Effect of Arylhydroxylamine Metabolites of Sulfamethoxazole and Dapsone on Stress Signal Expression in Human Keratinocytes

Farah D. Khan,¹ Piyush M. Vyas,² Anthony A. Gaspari, and Craig K. Svensson

Division of Pharmaceutics, The University of Iowa, Iowa City, Iowa (F.D.K., P.M.V., C.K.S.); Department of Dermatology, University of Maryland School of Medicine, Baltimore, Maryland (A.A.G.); and Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, Indiana (C.K.S.)

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ABSTRACT

The initiation of an immune response to small molecules is believed to require the release of stress/danger signals that activate resident dendritic cells, presumably secondary to the formation of reactive metabolites. We hypothesized that exposure to arylhydroxylamine metabolites of dapsone and sulfamethoxazole lead to the expression/release of numerous stress signals in the skin. To test this hypothesis, we examined the effect of these metabolites on the expression of selected heat shock proteins, uric acid, cytokines, adhesion molecules, and costimulatory molecules in normal human epidermal keratinocytes (NHEKs). NHEKs showed a time-dependent up-regulation of heat shock protein 70 and translocation of heat shock protein 27 when exposed to the arylhydroxylamine metabolites. In addition, the secretion of several proinflammatory cytokines was increased upon incubation of these cells with metabolite. In contrast, the uric acid concentration was not altered. Moreover, intercellular adhesion molecule-1, CD80, and CD86 expressions did not change when NHEKs were exposed to these reactive metabolites. Our data suggest that NHEKs selectively up-regulate certain danger signals when exposed to arylhydroxylamine metabolites. These signals may subsequently activate dendritic cells and initiate an immune response within skin.

Cutaneous drug reactions (CDRs) are the most common adverse drug reactions associated with sulfamethoxazole (SMX) and dapsone (DDS). In addition, these agents are among those most commonly associated with severe CDRs (Svensson et al., 2001). Exhibiting several characteristics of delayed-type hypersensitivity, these reactions seem to be immune-mediated, involving T-cell activation (Khan et al., 2006). Reaction of the immune system to such drugs may occur in a framework consistent with the hapten hypothesis (Park et al., 1987), as numerous studies have suggested a role for metabolic activation of drugs to reactive metabolites. These signals may subsequently activate dendritic cells and initiate an immune response within skin.

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¹ Current affiliation: Rutgers University, Department of Pharmacology and Toxicology, Piscataway, New Jersey.
² Current affiliation: Riley Hospital for Children, Wells Center for Pediatric Research, Indianapolis, Indiana.

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ABBREVIATIONS: CDR, cutaneous drug reaction; SMX, sulfamethoxazole; DDS, dapsone; S-NOH, sulfamethoxazole hydroxylamine; D-NOH, dapsone hydroxylamine; APC, antigen-presenting cell; LC, Langerhans cell; Hsp, heat shock protein; ICAM-1, intercellular adhesion molecule-1; IFN-α, interferon-α; NHEK, normal human epidermal keratinocyte; KGM-2, keratinocyte growth medium; DAPI, 4,6-diamidino-2-phenylindole; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide.
their unique migratory ability, LCs can transport antigen from the epidermis to regional lymph nodes, where they can activate T cells and initiate systemic immune responses (Cumberbatch et al., 2000; Jakob et al., 2001).

The list of molecules believed to be capable of acting as danger signals continues to expand. Examples include uric acid (Shi et al., 2003), heat shock proteins (Srivastava, 2002), adenosine 5′-triphosphate (la Sala et al., 2003), mammalian DNA, interferon-γ, interleukin-1β, CD-40 ligand, the breakdown product of hyaluron, lipopolysaccharide (Medzhitov and Janeway, 2000), unmethylated cytosine-guanine (CpG) sequences (Kandimala et al., 2003), and viral RNA. The inducible heat shock protein (Hsp) 70 has been recently characterized as a potent maturation stimulus for dendritic cells (Kuppern et al., 2001). Evidence to date indicates that heat shock proteins are released from necrotic but not apoptotic cells and that their release into the extracellular environment indicates nonphysiological tissue damage, which in turn induces a range of inflammatory responses. In particular, Sauter et al. (2000) demonstrated that necrotic cell lysates, but not apoptotic cells, provoke the maturation of APCs. Basu et al. (2000) also demonstrated that Hsp70, Hsp90, gp96, and calreticulin are released from cells as a result of necrotic but not apoptotic cell death.

