are only formed by UV-B [or shorter waves] from both UCA isomers, and not by UV-A [12]. Second, antioxidants have an abrogatory effect on the UV-induced and cis-UCA induced immunosuppression [14,15].

To demonstrate a possible role for UCA oxidation products in the phenomenon of UV-induced immunosuppression, the suppressive potency of UCA oxidation products was tested in a mouse model and compared with that of the UCA isomers themselves. The inhibition of induction of contact hypersensitivity (CHS) was used as a test, measuring ear swelling reductions after elicitation with the contact sensitizer picryl chloride.
Suppression of contact hypersensitivity response

2. Materials and Methods

2.1 Materials

*Trans*-UCA, imidazole-4-carboxylic acid, imidazole-4-carboxaldehyde, imidazole-4-acetic acid (sodium salt), glyoxylic acid monohydrate and oxalic acid dihydrate were supplied by Sigma-Aldrich/Fluka Chemie BV [Zwijndrecht, The Netherlands]. *Cis*-UCA was kindly offered by dr. WMPB Menge of the Free University, Department of Pharmacology, Amsterdam, The Netherlands. Picryl chloride (PCI; Chemotronix, Swannanoa, NC, USA) was used as contact sensitizer in all experiments.

2.2 Animals

Male BALB/c mice (8-10 weeks of age) were obtained from the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). The animals (BALB/c/Rivm) were kept in light-controlled, humidity-controlled and temperature-controlled rooms in the animal facility, already two weeks prior to the experiment. They were fed with water and Hope Farm Chow (SRM-A) ad libitum.

2.3 Contact Hypersensitivity

Picryl chloride (PCI) was recrystallized three times from methanol/water before use and was protected from light and humidity during storage at 4° C. For active contact sensitization, mice were sensitized with PCI by epicutaneous application of 25 μl 5 % PCI in ethanol/acetone (3:1) on each skin location of the abdomen, thorax and each foot (150 μl/mouse). Four days after sensitization, mice were challenged on both sides of each ear by topical application of one drop of 0.8 % PCI in olive oil. Duplicate measurements of ear thickness were made before elicitation and at 24 h after ear challenge with an engineer’s micrometer (Mitutoyo model 193-10, Tokyo, Japan). The gain in ear thickness was expressed as the mean ± SEM in micrometers and as a relative expression in percent of the positive control value (= 100 %, derived from non-treated sensitized mice). Nonsensitized (negative) control animals were challenged and measured similarly. These backgrond ear-swelling responses were subtracted from the other measured responses to obtain the net ear-swelling responses.

2.4 Test solutions

*Cis*-UCA and UCA oxidation products were dissolved in sterile phosphate buffered saline (PBS). 200 μl of freshly dissolved test material was given to each mouse subcutaneously, equally divided in two portions and 1 h prior to sensitization with PCI.

Part III: UCA in immunological models
2.5. The UCA photooxidation mixture (PO-mix)

*Trans*-UCA (8 mmol) was photooxidized in the presence of hydrogen peroxide (40 mmol) by xenon-arc radiation that was tuned by UG11 and WG305 optical filters, affording simulated solar UV with a spectral distribution in the UV-range from 290-400 nm. It was previously shown (12) that UV-radiation < 320 nm induces UCA photo-oxidation. The mixture of photooxidized products to be tested here is referred to as PO-mix. Because the UV-irradiation also contained the effective UV-wavebands to evoke photoisomerization, *cis*-UCA was formed in the mixture as well. However, the PO-mix contained less than 3 % of each UCA isomer, due to breakdown of UCA into known and unknown photooxidation products. The PO-mix, a light-yellow product, is readily soluble in water media and contains the previously identified three imidazolic UCA oxidation products, 'the imidazoles' (12). The imidazoles were tested for their CHS response in pure form, separately and in combination with each other.

2.6. Statistics

Levels of significance were calculated using the one-tailed Student's *t*-test; *p* < 0.05 and *p* < 0.01 were taken as significant and very significant differences, respectively. Each similarly treated group consisted of three mice, giving 18 ear thickness measurements. The data of the CHS experiment for the separate imidazoles (results of Fig.2) were derived from two sets of 3 mice (total *n* = 6).

3. Results

3.1. Suppression of CHS by photooxidized products of UCA

A mixture of photooxidized products of UCA (PO-mix), *trans*-UCA and *cis*-UCA were tested for their capacity to suppress CHS responses, using picryl chloride as the contact sensitizer. The test compounds, dissolved in PBS, were subcutaneously injected into the mice one hour prior to sensitization with PCI and the results were compared with those of PBS-treated, sensitized mice (positive controls). The PO-mix at concentrations of 0.2 and 2 g/l strongly decreased the CHS response in a dose-dependent fashion compared with that of the positive control (= 100 % swelling): 29 and 19 % ear swelling, respectively (Fig. 1). Although the CHS response after administration of the PO-mix (2 g/l) was only 19 % of the positive control, the difference with the ‘classical’ well-known inhibitor *cis*-UCA (31 % of positive control), was not significant.

