Characterization of the Formation and Localization of Sulfamethoxazole and Dapsone-Associated Drug-Protein Adducts in Human Epidermal Keratinocytes

Sanjoy Roychowdhury, Piyush M. Vyas, Timothy P. Reilly, Anthony A. Gaspari, and Craig K. Svensson

Division of Pharmaceutics, College of Pharmacy, University of Iowa, Iowa City, Iowa (S.R., P.M.V., C.K.S.); Immunotoxicology, Drug Safety Evaluation, Pharmaceutical Research Institute, Bristol-Myers-Squibb Company, Syracuse, New York (T.P.R.); and Department of Dermatology, University of Maryland School of Medicine, Baltimore, Maryland (A.A.G.)

Received March 8, 2005; accepted March 21, 2005

ABSTRACT
Sulfonamide- and sulfone-induced hypersensitivity reactions are thought to be mediated through bioactivation of parent drug molecule(s) to their respective reactive metabolite(s). Recent studies have demonstrated that keratinocytes can bioactivate sulfonamides and sulfones. Using enzyme-linked immunosorbent assay and hapten-specific rabbit antisera developed in our laboratory, we found that incubation of either normal human epidermal keratinocytes (NHEKs) or an immortalized human keratinocyte cell line (HaCaT) with sulfamethoxazole (SMX) or dapsone (DDS) resulted in the formation of drug/metabolite protein adducts. The formation of these adducts with SMX was increased in the presence of ascorbic acid, whereas N-acetyl-cysteine decreased adduct formation with both SMX and DDS. Adduct formation was confirmed using confocal microscopy when NHEKs were incubated with SMX, DDS, or their respective arylhydroxylamine metabolites. Cellular distribution of adducts was compared in permeable versus nonpermeable NHEKs. Exposure to SMX, DDS, or dapsone hydroxylamine resulted in the formation of intracellular adducts, whereas SMX hydroxylamine also resulted in the presence of adducts on the cell surface. In summary, our work shows that keratinocytes can bioactivate SMX/DDS to form drug-protein adducts, which may be acquired by antigen-presenting cells upon keratinocyte cell death, evoking an immune response. In addition, keratinocytes may themselves present antigen to hapten-specific cytotoxic T lymphocytes. Furthermore, our results also suggest that different sulfonamides/sulfones may have different protein targets for in situ haptenation in keratinocytes.

The use of sulfonamides is limited by their association with cutaneous drug reactions (CDRs) (Cribb et al., 1996a). These reactions range from a limited morbilliform rash to the development of toxic epidermal necrolysis, which may be life-threatening (Svensson et al., 2001). Despite intense research over the past decade, the exact mechanism of CDR associated with these drugs is still not clearly understood. Several lines of evidence indicate a direct involvement of the immune system in the development of sulfonamide-induced CDR (Warrington et al., 1983; Hertl et al., 1995; Mauri-Hellweg et al., 1995; Schnyder et al., 1997; Svensson et al., 2001). Although most drugs, including sulfonamides and sulfones, are not inherently immunogenic, they may be recognized as an antigen/immunogen after conjugation with protein (Pohl et al., 1988; Park et al., 1998; Uetrecht, 1999; Naisbitt et al., 2003). Immune responses may then be targeted against either the drug moiety of the conjugate or the carrier protein molecule of the adduct (Park and Kitteringham, 1990). In most cases, however, drugs must undergo intracellular bioactivation-generating reactive metabolites, which then bind to cellular proteins to form adducts (Svensson, 2003). These drug-protein adducts may then be processed by antigen-presenting cells and presented to T-cells as neoantigens.

Bioactivation of sulfonamides [e.g., sulfamethoxazole (SMX)] and sulfones [e.g., dapsone (DDS)] via oxidative metabolism has been proposed to be a critical step for the initiation of CDR associated with these drugs (Shear et al., 1986; Cribb et al., 1996a; Svensson, 2003). Studies by Cribb et al. (1996b) have demonstrated that bioactivation of SMX by liver microsomes...
results in the formation of covalent metabolite-protein adducts. We have previously demonstrated that incubation of normal human epidermal keratinocytes (NHEKs) with the arylhydroxylamine metabolite of SMX or DDS resulted in the formation of metabolite-protein adducts (Reilly et al., 2000). Manchanda et al. (2002) have also shown that reactive metabolites of SMX resulted in the formation of drug-protein adducts in T lymphoblastic cells exposed in vitro. Similarly, Naisbitt et al. (2002) demonstrated cell surface protein haptenation of rat antigen-presenting cells incubated with the nitroso metabolite of SMX. In addition to these in vitro results, an SMX-induced immune response has been shown to be associated with metabolism-dependent haptenation of the drug in vivo in the rat (Naisbitt et al., 2001a). Hence, studies to date have shown that exposure to exogenously generated putative reactive metabolites of SMX and DDS may result in haptenation with cellular proteins. It is, however, unclear whether or not important target cells for these reactions are capable of bioactivating these drugs resulting in intracellular haptenation, which may be more apt to lead to local presentation of antigens and activation of an immunological cascade.