The importance of cytokines in LC migration is also well established (Wang et al., 1999; Kimber et al., 2000). Keratinocytes have been shown to secrete a variety of cytokines that provide essential signals for migration of epidermal LCs (Barraud et al., 1997). They also have been shown to activate intercellular adhesion molecule (ICAM-1) and CD80 (also known as B7.1) on keratinocytes. Keratinocytes under stress also express adhesion molecules themselves, such as ICAM-1, that help in T-lymphocyte infiltration to damaged sites (Arnold-Schild et al., 1999).

These observations provide evidence that keratinocytes have the ability to release signals that can initiate and modulate the cutaneous immune response (Shiohara et al., 1989; Nickoloff and Turka, 1994; Stoff et al., 1994; Cumberbatch et al., 2000). Hence, stress generated within keratinocytes as a result of exposure to reactive metabolites may result in the release of "danger signals," which in turn may act as initiators of LC maturation and migration. Therefore, we tested the hypothesis that keratinocytes exhibit induced expression or release of such signals when exposed to the reactive metabolites of SMX or DDS, S-NOH and D-NOH. Our results suggest that exposure to metabolites results in an up-regulation of certain signals, indicating that keratinocytes have the potential to activate LCs upon exposure to these metabolites.

Materials and Methods

Materials and Cell Culture. S-NOH and D-NOH were synthesized and purified as described previously, with purity found to be >97% (Vyas et al., 2005). IFN-α was obtained from R&D Systems (Minneapolis, MN). All other chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO) unless specified otherwise. Adult normal human epidermal keratinocytes (NHKs) were obtained from Cambrex Bio Science Walkersville (Walkersville, MD) as cryopreserved first passage cells and subcultured to subsequent passages as described previously (Reilly et al., 2000). All experiments were performed on fourth passage cells. Cells from three different patients were used unless stated otherwise.

Immunofluorescence Microscopy. Expressions of Hsp70, Hsp27, ICAM-1, and CD80 were determined individually using Alexa Fluor 488 (Invitrogen) for 1 h at 37°C. The coverslips were washed three times with phosphate-buffered saline, treated with propidium iodide (0.5 mg/ml) for 5 min at room temperature in the dark, and mounted on glass slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA). For colocalization studies of Hsp27, mounting medium containing DAPI (Vector Laboratories) was used as a nuclear marker. The experiments for the individual markers were conducted in cells from three patients.

Uric Acid Assessment Using Spectrofluorometry. An Amplex uric acid kit from Invitrogen was used for the assessment of uric acid. In brief, (1 × 10⁵) cells were incubated with the metabolites (S-NOH and D-NOH) for 6 or 24 h at 37°C. After treatments, supernatants were collected, and aliquots kept at −86°C until assayed. The uric acid was determined using a fluorescence plate reader (SpectraMax) with excitation at 530 nm and emission at 590 nm. Sample values were calculated by subtracting the readings with no uric acid present. Sample concentrations were calculated from a standard plot. Results are presented as mean (S.D.) of incubations from three individual patients.

Cytokine Release Using Bio-Plex System. Release of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), granulocyte macrophage–colony-stimulating factor, and IFN-γ was determined using a multiplexed bead-based immunoassay kit (Bio-Rad, Hercules, CA) and read using a Luminex 100 analyzer (Bio-Rad). In brief, 5 × 10⁵ cells were incubated with phorbol 12-myristate 13-acetate (PMA) (20 ng/ml), 50 μM D-NOH, 150 μM S-NOH or DMSO for 24 h in six-well plates. Untreated samples incubated concurrently were used as control. To measure the cytokines released into the media, supernatant was collected, and an aliquot was removed and stored at −86°C until assayed. The treated cells were then scraped in 0.1% Triton X-100 on ice to measure the intracellular formation of cytokines. The Bio-Plex assay was performed according to the manufacturer’s protocol. In brief, a filter-bottom 96-well microplate was prewetted with assay buffer after which the buffer was removed using vacuum filtration, and 50 μl of polystyrene beads precoated with antibodies against the four cytokines was added into each well. The plate was washed twice with the washing buffer provided in the kit, and the buffer was removed by vacuum filtration. Cytokine standards (10–1000 pg/ml) prepared in KGM-2 and samples were added and allowed to incubate on a shaker for 30 min. After washing three times with wash buffer to remove unbound protein, 25 μl of Bio-Plex detection antibody specific for a different epitope on the cytokine was added, and the
mixture was allowed to incubate for 30 min. After further washings (three times), 50 μl of streptavidin-phycocerythrin was added, and the mixture was allowed to incubate for 10 min. The plate was washed (three times) to remove any unbound dye, suspended in 125 μl of assay buffer, and analyzed on a Luminex 100 analyzer, set to run a 50-μl sample and minimum of 100 events per cytokine. All incubations were performed at room temperature, and assay plates were covered with sealing tape and aluminum foil while shaking (300 rpm). After each wash step, the underside of the plate was blotted with a paper towel. Data were calculated with Bio-Plex Manager software (Bio-Rad) using a five-parameter logistic regression along with a weighting model (used when six or more standards are found to be within 70–130% of expected value). Standards, positive controls, and samples are expressed as picograms per milliliter. All results were obtained.

