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

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JPET #126615

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Abbreviations: ADR– adverse drug reaction; CDR– cutaneous drug reaction; DDS– dapsons; D-NOH- dapsons hydroxylamine; GM-CSF- granulocyte macrophage colony stimulating factor; Hsp- heat shock protein; Hsp70- heat shock protein 70; Hsp27- heat shock protein 27; IL-1 β - Interleukin 1 β ; ICAM-1- Intercellular adhesion molecule-1; LCs- Langerhans cells; NHEK- normal human epidermal keratinocytes; PI - propidium iodide; ROS– reactive oxygen species; SMX- sulfamethoxazole; S-NOH- sulfamethoxazole hydroxylamine; TNF- α - tumor necrosis factor- α

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JPET #126615

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 co-stimulatory molecules in normal human epidermal keratinocytes (NHEK). NHEK 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 pro-inflammatory cytokines was increased upon incubation of these cells with metabolite. In contrast, uric acid concentration was not altered. Moreover, intercellular adhesion molecule-1, CD80 and CD86 expression did not change when NHEK were exposed to these reactive metabolites. Our data suggests that NHEK 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.

Introduction

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 appear 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 in initiating delayed-type hypersensitivity reactions to drugs in the skin (Reilly et al., 2000; Park et al., 2001; Ju and Utrecht, 2002). These chemically reactive and unstable intermediates are able to bind to cellular proteins and other macromolecules, thereby generating haptenated proteins recognizable by critical immune cells. We have demonstrated that keratinocytes bioactivate SMX and DDS to reactive arylhydroxylamine metabolites (S-NOH and D-NOH, respectively) and form detectable covalent adducts with cellular macromolecules (Reilly et al., 2000; Roychowdhury et al., 2005).

Formation of haptenated proteins is, however, in itself not sufficient to provoke an immune response. Expression of co-stimulatory signals is necessary to initiate activation and maturation of dendritic cells; an essential step in T-cell recruitment (Hari et al., 2001). This co-stimulation may come from molecules or molecular structures released by cells undergoing stress or abnormal cell death. These molecules are detected by resting antigen presenting cells (APCs) as alarm signals of danger resulting in activation and maturation of these cells. In the skin, Langerhans cells (LCs) are APCs

JPET #126615

that monitor the epidermal microenvironment by taking up antigen and processing it into fragments that are readily presented on the cell surface by the major histocompatibility complex (MHC) molecules (Cumberbatch et al., 2000). Because of 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-1 β , CD-40 ligand, breakdown product of hyaluron, lipopolysaccharide (Medzhitov and Janeway, 2000), unmethylated cytosine-guanine (CpG) sequences (Kandimalla et al., 2003) and viral RNA. The inducible heat shock protein 70 (Hsp70) has been recently characterized as a potent maturation stimulus for dendritic cells (Kuppner 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 non-physiological tissue damage which in turn induces a range of inflammatory responses. In particular, Sauter and colleagues demonstrated that necrotic cell lysates, but not apoptotic cells, provoke the maturation of APCs (Sauter et al., 2000). Basu and associates also demonstrated that Hsp70, Hsp90, gp96, and calreticulin are released from cells as a result of necrotic but not apoptotic cell death (Basu et al., 2000).

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

JPET #126615

cytokines that provide essential signals for migration of epidermal LCs (Barbaud 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; Stoof 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. We, therefore, 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.

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 (NHEK) were obtained from Cambrex (Walkersville, MD) as cryopreserved 1st passage cells and sub-cultured to subsequent passages as described previously (Reilly et al., 2000). All experiments were performed on 4th passage cells. Cells from three different patients were used unless stated otherwise.