A competing theory for drug-induced delayed-type hypersensitivity reactions has been proposed by Pichler and associates (Schnyder et al., 1997; Pichler et al., 2002a,b), wherein noncovalent association of parent drug with major histocompatibility complex (MHC) on the cell surface of antigen-presenting cells is able to induce proliferation of drug-specific T-cells. Although in vitro studies have shown such T-cell activation by parent drug in T-cells isolated from sensitized individuals (i.e., those with a history of CDR to drug in question), no clear explanation has been provided for how nonadducted drug may sensitize subjects, a necessary prerequisite for the provocation of these reactions. Hence, the relative importance of bioactivation in the afferent and efferent phases of CDR remains unclear.

Because we have previously demonstrated that NHEKs are capable of metabolizing SMX and DDS to their respective arylhydroxylamine metabolites (Reilly et al., 2000), we tested the hypothesis that such bioactivation gives rise to intracellular haptenation with a cellular localization pattern that differs from haptenation arising from exposure to exogenously generated metabolite. Our results demonstrate that intracellular formation of metabolites results in haptenation when NHEKs are exposed to either SMX or DDS. Moreover, the cellular localization of protein adducts generated by SMX differs upon exposure to metabolite generated intracellularly versus that generated extracellularly.

Materials and Methods

Materials. Arylhydroxylamine metabolites of DDS and SMX were synthetized as described previously (Rieder et al., 1988; Vage et al., 1994; Reilly et al., 2000) and determined by high-performance liquid chromatography to be >97% pure. Rabbit antiserum was raised against SMX- and DDS-keyhole limpet hemocyanine conjugates and specificity assessed as described previously (Reilly et al., 2000). Rat tail collagen (type I) was obtained from Sigma-Aldrich (St. Louis, MO). Normal human epidermal keratinocytes (as first passage cells) and keratinocyte culture media were obtained from Cambrex Bio Science Walkersville (Walkersville, MD). HaCaT cells were obtained from Dr. N. Fusenig (DKFZ Heidelberg, Heidelberg, Germany). Microtiter ELISA plates (96-well) were obtained from Rainin Instruments (Woburn, MA). Goat-anti-rabbit IgG conjugated with Alexa fluor488, goat anti-mouse IgG conjugated with Alexa fluor568, and goat anti-rabbit antibody conjugated with alkaline phosphatase were purchased from Molecular Probes (Eugene, OR). Anti-HLA-ABC antibody was purchased from eBioscience (San Diego, CA). Bradford assay reagent was purchased from Pierce Chemical (Rockford, IL). Immunomount was obtained from Vector Laboratories (Burlingame, CA). All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific Co. (Pittsburgh, PA).

Cell Culture. Adult NHEKs were cultured as detailed previously (Reilly et al., 2000). In brief, cells were propagated in 75-cm² flasks using basal media (KBM-2) supplemented with bovine pituitary extract (7.5 mg/ml), human epidermal growth factors (0.1 ng/ml), insulin (5 µg/ml), hydrocortisone (0.5 µg/ml), epinephrine, transferrin, gentamicin (50 µg/ml), and amphotericin (50 ng/ml) at 37°C in an atmosphere containing 5% CO₂. Media was replaced every 2 to 3 days. When cell cultures reached near confluence (70–90%), cells were disaggregated using 0.025% trypsin/0.01% EDTA in HEPES followed by neutralization with 2 volumes of trypsin-neutralizing solution. Cell suspensions were then centrifuged at 220g for 5 min followed by washing in basal media and resuspension in KGM-2 (supplemented growth medium). Cells were then either subjected to subculturing or cryopreservation for further purposes. All experiments were performed using third to fourth passage cells.

Immortalized HaCaT cells were grown in RPMI containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). When cells reached confluence, they were subjected to trypsinization and subculturing as described above for NHEKs.