Statistical Analysis. Data were statistically analyzed using SigmaStat software (Systat Software Inc., San Rafael, CA). For comparing groups, a Kruskal-Wallis analysis of variance on ranks was used along with a Holm-Sidak test for multiple comparisons. p < 0.05 was considered significant.

Results

Effect of Metabolites on Hsp70 Expression. We examined the ability of NHEKs to induce Hsp70 expression when exposed to the arylhydroxylamine metabolites (D-NOH and S-NOH) using confocal microscopy. NHEKs heat-shocked to 42°C demonstrated an up-regulation of Hsp70 compared with control cells, which showed no detectable constitutive expression of Hsp70 (Fig. 1A). We observed a time-dependent induction in Hsp70 expression in NHEKs exposed to D-NOH (Fig. 1B) and S-NOH (Fig. 1C). A higher concentration of S-NOH (150 μM) was used compared with D-NOH (50 μM) because our previous work has shown that S-NOH is less cytotoxic and induces less oxidative stress than D-NOH (Reilly et al., 1998, 1999, 2000; Vyas et al., 2005). This experiment was repeated in cells obtained from three patients, and similar results were obtained (data not shown).

Effect of Metabolites on Hsp27 Expression. Time-dependent assessment of Hsp27 expression in NHEKs revealed metabolite-specific effects. As shown in Fig. 2, Hsp27 is constitutively expressed in NHEKs and appeared to translocate from the cytoplasm to the nucleus/perinuclear region when NHEKs were treated with D-NOH. This translocation was observed within 6 h of treatment with D-NOH and showed almost complete reversal of the translocated Hsp27 back to the cytoplasm 24 h after addition of metabolite. NHEKs treated with S-NOH showed no difference in Hsp27 localization compared with cells treated with DMSO. To confirm the nuclear translocation of Hsp27 in response to D-NOH, we performed a colocalization study with a nuclear marker, DAPI. Figure 3 shows that NHEKs treated with D-NOH for 6 h induced translocation of Hsp27 to the nuclear region, which colocalized with DAPI, giving the appearance of a white-light blue color in the perinuclear region.

Effect of Metabolites on Uric Acid Release. Because we were able to demonstrate an up-regulation in Hsp70 at 24 h and Hsp27 at 6 h, we conducted our experiments with a 6- and a 24-h exposure to the metabolite and found that within these time frames uric acid did not change significantly in the supernatant (data not shown). We also determined whether the concentration of uric acid was altered in the lysate obtained from cells treated with the metabolites but found that the lysate concentration did not change significantly from control cells (data not shown). The negative findings have been summarized in Table 1.

Fig. 1. Effect of arylhydroxylamine metabolites on Hsp70 expression in NHEKs using confocal microscopy. Cells were treated with either 50 μM D-NOH or 150 μM S-NOH for 6, 12, or 24 h. As a positive control, cells were heat shocked for 1 h at 42°C and allowed to recover at 37°C-5% CO₂ for 5 h. As a negative control, cells were treated with media alone (24 h) or DMSO (vehicle, for 24 h). Treatments were made such that all incubations ended at the same time. Nuclei are stained with propidium iodide and appear as red. Green fluorescence represents Hsp70 expression. The experiment was repeated using two additional patients, and similar results were obtained.
Effect of Metabolites on the Release of Cytokines by NHEKs.