Immunofluorescence microscopy. Expression of Hsp70, Hsp27, ICAM-1 and CD80 were determined individually using laser scanning confocal microscopy. Fluorescence images were collected with a Zeiss Laser Scanning Microscope, LSM 510, Axiovert stand, 40 or 63 x oil lens. Briefly, cells grown in 75 cm² flasks were harvested and 0.5×10^5 cells were plated on collagen-coated (0.1 mg/ml) 18 mm coverslips placed in 12 well plates. After a recuperation period of 24 h in 2 ml of keratinocyte growth media (KGM-2) at 37°C/5%CO₂, media was removed and replaced with warm KGM-2 and treatments were performed for indicated time periods. After treatments, the cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and fixed with para-formaldehyde (PFA, final conc. 2%) at room temperature for 20 min. This was followed by incubation with tris-casein buffer (pH 7.6) containing 0.3% triton x-100 at room temperature for 30 min to block non-specific binding and permeabilize the cell membrane. Fixed cells were treated with (1:150) mouse antibody against Hsp70, Hsp27, ICAM-1 or (1:10) CD80 and CD86 (Invitrogen, Carlsbad, CA) at 37°C for 1 h.

JPET #126615

After three washings, this was followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 488 (Invitrogen, Carlsbad, CA) for 1 h at 37°C. The coverslips were washed thrice with PBS and 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 co-localization studies of Hsp27, mounting media containing DAPI (Vector laboratories, Burlingame, CA) was used as a nuclear marker. The experiments for the individual markers were conducted in cells from three patients.

Uric acid assessment using spectrofluorometry. Amplex uric acid kit from Invitrogen (Carlsbad, California) was used for the assessment of uric acid. Briefly, (1×10^6) 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 assay involved catalytic oxidation of uric acid in the presence of uricase to allantoin and H_2O_2 . The concentration of H_2O_2 , which is directly proportional to the concentration of uric acid, is determined by addition of amplex red reagent. Each reaction mixture contained 50 μ M amplex red reagent, 0.2 U/mL horseradish peroxidase, 0.24 U/mL uricase in reaction buffer. Reactions were incubated for 30 min at 37°C in the dark and fluorescence measured in a fluorescence plate reader (Spectra Max) using excitation λ 530 nm and emission λ 590 nm. Sample values were calculated by subtracting the readings with no uricase present. Sample concentrations were calculated from a standard plot. Results are presented as mean (SD) of incubations from three individual patients.

JPET #126615

Cytokine release using Bio-plex system. Release of interleukin 1- β (IL-1 β), tumor necrosis factor- α (TNF- α), granulocyte macrophage colony stimulating factor (GM-CSF) and interferon- γ (IFN- γ) was determined using multiplexed bead-based immunoassay kit (Bio-Rad, Hercules, CA) and read using a Luminex 100TM analyzer (Bio-Rad, Hercules, CA). Briefly, 5×10^5 cells were incubated with phorbol 12-myristate 13-acetate (PMA, 20 ng/mL), 50 μ M D-NOH, 150 μ M S-NOH or DMSO for 24 h in 6-well plates. Untreated samples incubated concurrently were used as control. To measure the cytokines released into the media, supernatant were collected, an aliquot 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. Briefly, a filter-bottom 96-well microplate was pre-wetted with assay buffer following which the buffer was removed using vacuum filtration. Fifty μ L of polystyrene beads pre-coated with antibodies against the four cytokines were added into each well. The plate was washed twice with the washing buffer provided in the kit and buffer 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 thrice with wash buffer to remove unbound protein, 25 μ L of Bio-Plex detection antibody specific for a different epitope on the cytokine was added and allowed to incubate for 30 min. After further washings (3x), 50 μ L of streptavidin-phycoerythrin was added and allowed to incubate for 10 min. The plate was washed (3x) to remove any unbound dye and suspended in 125 μ L of assay buffer and analyzed on a Luminex 100TM analyzer, set to run a 50 μ L sample and minimum of 100 events per cytokine. All incubations were performed at room

JPET #126615

temperature and assay plates 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 was calculated using Bio-Plex ManagerTM software (Bio-Rad) using a five-parameter logistic regression along with weighting model (used when 6 or more standards are found to be within 70-130 % of expected value). Standards, positive controls and samples were expressed as pg/mL. All concentrations were rounded to next whole digits. Values of less than 0.5 were taken as 0 and represented as N.D. (not detected). Results are presented as mean (SD) of five incubations for each treatment from three patients.