ELISA Analysis of Drug/Metabolite-Protein Adducts. Formation of covalent adducts following SMX or DDS exposure, in the presence or absence of ascorbic acid (2 mM) or N-acetylcyesteine (2 mM), was determined by cultivating NHEK or HaCaT cells (1 × 10⁶ cells) for 24 h in 50-ml centrifuge tubes containing 10 ml of complete growth medium. Cells were then incubated with SMX or DDS (800 µM) in the presence or absence of 2 mM ascorbic acid. After 24 h, tubes were centrifuged at 220g for 5 min to pellet the cells. The supernatant containing the medium was drained off, and the cell pellets were lysed in 1 ml of deionized water, using repeated cycles of freezing and thawing (three times) and ultrasonication to ensure complete lysis. The cell suspension was then thoroughly vortexed and centrifuged at 220g for 5 min, and the pellets containing the cell debris were discarded. The supernatant containing cellular soluble proteins was collected for protein assay and subsequent ELISA.

ELISA analysis for detection of covalent adduct formation was performed as described previously (Reilly et al., 2000) with minor modifications. Following protein content measurement using the Bradford reagent kit, all samples were diluted to contain 250 µg/ml protein. An aliquot of 100 µl was adsorbed onto 96-well polystyrene microtiter plates for 16 h at 4°C. Wells were washed three times using Tris buffer (0.5% casein, 0.9% NaCl, 0.01% thimerosal, and 10 mM Tris-HCl, pH 7.6) and then blocked with Tris-casein buffer containing V₉₉₉ max kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

Metabolite Formation. NHEK suspensions (1 × 10⁶ cells/ml) were incubated in glass-capped tubes with 800 µM drug in the presence or absence of 2 mM ascorbic acid or N-acetylcysteine (NAC) for 1 h at 37°C. At the completion of the incubation, ethyl acetate (5 ml) was added, samples extracted, and S-NOH content quantified using high-performance liquid chromatography as described previously (Reilly et al., 2000).
**Immunocytochemistry**. Drug/metabolite-protein covalent adduct formation was visualized using confocal microscopy. Cells were grown on collagen-coated (0.1 mg/ml) coverslips placed in Petri dishes containing 2 ml of complete growth medium. After 24 h, cultures were subjected to different drug treatments for varying periods of time (specified under **Results**) followed by washing (three times) with phosphate-buffered saline (PBS; 0.05 M sodium phosphate and 0.15 M NaCl, pH 7.4) and fixation for 20 min with 4% paraformaldehyde in PBS. After fixation, cultures were washed three times with PBS followed by blocking for 60 min with Tri-casein buffer containing 0.3% Triton X-100 and overnight incubation with the anti-DDS or anti-SMX antisera (1:500 diluted in blocking buffer) at 4°C. Coverslips were then washed with PBS, incubated for 3 h at 37°C with the fluorochrome-conjugated secondary antibody (Alexa fluor-488-labeled goat-anti-rabbit IgG, 1:500 diluted in blocking buffer), and mounted on glass slides using Immunomount containing antifade reagent.

To differentiate between intracellular and cell surface drug-protein adducts, cells were treated with the native drug (DDS or SMX, 800 μM, 24 h) or its arylhydroxylamine metabolite (D-NOH or S-NOH, 100 μM, 3 h) followed by routine immunocytochemical procedure with the exception that the permeabilization step was deleted.

Fluorescence images were acquired with a Zeiss Laser Scanning Microscope (LSM 510, Zeiss Axiostand, Zeiss 63× oil lens; Carl Zeiss GmbH, Jena, Germany) using excitation at 488 nm. Emission was set to a long-pass filter at 505 nm.

To assess colocalization, NHEKs treated with S-NOH at 100 μM/3 h or dimethyl sulfoxide only were subjected to fixation with 4% paraformaldehyde as described above. After fixation, cultures were washed three times with PBS followed by blocking for 60 min with Tri-casein buffer and overnight incubation with the anti-SMX antisera (1:500 diluted in blocking buffer) and anti-HLA-ABC antibody (1:250 diluted in blocking buffer) at 4°C. Coverslips were then washed with PBS, incubated for 3 h at 37°C with the fluorochrome-conjugated secondary antibodies (Alexa fluor-488-labeled goat-anti-rabbit IgG and anti-mouse Alexa fluor 568, 1:500 diluted in blocking buffer), and mounted on glass slides using Immunomount containing antifade reagent. Double-channel fluorescence images were acquired with a laser scanning microscope (LSM 510, Zeiss Axiostand, Zeiss 63× oil lens) using excitation at 488 and 543 nm. Emission was set to band pass filter at 505 to 525 (for green channel) and a long-pass filter at 570 nm (for red channel).