Although the overall concentration of the PO-mix was twice that of *cis*-UCA, it must be noted that the suppression-inducing compounds in that
Suppression of contact hypersensitivity response

Suppression of CHS by photooxidized products of UCA

<table>
<thead>
<tr>
<th></th>
<th>% Ear swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative control</td>
<td>0</td>
</tr>
<tr>
<td>positive control</td>
<td>100</td>
</tr>
<tr>
<td>cis-UCA 1 g/l</td>
<td>31</td>
</tr>
<tr>
<td>PO-mix 2 g/l</td>
<td>19</td>
</tr>
<tr>
<td>PO-mix 0.2 g/l</td>
<td>29</td>
</tr>
</tbody>
</table>

Figure 1. Net (Ag-specific) ear swelling response 24 h after elicitation of the ear pinnae with one drop of 0.8 % picryl chloride (PCI) in olive oil. The background swelling, induced by PCI application of non-sensitized animals (< 10 μm), was subtracted from the swelling in PCI-sensitized animals. The positive control revealed a net ear swelling of 115 μm and was set to 100 %. The other values (dark grey) were converted into percentages by multiplying the particular net ear swelling by 100 and dividing by the net ear swelling of the positive control. The double asterisk means a very significant difference of p < 0.005, compared to positive control.

Cis-UCA and both PO-mix solutions showed very significant CHS reductions (p < 0.005) compared to the positive control (Fig. 1).

Part III: UCA in immunological models
Suppression of CHS by imidazolic UCA oxidation products

<table>
<thead>
<tr>
<th>Product</th>
<th>Concentration (g/l)</th>
<th>% Ear swelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>Positive control</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>cis-UCA</td>
<td>1.0</td>
<td>67</td>
</tr>
<tr>
<td>ImCOOH</td>
<td>1.0</td>
<td>66</td>
</tr>
<tr>
<td>ImCOOH</td>
<td>0.2</td>
<td>74</td>
</tr>
<tr>
<td>ImCHO</td>
<td>1.0</td>
<td>59</td>
</tr>
<tr>
<td>ImCHO</td>
<td>0.2</td>
<td>48</td>
</tr>
<tr>
<td>ImAc</td>
<td>1.0</td>
<td>59</td>
</tr>
<tr>
<td>ImAc</td>
<td>0.2</td>
<td>68</td>
</tr>
</tbody>
</table>

Figure 2. Same conditions as in Figure 1, except that the positive control showed a net ear swelling of 111 μm and that the single and the double asterisks refer to a significant reduction, compared to the positive control, of p < 0.05 and p < 0.01, respectively.

3.2. Suppression of CHS by imidazolic UCA oxidation products

Within the mix of UCA photooxidation products three imidazolic compounds were identified, i.e. imidazole-4-carboxylic acid (ImCOOH), imidazole-4-carboxaldehyde (ImCHO) and imidazole-4-acetic acid (ImAc), the ‘imidazoles’. These three imidazoles were separately tested for the CHS response.
Suppression of CHS by combinations of imidazolic UCA oxidation products

**Figure 3.** Same conditions as in Figure 1, except that the positive control showed a net ear swelling of 70 µm and that the single and double asterisks refer to very significant differences, compared to positive control, of p < 0.005 and p < 0.0005, respectively.

ImCOOH 1.0 g/l and ImAc at 1.0 and 0.2 g/l showed significant (p < 0.05) reductions in CHS responses, similar to that of cis-UCA (Fig. 2). However, ImCOOH at a concentration of 0.2 g/l did not show a significant reduction compared to that of the positive control. In this experiment ImCHO at both 1.0 and 0.2 g/l showed the strongest reductions in CHS response with a very significant level (p < 0.01) (Fig. 2).

Part III: UCA in immunological models
Another identified UCA oxidation product, glyoxylic acid, and its proposed oxidation product, oxalic acid, were also tested as neutral ammonium salts for their effects on the CHS response, but they did not show any significant suppressive effects (data not shown). Other identified UCA oxidation products were not tested yet, either because of unavailability, e.g. UCA dimers, or because of expected lack of relevance in this context, e.g. glycine and aspartic acid.

3.3. Suppression of CHS by combinations of imidazolic UCA oxidation products

The three imidazoles, ImCOOH, ImCHO and ImAc (here: A, B, C, respectively) are concurrently generated in the skin upon oxidative stress (12). Therefore, the three imidazoles were also tested in several combinations to aim at reinforcement of the suppression of CHS response. In the double (AB, BC, AC) or triplet (ABC) combinations each imidazole was kept at a concentration of 0.33 g/l. For the triplet combination each imidazole was also used at the 4-fold lower concentration of 0.08 g/l.