Statistical analysis. Data was statistically analyzed using SIGMASTAT software (San Rafael, CA). For comparing groups, Kruskal Wallis ANOVA on ranks was used along with Holm-sidak test for multiple comparisons. A p value of <0.05 was considered significant.

Results

Effect of metabolites on Hsp70 expression. We examined the ability of NHEK to induce Hsp70 expression when exposed to the arylhydroxylamine metabolites (D-NOH and S-NOH) using confocal microscopy. NHEK heat shocked to 42°C demonstrated an up regulation of Hsp70 compared to control cells, which showed no detectable constitutive expression of Hsp70 (Figure 1, panel A). We observed a time-dependent induction in Hsp70 expression in NHEK exposed to D-NOH (Figure 1, panel B) and S-NOH (Figure 1, panel C). A higher concentration of S-NOH (150 μ M) was used as compared to D-NOH (50 μ M) because our previous work has shown that S-NOH is less cytotoxic and induces less oxidative stress than D-NOH (Reilly et al., 1998; Reilly et al., 1999; Reilly et al., 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 NHEK revealed metabolite-specific effects. As shown in Figure 2, Hsp27 is constitutively expressed in NHEK and appeared to translocate from the cytoplasm to the nucleus/perinuclear region when NHEK 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. NHEK treated with S-NOH showed no difference in Hsp27 localization compared to cells treated with DMSO. To confirm the nuclear translocation of Hsp27 in response to D-NOH, we performed a co-localization study with a nuclear marker, DAPI. Figure 3 shows that NHEK treated with D-NOH for 6 h induced translocation of Hsp27

JPET #126615

to the nuclear region which co-localized with DAPI, giving the appearance of a white-light blue color in the perinuclear region.

Effect of metabolites on uric acid release. Since 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 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 lysate concentration did not change significantly from control cells (data not shown). The negative findings have been summarized in table 1.

Effect of metabolites on the release of cytokines by NHEK. To assess the appropriate time frame for evaluating cytokine release, NHEK were exposed to phorbol myristate acetate (PMA) and cytokine concentrations in supernatant determined. PMA significantly increased all four cytokines studied; indicating that the time frame of incubation was sufficient to induce measurable cytokine secretion (Figure 4A). We found that 24 h exposure period to metabolite significantly increased the cytokine production by both D-NOH and S-NOH (Figure 4B), though to a substantially lower level than that seen with PMA. Assessment of cytokine release using a 48 h exposure time demonstrated a similar level of increase in cytokine secretion as that observed with 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

JPET #126615

NHEK were exposed to D-NOH and S-NOH, the intracellular pools of TNF- α (Figure 5A) and IL-1 β (Figure 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 co-stimulatory molecules in NHEK.

Exposure of NHEK to IFN- γ for 24 h (positive control) showed the expected increase in ICAM-1 expression on the surface (data not shown). In contrast, NHEK incubated with either D-NOH or S-NOH showed no induction in ICAM-1 expression over control incubations (data not shown). Similarly, these metabolites had no effect on CD86 and CD80 expression in NHEK (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 (Utrecht, 1999). Our current insight into the mechanism of such reactions has largely been based on *in vitro* models utilizing cell cultures (monolayer or co-cultures) 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 NHEK 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 initiating CDRs by 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; Todryk et al., 2000; Kuppner 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 non-cytotoxic 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