**Image Analysis.** For imaging with the confocal laser scanning microscope, laser attenuation, pinhole diameter, photomultiplier sensitivity, and offset were kept constant for every set of experiments. Images were acquired from three different view fields of each slide. The obtained images were quantitatively analyzed for changes in fluorescence intensities within regions of interest (boxes drawn over cell somata) using the Image J software. Fluorescence values from minimum of three view fields from three different slides of each treatment were averaged and expressed as mean (S.D.) fluorescence intensity.

**Statistical Analysis.** Data are presented as mean (S.D.). Data were analyzed using SigmaStat (SPSS Inc., Chicago, IL). Statistical comparison between groups was made using ANOVA and the Holm-Sidak method for multiple pair-wise comparisons. p < 0.05 was considered to be significant.

**Results**

**Effect of Ascorbic Acid and N-Acetylcysteine on Protein Haptenation in NHEKs Treated with SMX or DDS.** Because NHEKs have been shown to bioactivate SMX and DDS to their respective arylhydroxylamine metabolites, which have been demonstrated to form covalent adducts when incubated directly with NHEKs, we sought to determine whether an extended period of incubation (24 h) of these cells with the parent compounds results in protein haptenation. Using ELISA analysis, significant covalent adduct formation in NHEKs was found when NHEKs were incubated with either SMX or DDS (Fig. 1, A and B). In comparison with the SMX-treated cells, NHEKs treated with DDS seemed to show greater covalent adduct formation, although this may reflect differences in the affinity of

![Fig. 1](image-url)
antsera for the respective adducts and not absolute ad
duct amount. The specificity of the antisera for SMX or
DDS adducts in NHEKs has been previously demonstrated
by competitive inhibition with SMX or DDS (Reilly et al.,
2000). Interestingly, covalent adducts in NHEKs increased
by 3-fold when incubated with SMX in the presence of
ascorbic acid (AA, 2 mM) compared with SMX alone (Fig.
1A). When added to incubations containing DDS, AA did
not result in a significant increase in adduct formation
(Fig. 1B). In contrast to AA, NAC significantly decreased
covalent adducts in both SMX- and DDS-treated cells (Fig.
1, A and B). The concentration of AA and NAC used in
these studies stabilizes the arylhydroxylamine metabolites
(Vage et al., 1994; Vage and Svensson, 1994). Concentra-
tions of these antioxidants up to 5 mM had no effect on
NHEK cell viability (data not shown). Evaluation of meta-
bolite formation demonstrated that the recovery of S-
NOH was higher in the presence of AA or NAC, with AA
providing greater recovery than NAC (Fig. 2).

Covalent Adduct Formation in HaCaT Cells. HaCaT
cells are a commonly used immortalized keratinocyte cell line
whose metabolic capacities are relatively unknown. Because
these are a commonly used alternative model to NHEK, we
evaluated the ability of SMX and DDS to yield protein hapte-
nation in HaCaT cells. As seen with NHEKs, covalent adducts
were detected in immortalized HaCaT cells treated with either
SMX or DDS (Fig. 3). Coincubation with AA increased covalent
adducts in both SMX- and DDS-treated HaCaT cells compared
with drug alone, although in a much more substantial manner
with SMX (~0.5× increase versus DDS alone, compared with
~10× increase versus SMX alone).

**Immunocytochemical Detection of Covalent Add-
ducts in NHEKs Treated with DDS.** In addition to ELISA,
immunocytochemical techniques coupled with confocal mi-
croscopy were used to visualize and semiquantify the hapte-
nated proteins formed in DDS-treated NHEKs. First, adduct
formation was assessed after exposure to varying concentra-
tions of DDS for 24 h in permeabilized cells. As illustrated in
Fig. 4A, DDS pretreatment resulted in the formation of ad-
ducts that were readily detected at all concentrations exam-
ined (100, 250, and 800 μM). Adduct formation in NHEKs
treated with 800 μM DDS showed significantly higher adduct
formation compared with the cells treated with 100 and 250
μM DDS. Incubation of NHEKs fixed prior to addition of DDS
exhibited a marked reduction in fluorescence intensity com-
pared with incubation of cells without prior fixation (data not
shown). The time dependence of adduct formation was also
evaluated using similar incubation conditions and assess-
ment. As illustrated in Fig. 5, detectable adducts were
formed within 0.5 h of drug application and reached a max-
imum level between 3 and 6 h of exposure. This time course
of protein haptenation is similar to the time course of aryl-
hydroxylamine metabolite formation that we have reported
previously with DDS (Reilly et al., 2000).