In contrast to the results of the separately tested imidazoles (previous experiment), very significant reductions in ear swelling (p < 0.005) resulted from paired combinations of the imidazoles (AB, BC and AC) compared to that of the positive control (Fig. 3). However, the strongest reductions in CHS response were obtained when the three imidazoles were administered in combination. The triplet combinations of the imidazoles (ABC), resulted in a CHS response of 24 % of positive control, which is a very significant reduction (p < 0.0005). Moreover, a similarly strong reduction in CHS response (24 %) was obtained with a four times lower concentration (Fig. 3). Consequently, the strong suppressive abilities of the triplet combinations of the imidazoles (ABC) must have been retained across a concentration range, of which the limits are not yet defined.

4. Discussion

Trans-UCA and cis-UCA were tested in CHS models many times before during the past two decades and cis-UCA was unanimously found to exert the most potent suppression of CHS responses, as compared to trans-UCA. Similar results were also obtained in an analogous model, the delayed type hypersensitivity model (DTH), using herpes simplex virus (HSV) as antigen (16). It should be noted that the molecular structures of UCA isomers are not unique in causing suppressive effects in these CHS and DTH models. Other imidazolic compounds have been shown previously to suppress DTH to HSV as well (16,17). It seems that the strongest suppressive effects were found with compounds having a substituent at the
4-position of the imidazole ring (e.g., dihydroUCA, histamine and cis-UCA). The three imidazolic UCA oxidation products tested in this study have '4'-substituted imidazole rings as well. In this respect, the suppressive ability of the imidazolic UCA oxidation products is in line with this concept. In particular ImCHO, alone or combined with the other imidazoles, showed potent suppression of the CHS response. Related compounds with other molecular modalities, such as other 5-membered heterocyclic rings, were less able to cause suppression of DTH to HSV (16).

Histamine, or 2-[imidazol-4-yl]ethylamine, is a '4'-substituted imidazole as stated above and since histamine was show to suppress the immune response to HSV (16) and to regulate a variety of immune effector cells by interaction with H1- and H2-receptors, cimetidine (an H2-receptor antagonist) and terfenadine (an H1-receptor antagonist) were tested in the model of DTH response in the mouse (17). Both pharmacas proved to be able to reduce or abrogate the cis-UCA-induced immunosuppression. However, thioperamide, an H3-receptor antagonist, could not reverse the suppression of the DTH response (18). These results indicate that cis-UCA may act through histamine H1 receptors and histamine H2 receptors in the skin. Accordingly, we thought it would be worthwhile to investigate the interaction between histamine receptors or histamine-like receptors and the three imidazolic UCA oxidation products. However, cimetidine is also a good hydroxyl radical scavenger (19) and may therefore compete with the UCA isomers, leading to a reduced formation of UCA oxidation products. This observation may form an alternative explanation for the antagonistic effects of cimetidine in cis-UCA induced immunosuppression.

The strong reduction in CHS response exerted by the PO-mix, is a phenomenon for further investigation. The PO-mix consists of identified compounds and unidentified compounds, each of them at concentrations that must be lower than any test concentration in the performed experiments (< 0.2 g/l), and yet a strong reduction in CHS response resulted, at least as strong as that of cis-UCA at the higher concentration of 1 g/l (Fig. 1). The three identified imidazoles may contribute to this suppressive effect, but it is plausible that other, even more powerful immunosuppressants may be present among the unidentified compounds. ImCOOH and ImAc showed moderate suppressive effects on CHS response, while ImCHO was the strongest suppressant (Fig. 2). The reductions in CHS response by the triplet combinations of the imidazoles seem to be stronger than that of cis-UCA, which finding is reinforced by a similar large reduction in CHS response, caused by a 4-fold diluted triplet combination (Fig. 3).

In our experiments, testing of the individual imidazoles resulted in smaller reductions in CHS responses than observed with combinations of the
imidazoles at similar or lower concentrations. However, it is too premature to draw conclusions on synergistic effects already, because the results of the separate and combined actions of the imidazoles were derived from different experiments. As yet unanswered questions in this respect are: 1. the degree of suppression that would be observed at lower concentrations of the test compounds, and 2. the possible occurrence of synergy from combinations of the test compounds. The first question is in particular relevant to the expected levels in sun-exposed skin. Our previous study [8] showed that the concentrations of the three imidazolic UCA oxidation products are lower than those of the UCA isomers in the UV-B exposed corneal layer of the skin. Accurate estimations cannot be made because of different routes of gaining the imidazoles [photooxidative induction at a certain skin area versus subcutaneous injection].

Adopting the view of an active role of UCA oxidation products in skin immunity, we have no explanation yet for the more intense immunosuppressive action of cis-UCA over trans-UCA, as similar chromatographic patterns emerged from oxidized trans-UCA and oxidized cis-UCA after HPLC-analysis. As with cis-UCA, the relation of the immunosuppressive effects of the imidazoles with health hazards such as skin cancer should become known in the future. Our future research will be focussed on the identification of other UCA oxidation products, on their assessment in vivo under various conditions of oxidative stress, and on further testing of their immune functions.

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