JPET #126615

the primary signal of stress in response to the metabolite-induced injury. There are several pieces of evidence that suggest 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 pro-inflammatory cytokines and activate dendritic cells in the same manner as lipopolysaccharide (Campisi et al., 2003). Work by Asea and colleagues has specifically shown that Hsp70 secreted from viable cells can act as a cytokine and stimulate monocytes to further secrete pro-inflammatory cytokines (Asea et al., 2000). Their work suggests that Hsp70 can act as adjuvant for inducing immune responses. Ongoing work in our lab 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 (Khan FD, Svensson CK, personal communication). Our previous results have shown that metabolites bind to cellular proteins (generating neoantigens), while 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 if skin biopsies from patients suffering from 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 response between metabolites. Translocation of Hsp27 is associated with oxidative stress whereas expression of Hsp27 is correlated with increased cell survival (Nishioka

JPET #126615

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 indicates 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 if this translocation can serve as a potential marker for CDRs.

While 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 NHEK treated with metabolites and observed no significant change when compared to untreated NHEK. Interestingly, uric acid is also known as an antioxidant that has been shown to be present in human skin (Shindo et al., 1994). This 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 (Wilmer et al., 1994). Moreover, it is known that pro-inflammatory cytokines up regulate the expression of co-stimulatory and adhesion molecules on antigen presenting cells (Hulette et al., 2002). Keratinocytes can themselves express the co-stimulatory,

JPET #126615

adhesion and MHC molecules when stimulated with pro-inflammatory cytokines such as IFN- γ or TNF- α (Gueniche et al., 1994; Wikner 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 (HLA-ABC, which is involved in antigen presentation) when exposed to IFN- γ , D-NOH and S-NOH, indicating that metabolites may enhance antigen presenting ability of these cells (Roychowdhury S and Svensson CK, personal communication). Our present data suggests that these metabolites, by inducing cytokine expression both 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 KCs; 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 pro-inflammatory cytokines. This may suggest that metabolites provide danger signals needed to activate LCs in the skin and initiate an immune response. At the same time, there may be other danger signals involved other than those studied here that are released by stressed keratinocytes. Studies using biopsy samples from patients experiencing CDRs will be necessary to determine the clinical relevance of these findings.

JPET #126615

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JPET #126615

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JPET #126615

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JPET #126615

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JPET #126615

Footnotes

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Legends for figures

Figure 1: Effect of arylhydroxylamine metabolites on Hsp70 expression in NHEK using confocal microscopy. Cells were treated either with 50 μ M D-NOH or 150 μ M S-NOH for 6, 12 or 24 h. As a positive control, cells were heat shocked (HS) 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.

Figure 2: Effect of arylhydroxylamine metabolites on Hsp27 expression in NHEK using confocal microscopy. Cells were treated with either D-NOH (50 μ M) or S-NOH (150 μ M) for 6, 12 or 24 h or DMSO (vehicle, for 24 h) and probed using confocal microscopy using an anti-Hsp27 antibody as described in Materials and Methods. Green fluorescence represents Hsp27 expression.

Figure 3: Assessment of the effect of arylhydroxylamine metabolites on Hsp27 translocation in NHEK using confocal microscopy. Cells were treated with either 50 μ M D-NOH or 150 μ M S-NOH for 6 h or DMSO (vehicle, for 6 h). Translocation of Hsp27 (green) to nucleus is co-localized in cells exposed to D-NOH with the nuclear dye, DAPI (blue), giving rise to a white-light blue color in the perinuclear region. Note: cells did not appear to change shape/size when observed under phase contrast in all treatments (data not shown).

JPET #126615

Figure 4: Effect of arylhydroxylamine metabolites on cytokine secretion in NHEK.