**Detection of Covalent Adducts on the Cell Surface of
NHEKs.** To determine whether covalent adducts of SMX
and/or DDS were present on the cell surface as well as intra-
cellularly, cells were incubated with parent drug or the cor-
responding arylhydroxylamine metabolite and subjected to
immunocytochemical analysis using permeabilized and non-
permeabilized NHEKs. In permeabilized cells, antisera
are able to detect adducts formed inside the cells, as well as those

---

**Fig. 2.** Formation of S-NOH in NHEKs incubated in the presence or absence of AA or NAC. NHEKs were seeded at a density of 1 × 10^5 cells/ml and incubated with 800 μM SMX in the presence or absence of antioxidants at 2 mM for 1 h. Results shown are mean (±S.D.) of four incubations for each condition. *, significantly different (p < 0.05) from control; **, significantly different from control and NAC using ANOVA with the Holm-Sidak method for multiple pair-wise comparisons.

**Fig. 3.** Covalent binding of SMX and DDS in HaCaT cells. HaCaTs were seeded at a density of 1 × 10^5 cells/ml and incubated in the presence or absence of (A) 800 μM DDS or (B) 800 μM SMX with or without AA at 2 mM. SMX- and DDS-protein adducts were determined using a hapten-specific ELISA assay. Results shown are the optical density (OD) mean (± S.D.) from four separate incubations for each condition, except for control for Fig. 3B, where data are average of two incubations. *, p < 0.05 compared with control; **, p < 0.05 compared with control and DDS/SMX without AA using ANOVA with the Holm-Sidak method for multiple pair-wise comparisons.
on the cell surface, whereas in nonpermeabilized cells, antisera can only detect those expressed on the cell surface. As seen in Fig. 6A, adduct formation was readily detected in permeabilized NHEKs that had been treated with either DDS or D-NOH. In contrast, studies in nonpermeable cells indicated that neither DDS nor D-NOH gave rise to adducts on the surface of NHEKs under these culture conditions (Fig. 6B). As seen with DDS/D-NOH, preincubation of NHEKs with SMX or S-NOH resulted in the formation of detectable protein adducts when cells were permeabilized prior to incubation with antisera (Fig. 7A). When quantified, protein haptenation was found to be significantly higher in NHEKs incubated with 100 μM S-NOH for 3 h compared with that found when cells were incubated with 800 μM SMX for 24 h (Fig. 7B). In contrast to the pattern seen when cells were incubated with D-NOH (Fig. 6B), the nonpermeable NHEKs pretreated with S-NOH gave evidence of drug-protein adduction on the cell surface (Fig. 7C). Such adducts were not clearly detected on the surface of cells that were treated with SMX and not permeabilized (Fig. 7C).

Colocalization of MHC-I and S-NOH-Induced Cell Surface Adducts. To confirm the observations illustrated in Fig. 7C indicating surface localization of S-NOH-protein adducts in nonpermeable cells, the colocalization of MHC-I (HLA-ABC) and SMX/S-NOH protein adducts was evaluated in nonpermeable NHEKs. As shown in Fig. 8, we confirmed previous reports that HLA-ABC is expressed on the surface of cultured NHEKs (Wikner et al., 1986). When cells were incubated with NHEKs S-NOH 100 μM for 3 h and probed with anti-HLA-ABC antibody and anti-SMX antisera with-

Fig. 4. Assessment of covalent adduct formation in permeable NHEKs pretreated with varying concentrations of DDS assessed by confocal microscopy. A, NHEK cells incubated with DDS (0, 100, 250, or 800 μM) for 24 h. Controls were only exposed to the vehicle (dimethyl sulfoxide). At the end of the incubation, cells were fixed with paraformaldehyde, permeabilized, immunostained, and imaged on a confocal microscope for DDS-specific covalent adduct formation. B, images analyzed as detailed under Materials and Methods, and fluorescence intensity from a minimum of three view fields of three different slides of each treatment (with 5–10 cells per field) averaged and expressed as mean (+S.D.) fluorescence intensity (arbitrary units). Results were analyzed using ANOVA with Holm-Sidak method for multiple pair-wise comparisons. *, p < 0.05 compared with control group; **, p < 0.05 compared with control, DDS 100, and DDS 250.