To assess the appropriate time frame for evaluating cytokine release, NHEKs were exposed to PMA and the cytokine concentrations in the supernatant were determined. PMA significantly increased all four cytokines studied; indicating that the time frame of incubation was sufficient to induce measurable cytokine secretion (Fig. 4A). We found that a 24-h exposure period to the metabolite significantly increased the cytokine production by both D-NOH and S-NOH (Fig. 4B), although to a substantially lower level than that seen with PMA. Assessment of cytokine release using a 48 h exposure time demonstrated a level of increase in cytokine secretion similar to that observed with a 24 h exposure (data not shown). Secretion of cytokine into the extracellular space is critical for activation of Langerhans cells in the skin; however, in the event of necrotic cell death (such as that caused by the metabolites) the intracellular signals will

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<tr>
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<td>Uric acid secretion</td>
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GM-CSF, granulocyte macrophage–colony-stimulating factor.

**Effect of Metabolites on the Release of Cytokines by NHEKs.** To assess the appropriate time frame for evaluating cytokine release, NHEKs were exposed to PMA and the cytokine concentrations in the supernatant were determined. PMA significantly increased all four cytokines studied; indicating that the time frame of incubation was sufficient to induce measurable cytokine secretion (Fig. 4A). We found that a 24-h exposure period to the metabolite significantly increased the cytokine production by both D-NOH and S-NOH (Fig. 4B), although to a substantially lower level than that seen with PMA. Assessment of cytokine release using a 48 h exposure time demonstrated a level of increase in cytokine secretion similar to that observed with a 24-h exposure (data not shown). Secretion of cytokine into the extracellular space is critical for activation of Langerhans cells in the skin; however, in the event of necrotic cell death (such as that caused by the metabolites) the intracellular signals will
be released and able to provoke activation and migration of resident LCs in the surrounding area. When NHEKs were exposed to D-NOH and S-NOH, the intracellular pools of TNF-α (Fig. 5A) and IL-1β (Fig. 5B) were significantly increased. Moreover, the resultant intracellular concentrations were found to be inversely related to the cytotoxic potency of the metabolites. S-NOH, which is comparatively less cytotoxic than D-NOH, showed higher levels of intracellular cytokines, suggesting that although both metabolites stimulate the synthesis of the cytokines, cell death may play some role in the release of these cytokines.

**Effect of Metabolites on Adhesion and Costimulatory Molecules in NHEKs.** Exposure of NHEKs to IFN-γ for 24 h (positive control) showed the expected increase in ICAM-1 expression on the surface (data not shown). In contrast, NHEKs incubated with either D-NOH or S-NOH showed no induction in ICAM-1 expression over control incubations (data not shown). Likewise, these metabolites had no effect on CD86 and CD80 expression in NHEKs (data not shown). The negative findings have been summarized in Table 1.

**Discussion**

The relatively low occurrence of the CDRs and the absence of viable animal models has hindered mechanistic studies of these reactions (Uetrecht, 1999). Our current insight into the mechanism of such reactions has largely been based on in vitro models using cell cultures (monolayer or cocultures) or skin explants. As a wide variety of studies have demonstrated that epidermal keratinocytes play a critical role in regulating immune responses in the skin (Nickoloff et al., 1995; Gaspari, 1997), we have utilized these cells in our efforts to elucidate the mechanism of CDRs provoked by sulfonamides and sulfones. To date, we have been able to demonstrate that NHEKs are able to bioactivate sulfonamides and form metabolite-protein adducts and that these metabolites generate oxidative stress within keratinocytes (Reilly et al., 2000; Roychowdhury et al., 2005; Vyas et al., 2005). In this investigation, we sought to determine whether exposure of NHEK to D-NOH and S-NOH leads to the expression of danger signals, which may play a role in activating LCs or T cells.