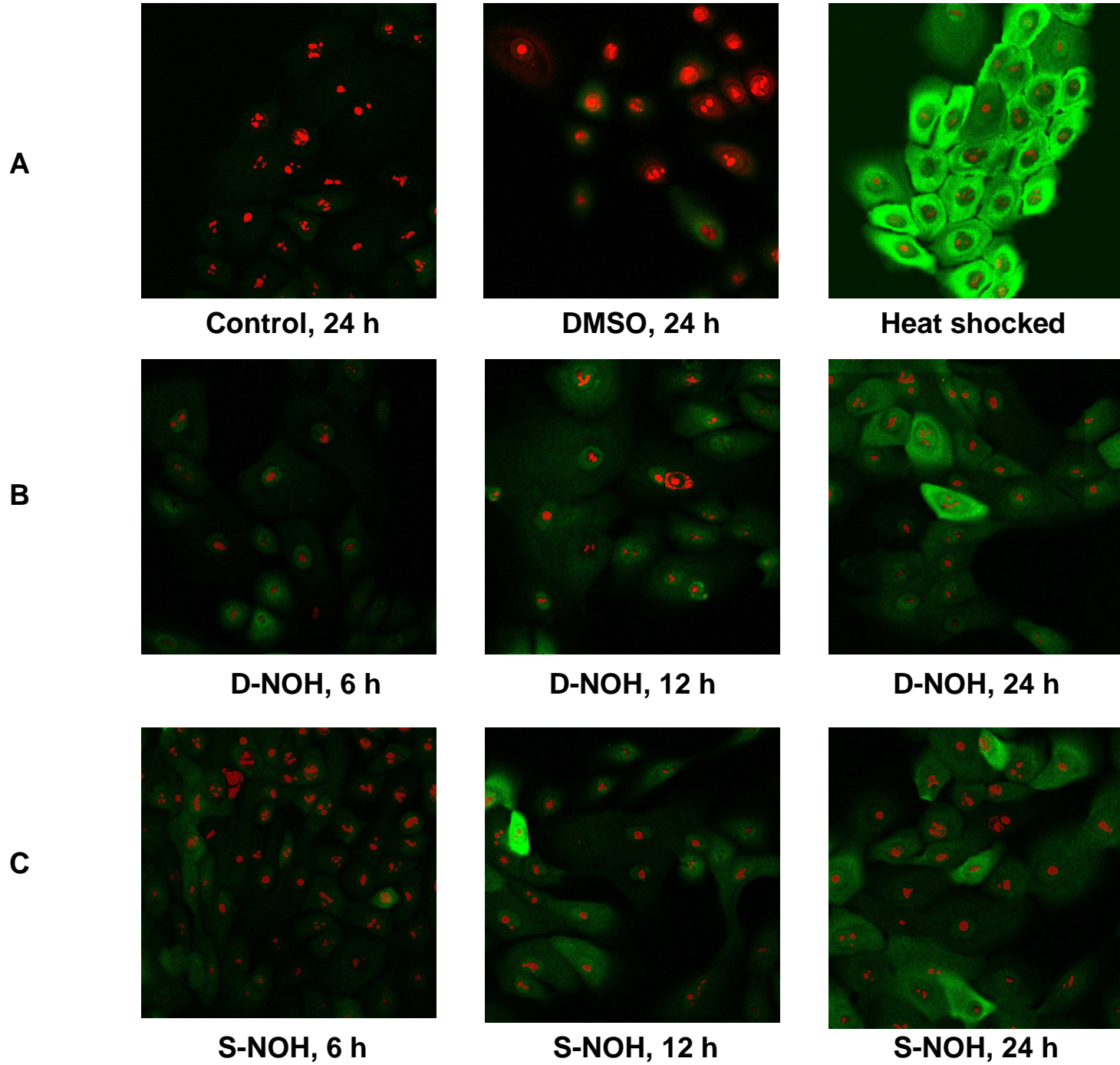
A: Cytokine concentrations in media obtained from NHEK treated with phorbol 12-myristate 13-acetate (PMA). Keratinocytes (5×10^5 /mL) were treated with PMA (20 ng/mL) or DMSO (vehicle) or for 24 h at 37°C. **B:** Cytokine concentrations in media obtained from NHEK treated with metabolites. Keratinocytes (5×10^5 /mL) were exposed to 50 μ M D-NOH, 150 μ M S-NOH or DMSO (vehicle) for 24 h. Results are expressed as mean (SD) of five incubations for each treatment from three patients. Cytokine concentration below 0.5 were taken as zero and represented as N.D. Data was analyzed statistically using t-test (PMA versus DMSO for panel A) and ANOVA (for panel B). * $p < 0.001$ compared to DMSO treated control for corresponding panel. # $p < 0.05$ compared to corresponding S-NOH incubation in panel B.