Fig. 5. Time course of protein haptenation in DDS-treated NHEKs. NHEK cells were treated with 800 μM DDS for 0 to 24 h. Controls were only exposed to the vehicle (dimethyl sulfoxide) for 24 h. At the end of the incubation, cells were fixed with paraformaldehyde, permeabilized, immunostained, and imaged on the confocal microscope for DDS-specific covalent adduct formation. Images were analyzed as detailed under Materials and Methods, and fluorescence intensity from a minimum of three view fields (5–10 cells per field) of three different slides of each treatment was averaged and expressed as mean (+S.D.) fluorescence intensity (arbitrary units). Results were analyzed using ANOVA with Holm-Sidak method for multiple pair-wise comparisons. *, p < 0.05 compared with control incubations; **, p < 0.05 compared with control and 0.5-h incubation.
Fig. 6. Detection of protein haptenation in permeable or nonpermeable NHEKs after incubation with DDS or D-NOH. Cells were incubated with 800 μM DDS for 24 h or 100 μM D-NOH for 3 h and fixed with paraformaldehyde. After incubation and fixation, one group of cell cultures from each treatment was permeabilized (A), whereas the other group remained nonpermeable (B). Cells were subjected to the standard immunostaining procedure as described under Materials and Methods, and images were acquired using a confocal microscope. Figures represent results from three separate experiments with essentially identical results.

Fig. 7. Detection of protein haptenation in permeable and nonpermeable NHEKs treated with SMX or S-NOH. Cells were incubated with 800 μM SMX for 24 h or 100 μM S-NOH for 3 h and fixed with paraformaldehyde. At the end of the incubation period, one group of cell cultures from each treatment was subjected to permeabilization (A), whereas the other group remained nonpermeabilized (C). Cells were subjected to the standard immunostaining procedure as described under Materials and Methods, and images were acquired using a confocal microscope. Adduct formation was quantified for the permeable cells as described under Materials and Methods (B). Control fluorescent intensity represents the average of two incubations, whereas the results for SMX and S-NOH represent the results of three separate incubations. * p < 0.05 compared with SMX fluorescence intensity. Figures in impermeable cells represent results obtained with three separate cultures with essentially identical results.
out prior permeabilization, colocalization was evident (Fig. 8).

Discussion

Sulfonamides and sulfones are often associated with CDR, yet the pathogenesis of these reactions is still poorly understood (Svensson, 2003). Work by several investigators has demonstrated that oxidation of these drugs to their arylhydroxylamine or arylnitroso metabolites render them capable of binding to cellular proteins, which may either exert a direct toxic effect or evoke an immune response against the newly formed drug-protein conjugate (Gruchalla et al., 1998; Reilly et al., 2000; Naisbitt et al., 2001b; Manchanda et al., 2002; Farrell et al., 2003). The covalent nature of this binding in human liver microsomes, keratinocytes, and a T-cell line has been confirmed using Western-blot analysis (Cribb et al., 1996b; Reilly et al., 2000; Manchanda et al., 2002). Although much work has focused on liver- or circulating cell-generated metabolite(s) (Uetrecht et al., 1993), we have hypothesized the importance of local bioactivation in the skin in the gen-

Fig. 8. Colocalization analysis of HLA-ABC- and S-NOH-induced protein adducts in nonpermeable NHEKs treated with 100 μM S-NOH or vehicle alone. Cells were treated with S-NOH or vehicle and fixed with paraformaldehyde. At the end of the incubation period, cells were subjected to the standard immunostaining procedure without permeabilization as described under Materials and Methods followed by image acquisition using a confocal microscope. Red, HLA-ABC staining; green, SMX-protein adduct; orange- and yellow-stained regions (exemplified with arrows), colocalization of red and green (HLA-ABC and SMX-protein adducts). Figure represents results from two separate sets of experiments with essentially identical results.

Fig. 9. Scheme for the bioactivation of SMX and the effect of antioxidants on metabolite disposition. Bioactivation of SMX is predominantly catalyzed by CYP2C9, although it may also be mediated via myeloperoxidase. The hydroxylamine metabolite auto-oxidizes to the nitroso compound, which is believed to be the species directly involved in covalent binding. Nonenzymatic reduction of the nitroso is mediated by ascorbate, glutathione, or N-acetylcysteine. In the presence of limiting thiol, the semimercaptal rearranges to a sulfinamide, whereas in excess thiol, the semimercaptal is converted to the hydroxylamine. Similar bioactivation/degradation pathways occur for DDS. Adapted from Trepanier et al. (2004).
eration of these reactions (Reilly et al., 2000). NHEKs were found to be capable of bioactivating SMX and DDS to their respective arylhydroxylamine metabolites. Short-term incubation of NHEKs with the arylhydroxylamine metabolites resulted in protein adduction readily detected using an ELISA method, whereas incubation with the parent compounds (3–24 h) did not give rise to detectable adducts, presumably as a result of the limits of analytical sensitivity under the conditions that were used.

