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Haptenation of Sulfonamide Reactive Metabolites to Cellular Proteins

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ABSTRACT

Adverse drug reactions are a major problem complicating medical therapy. The pathogenesis of many severe adverse drug reactions, notably hypersensitivity reactions, is poorly understood. The sulfonamides are associated with severe hypersensitivity reactions. The initial pathogenesis seems to be caused by bioactivation of the parent drug to a reactive intermediate and subsequent propagation by the immune system. The determinants of the immune response are not known. We explored the formation of sulfonamide haptens in Molt-3 and HEPA 1C1C7 cells after incubation with sulfamethoxazole (SMX), the hydroxylamine of sulfamethoxazole (SMX-HA), or the nitroso of sulfamethoxazole (SMX-NO). Haptenation was demonstrated with SMX-HA and SMX-NO but not SMX; this occurred at concentrations below that associated with toxicity (significant haptenation was seen at 25 to 50 μM). Thus, haptenation occurred presumably onto viable cells. Haptenation occurred rapidly; haptenation of cell surface proteins was demonstrated within 5 min. This did not occur indiscriminately; confocal microscopy demonstrated haptenation onto specific sites on the cell membrane. We found that haptenation was significantly inhibited by thiols and other antioxidants ($p < 0.05$). Sulfonamide-specific haptens were rapidly internalized by what seemed to be a caveolae-dependent process. It seems that sulfonamide reactive metabolites haptenated specific cell surface proteins that are rapidly internalized. Understanding the specific protein target(s) for haptenation and how these haptens are processed will be important in understanding the immune mediation of sulfonamide hypersensitivity adverse drug reactions.

One of the major risks of pharmacotherapy is adverse drug reactions (Weinshilboum, 1987; Rieder, 1993). Adverse drug reactions are a significant cause of morbidity and mortality, and hinder effective therapy (Goldstein et al., 1984). Approximately 5% of patients develop adverse reactions during therapy, and 5 to 10% of patients develop adverse events during hospitalization (Bates et al., 1997; Rieder, 1997; Pirmohamed and Park, 1999). In the US, adverse drug reactions are believed to be the fourth most common cause of death (Pirmohamed and Park, 1999).

Predictable adverse drug reactions account for more than 80% of all reactions (Pirmohammed and Park, 1999). They are dose-dependent, can be anticipated from the drug's pharmacology, and resolve when dose is reduced. Unpredictable or idiosyncratic adverse drug reactions are less common but account for some of the most serious adverse events. These reactions are not related to the known pharmacology of the drug, do not show any simple dose-response relation, resolve only when treatment is discontinued, and even then can sometimes progress (Park et al., 1992). Clinical manifestations are remarkably diverse and include fever, skin rash, and multiorgan involvement (Park et al., 1987; Rieder, 1993, 1994; Pirmohamed et al., 1996). Although uncommon, many of these reactions are life threatening.

It has recently been appreciated that drug metabolism may be a key factor in the pathogenesis of many unpredictable drug hypersensitivities. Drug metabolism is usually thought of as generating polar, inactive metabolites that are readily excreted from the body. However, phase I enzymes, particularly isozymes of cytochrome P450, may bioactivate drugs to chemically reactive or toxic metabolites. The initial pathogenesis of idiosyncratic hypersensitivity reactions seems to involve bioactivation of parent drugs to reactive metabolites (Spielberg et al., 1981; Shear et al., 1985). The further propagation of these reactions seems to be mediated by the immune system (Hess and Rieder, 1997).

The clinical manifestations of an adverse event seem to be

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ABBREVIATIONS: SMX, sulfamethoxazole; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SMX-HA, hydroxylamine of sulfamethoxazole; SMX-NO, nitroso of sulfamethoxazole; FITC, fluorescein isothiocyanate; TBS, phosphate-buffered saline and 0.05% Tween 20; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; GSH, glutathione.

largely determined by the immune response generated (Hess and Rieder, 1997; Coleman, 1998). Most drugs and metabolites are less than 1000 Da and are conventionally not considered immunogenic. A hapten is a small-molecular-weight species that is immunogenic when conjugated to protein but not in free form. Our current belief is that drug hypersensitivity is largely based on hapten formation, in that drug-protein conjugates are recognized as an immunogen rather than the small drug molecules themselves (Coleman, 1998). Immune responses may be directed against the drug, part of the carrier molecule, or both. Conjugation can lead to formation of two distinct types of antigenic determinants, the hapten itself or a structurally modified carrier molecule. The nature of the interaction between antigen and immune system will determine the type of tissue injury observed (Park and Kitteringham, 1990; Bolzacchini et al., 1998).