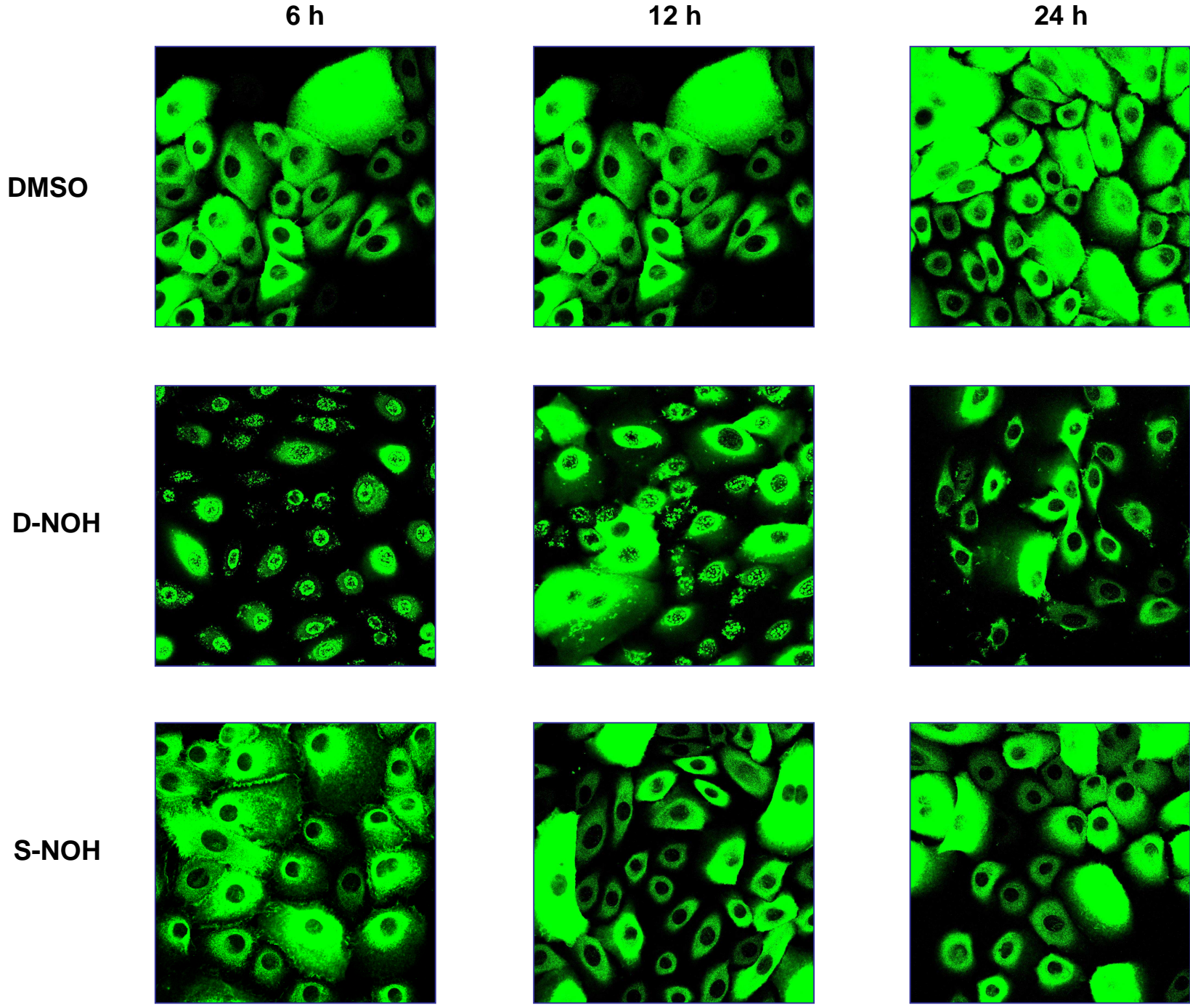
Figure 5 A: Effect of arylhydroxylamine metabolites on intracellular TNF- α concentration in NHEK. Keratinocytes (5×10^5 /mL) were exposed to 50 μ M D-NOH, 150 μ M S-NOH or DMSO (control) for 24 h. Results are expressed as mean (SD) of five incubations for each treatment from a single patient. * $p < 0.005$ compared to control. **B: Effect of arylhydroxylamine metabolites on intracellular IL-1 β concentration in NHEK.** Keratinocytes (5×10^5 /mL) were exposed to 50 μ M D-NOH, 150 μ M S-NOH or DMSO (control) for 24 h. Results are expressed as mean (SD) of five incubations for each treatment from a single patient. * $p < 0.005$ compared to control, ** $p < 0.005$ compared to control and D-NOH.