In the studies reported herein, protein haptenation was readily demonstrated via ELISA when NHEKs or HaCaTs were incubated with either SMX or DDS for 24 h (Figs. 1, A and B, and 3). The ability to detect such adducts in the present study is likely due to the use of higher protein concentrations in the ELISA assay compared with that used previously (250 versus 50 μg/ml). Interestingly, AA was found to increase (Figs. 1 and 3) and NAC decrease the level of adduct formed (Fig. 1, A and B). These observations differ from those reported by Manchanda et al. (2002), who found that both NAC and AA, as well as glutathione, decreased the haptenation of proteins when MOLT-3 cells were exposed to S-NOH. Importantly, their studies were conducted with exposure of cells to preformed metabolite, whereas the present investigation examined the effect of these antioxidants in cells exposed to the parent compound. MOLT-3 cells do not seem to be capable of oxidizing these amines, whereas NHEKs clearly form the respective arylhydroxylamine metabolites.

The contrasting effects of AA and NAC on protein adduction observed in the present study may be explained by a consideration of the effect of these two antioxidants on the fate of arylhydroxylamine metabolites, as exemplified with SMX (Fig. 9). After formation, the arylhydroxylamine metabolite rapidly auto-oxidizes to a nitroso form (Farrell et al., 2003), which is believed to be the penultimate form that covalently binds cellular macromolecules (Naisbitt et al., 1999; Manchanda et al., 2002). Alternatively, this metabolite may be converted to the nitro form or dimerize. In the presence of ascorbic acid, the nitroso is reduced to the hydroxylamine (Trepianier et al., 2004). This creates a cycling mechanism that may prolong the overall exposure of the cell to the reactive species. In contrast, NAC is able to form a semicarbazide conjugate with the nitroso metabolite (Cribb et al., 1991). Depending on the level of NAC (or glutathione), the semicarbazide may decompose to the arylhydroxylamine or be converted to SMX via a sulfonamide intermediate. It seems that in the conditions described in the present report, this latter pathway predominates, which would reduce the available metabolite for addition to cellular proteins. The greater effect of ascorbate on SMX-protein adduct formation compared with DDS-protein adduct formation may simply reflect the greater reactivity of D-NOH, such that addition with protein occurs at a faster rate and degradation of the metabolite has less effect on the mass balance of metabolite. Indeed, we have found that the reactivity of D-NOH in terms of methemoglobin formation (Reilly et al., 1999), cytotoxicity (Reilly et al., 1998; Reilly et al., 2000), and reactive oxygen species formation (P. M. Vyas, S. Roychowdhury, P. M. Woster, and C. K. Svensson, unpublished data) is substantially greater than that seen with S-NOH. Previous studies in our laboratory have shown that AA and NAC also stabilize D-NOH and enhance its recovery in liver microsomes incubated with DDS (Vage et al., 1994; Vage and Svensson, 1994). An alternative explanation for the observed increase in adduct formation in the presence of AA is an increased conversion to the acetoxy metabolite. Because in vitro studies indicate that this metabolite is more reactive than the arylhydroxylamine (Nakamura et al., 1995), increased conversion to this product may be expected to result in an increase in covalent binding. Because we have previously demonstrated the presence of N-acetyltransferase in NHEKs (Reilly et al., 2000), metabolic conversion of the arylhydroxylamine to the acetoxy would be expected in these cells. Importantly, Nakamura et al. (1995) have demonstrated that the conversion of S-NOH to the acetoxy metabolite via N-acetyltransferase is increased in the presence of glutathione. However, as we observed that NAC decreased the formation of covalent adducts, this seems to be an unlikely mechanism for the increased addition in ascorbic acid.

Cell surface expression of antigens following administration of hydroxylamine and nitroso metabolites of SMX have been demonstrated in various cell types, including splenocytes, lymphocytes, and epidermal keratinocytes, in an in vivo rat model (Naisbitt et al., 2001a; Naisbitt et al., 2002). However, parent drug administration failed to show any surface antigen presentation in any cell type examined. Such studies suggest that surface expression of covalent adducts does occur in vivo and that this form of presentation may complement the noncovalent association proposed by Pichler et al. (2002b). Hence, we used immunohistochemical methods to identify the localization of haptenated proteins in NHEKs.