Recent studies have suggested that heat shock proteins play an important role as danger signals in the activation of the immune system, especially in the pathophysiology of
immune-mediated skin diseases (Nishioka et al., 1999; Todorik et al., 2000; Kuppper et al., 2001). In addition, known contact sensitizing agents have been demonstrated to induce heat shock protein expression in human keratinocytes (Carroll and Wood, 2000). Hence, it was logical to assess the role of reactive metabolites on this important family of chaperone proteins. Our demonstration that Hsp70 is up-regulated upon exposure to noncytotoxic doses of D-NOH and S-NOH indicates that metabolites have the ability to stimulate keratinocytes to mount a stress response and may act as the primary signal of stress in response to the metabolite-induced injury. There are several pieces of evidence suggesting that intracellular Hsp may be released from cells dying necrotically and from cells undergoing stress (Asea et al., 2000; Basu et al., 2000; Sauter et al., 2000). Extracellular Hsp70 has also been shown to induce proinflammatory cytokines and activate dendritic cells in the same manner as lipopolysaccharide (Campisi et al., 2003). Work by Asea et al. (2000) has specifically shown that Hsp70 secreted from viable cells can act as a cytokine and stimulate monocytes to further secrete proinflammatory cytokines. Their work suggests that Hsp70 can act as an adjuvant for inducing immune responses. Ongoing work in our laboratory has shown that D-NOH causes necrotic cell death in keratinocytes, suggesting that in the event of cell death, Hsp may be released into the extracellular space and may be able to activate LCs (F. D. Khan and C. K. Svensson, personal communication). Our previous results have shown that metabolites bind to cellular proteins (generating neoantigens), whereas our current study demonstrates that these metabolites up-regulate Hsp70 (as a danger signal) (Roychowdhury et al., 2005). Hence, these cellular studies suggest possible participation of reactive metabolites in both arms of the danger hypothesis. An important next step will be to determine whether skin biopsies from patients with CDRs to these drugs show the presence of adduct in a complex with Hsp and whether this peptide-Hsp complex is detectable in LCs present in the skin. Our failure to see an effect on the induction of translocation of Hsp27 after exposure to S-NOH suggests differences in the regulation of stress responses between metabolites. Translocation of Hsp27 is associated with oxidative stress, whereas expression of Hsp27 is correlated with increased cell survival (Nishioka et al., 1999). We have previously shown that the level of oxidative stress generated by D-NOH in keratinocytes is considerably higher than that observed with S-NOH (Vyas et al., 2005). Hence, the differential effect of these metabolites on this chaperone protein may simply reflect their ability to induce reactive oxygen species. Our data with Hsp27 indicate that the translocation of this particular chaperone protein may have an involvement in the prevention of stress-induced damage to nuclear proteins and DNA repair processes. Further work is needed to determine whether this translocation can serve as a potential marker for CDRs.

Although uric acid is mainly associated with inflammatory gout, recently uric acid crystals were found to be potent activators of dendritic cells (Shi et al., 2003). We determined concentrations of uric acid released intracellularly and in the supernatant by NHEKs treated with metabolites and observed no significant change compared with untreated NHEKs. Interestingly, uric acid is also known as an antioxidant that has been shown to be present in human skin (Shindo et al., 1994). This finding may suggest that uric acid primarily plays a role in protection against oxidative stress generated by the metabolites.

Cytokines play an important role in the initiation and propagation of the cutaneous immune response by facilitating LC activation and their migration from the skin (Cumberbatch et al., 2000). Several studies have also reported an induction in keratinocyte-induced cytokines in response to contact allergens such as nickel (Wilmers et al., 1994). Moreover, it is known that proinflammatory cytokines up-regulate the expression of costimulatory and adhesion molecules on antigen-presenting cells (Hulette et al., 2002). Keratinocytes can themselves express the costimulatory, adhesion, and major histocompatibility complex molecules when stimulated with proinflammatory cytokines such as IFN-γ or TNF-α (Guénette et al., 1994; Winkler et al., 1994; Wakem et al., 2000). We have previously found that normal human epidermal keratinocytes can up-regulate the expression of human leukocyte antigen ABC (which is involved in antigen presentation) when exposed to IFN-γ, D-NOH, and S-NOH, indicating that metabolites may enhance the antigen-presenting ability of these cells (S. Roychowdhury and C. K. Svensson, personal communication). Our present data suggest that these metabolites, by inducing cytokine expression intracellularly and their secretion into the media, may participate in the induction of an immune response by inducing activation of LCs. Although the cellular mechanism or signaling cascade involved in the up-regulation of cytokine is not known, this increase in cytokine concentration may be due to the increase in Hsp70 expression. However, this increase in cytokine concentration was not sufficient to up-regulate the expression of either CD-80/CD86 or ICAM-1 on keratinocytes, suggesting that these metabolites do not play a direct role in lymphocyte infiltration in initiation of CDRs.

In the present study, we have demonstrated that D-NOH and S-NOH can induce danger signals in keratinocytes by induction/translocation of heat shock proteins and secretion of proinflammatory cytokines. This result may suggest that metabolites provide danger signals needed to activate LCs in the skin and initiate an immune response. At the same time, danger signals other than those studied here that are released by stressed keratinocytes may be involved. Studies using biopsy samples from patients experiencing CDRs will be necessary to determine the clinical relevance of these findings.

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