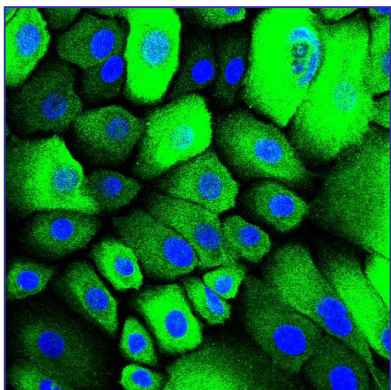
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Table 1: Summary of negative findings.

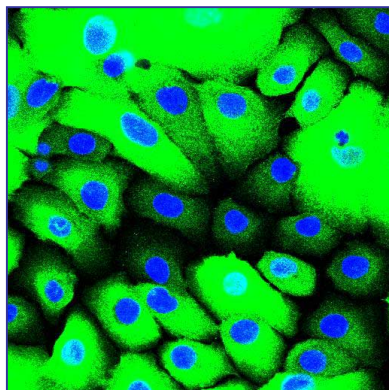
| Signal Studied | Observed Findings with metabolite treatment |
|-------------------------------------|--|
| Hsp27 translocation | No translocation observed with S-NOH |
| Uric acid secretion | No change observed with either metabolite |
| Intracellular uric acid | No change observed with either metabolite |
| Intracellular ICAM-1, CD80, CD86 | No change observed with either metabolite |
| Intracellular GM-CSF, IFN- γ | No change observed with either metabolite |



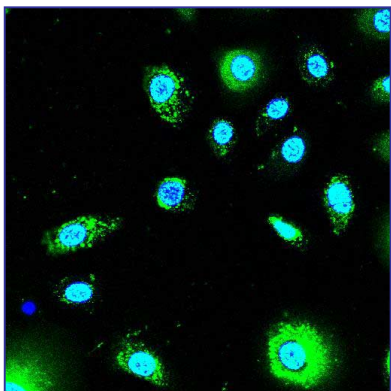




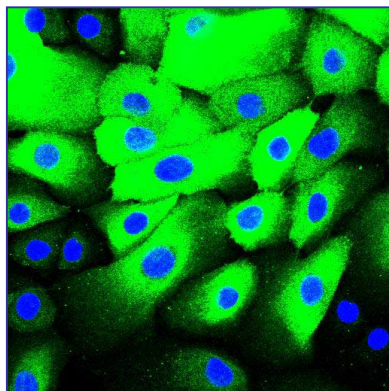
NHEK (6 h)



DMSO (6 h)



D-NOH (6 h)



S-NOH (6 h)