Incubation of NHEKs with DDS or SMX (in the absence of any antioxidant) resulted in haptenated proteins readily detectable via confocal microscopy (Figs. 4–7). Evaluation of cells that were made permeable prior to incubation with antisera revealed that adducts were detectable throughout the cytosolic space. Using a semiquantifiable comparison, it was found that the time course of DDS-protein adduct formation was similar to that which we have reported previously for the formation of both D-NOH and S-NOH in NHEKs (Fig. 5) (Reilly et al., 2000). For both drugs, incubation with the arylhydroxylamine metabolite for a shorter duration and at significantly lower concentrations resulted in a higher level of adduct formation (Figs. 6 and 7). When antisera were used to probe for adducts in nonpermeable cells, neither DDS nor D-NOH were found to give rise to adducts on the cell surface (Fig. 6B). In contrast, although a similar approach failed to detect cell surface adducts when NHEKs were incubated with SMX, cell surface adducts were detected when NHEKs were incubated with S-NOH (Fig. 7C). Further studies gave rise to evidence that HLA-ABC- and S-NOH-induced adducts were colocalized on the cell surface in nonpermeable cells (Fig. 8).

Our data demonstrate that NHEKs can bioactivate SMX and DDS to give rise to haptenated proteins. The death of such cells would permit adduct uptake by antigen-presenting cells (e.g., Langerhans cells) and the initiation of the cascade of events which that provoke a CDR. Although the reactive metabolites can themselves result in cytotoxicity, it is unclear if the concentrations achieved in the skin are capable of inducing cell death. Alternatively, secondary insults to cells possessing haptenated proteins (via trauma or infection) may result in the release of adduct and uptake by antigen-presenting cells.
A second consideration is how keratinocytes might be targeted for cell killing by activated T-cells recruited to the skin. It is known that in severe CDR, substantial keratinocyte cell death occurs (Pichler et al., 2002a,b). It would seem that surface localization and/or presentation of haptenated proteins is essential for targeting these cells. Our observation that exposure of NHEKs to preformed S-NOH (which could arise in the liver and distribute to the skin) can result in cell surface adduction suggests that such surface localization and/or presentation may occur. Although our data suggest these adducts are colocalized on the cell surface with HLA-ABC, these data only indicate that such adducts are present in a regional distribution similar to HLA-ABC and do not definitively demonstrate that drug-adducted peptide is being expressed in an MHC context. However, as keratinocytes can directly present haptens to hapten-specific MHC-I restricted CD8+ cytotoxic T cells, these data suggest that this may be the means by which keratinocytes are targeted for killing by cytotoxic T cells (Bour et al., 1995; Kehren et al., 1999). The higher surface adduction in S-NOH-treated NHEKs compared with D-NOH could be a plausible explanation for higher incidence of ADRs following SMX treatment in comparison with the DDS therapy (Medina et al., 1990; Pertel and Hirschtick, 1994). This may also explain why DDS administration is more highly associated with hematomal toxicity, whereas SMX is more commonly associated with hypersensitivity reactions. Studies are ongoing to assess the ability of these haptenated proteins in NHEKs to be presented in the context of MHC-I or MHC-II, as well as the basis for the differential surface localization between S-NOH and D-NOH.

In the presence of proinflammatory cytokines, NHEKs express MHC-II (Wikner et al., 1986; Wakita et al., 1996; Albanesi et al., 1998). Hence, it is possible that under inflammatory conditions, intracellular protein adducts formed in NHEKs are localized and/or presented on the cell surface in the context of MHC II, which would allow presentation to class II MHC-restricted CD4+ T-cells. Studies are currently underway to test this hypothesis.

In conclusion, the demonstration of protein haptenation in NHEKs exposed to SMX, DDS, or their respective arylhydroxylamine metabolites provides further support for our hypothesized role of events at the cutaneous level in the precipitation of CDR to these drugs (Reilly et al., 2000; Svensson et al., 2001). Further support of this hypothesis will necessitate the demonstration of such adducts in patients during the acute phase of CDR, as well as additional delineation of the cellular and/or molecular events linking drug-protein adduct formation in NHEKs with immunologic activation.

Acknowledgments

We thank William Wuister for technical assistance and the staff of the Central Microscopy Core Facility at The University of Iowa, which is supported by the Office of the Vice President for Research, for technical assistance.

References


Address correspondence to Dr. Craig K. Svensson, Division of Pharmaceutics, College of Pharmacy, The University of Iowa, 115 South Grand Avenue, S213 PHAR, Iowa City, IA 52242. E-mail: craig-svensson@uiowa.edu