A key characteristic for an agent to function as a hapten is the ability to form stable bonds with nucleophilic groups on proteins under aqueous conditions (Park et al., 1987). For most drugs, it is assumed that bioactivation of the drug to a chemically reactive metabolite, such as an epoxide, quinone, nitroso derivative, or acyl halide, acts as a hapten and is processed by antigen presenting cells as the ultimate immunogen (Park et al., 1995; Coleman, 1998; Coleman and Blanca, 1998). The specific immune response mounted is dependent on antigen processing and presentation. Presentation of a haptenated peptide can lead to an immune response in which the hapten is recognized as the major antigenic determinant or in which the peptide itself is the major determinant (Martin and Weltzien, 1994; Kalish, 1995; Weltzien et al., 1996).

The sulfonamides are antimicrobial agents associated with serious hypersensitivity adverse reactions (Shear et al., 1986; Rieder et al., 1989). Most of a sulfonamide dose ($\leq 95\%$) is metabolized by *N*-acetyltransferase to a nontoxic acetylated conjugate (Cribb et al., 1995). A small fraction of a sulfonamide dose, however, is also metabolized to a reactive hydroxylamine metabolite, primarily by CYP2C9 (Cribb et al., 1995). Evolution of these reactions seems to be caused by the immune system (Hess and Rieder, 1997). Naisbitt et al. (1999) have demonstrated that reactive sulfonamide derivatives can bind to the surface of white blood cells. The subsequent fate of these bound derivatives and the role of haptens in the immune component of sulfonamide hypersensitivity reactions are poorly understood; this research was conducted to define hapten formation in response to sulfonamides and their reactive metabolites (Naisbitt et al., 1999).

Materials and Methods

MOLT-3 Cell Line. MOLT-3 cells are a human, peripheral blood, T lymphoblast cell line maintained in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin and 50 μM 2-β-mercaptoethanol at 37°C in a humidified atmosphere containing 5% CO_2.

HEPA 1C1C7 Cell Line. HEPA 1C1C7 cells are a mouse liver hepatoma cell line. Cultures were maintained in Dulbecco's modified Eagle's medium with high glucose (Invitrogen) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 5% CO_2.

Cells of lymphoblastic origin, such as MOLT-3 cells, have been used as an in vitro model for a number of studies of drug toxicity (Shear et al., 1985; Rieder et al., 1989, 1992). It is probable that a great deal of bioactivation of drugs to reactive intermediates occurs in the liver; thus the HEPA 1C1C7 line was used to determine whether our findings were unique to nonadherent cells of hematoreticular lineage or if they could be generalized to other cell types.

Purification of SMX-Keyhole Limpet Hemocyanin Immunization Rabbit Serum. Post SMX-keyhole limpet hemocyanin immunization rabbit serum samples were previously collected and frozen at −20°C. Rabbit serum samples were purified using a Protein G Sepharose 4 Fast Flow column (Pharmacia LKB, Uppsala, Sweden) to select only IgG and its subclasses from the crude serum. Rabbit serum (10 ml) was dialyzed overnight in 2 liters of PBS, pH 7.2, using Spectra/Por 6 and 7 Molecularporous dialysis membrane (1.8 cm/ml) (Spectrum, Dallas, TX). The protein G column was equilibrated by filling with binding buffer (0.2 M sodium phosphate, pH 7.0) and allowing column to drain. After immunization, rabbit serum was diluted 1:1 with binding buffer to ensure proper ionic strength and pH for optimal binding and was then placed in 5-ml aliquots over the equilibrated column, allowing the serum to absorb into the gel. Binding buffer (~30 ml) was passed through the column to wash away any unbound materials. Elution buffer (1.0 M glycine-HCl, pH 2.7) was then added to the column to elute the bound IgG. The antibody fractions were collected in 1-ml aliquots in Eppendorf tubes containing 100 μl of neutralization buffer (1.0 M Tris-HCl, pH 9.0) which allows for immediate renaturing of the purified sample. The absorbance of each of the 1-ml collection fractions was measured using Bio-Rad (Hercules, CA) protein estimation assay at a wavelength of 650 nm. Antibody collections of (10 mg/ml) were collected, dialyzed overnight in PBS, and stored at −20°C until required.

Protein Estimation. Protein concentrations were obtained using a Bio-Rad DC protein assay applying the following colorimetric microplate assay protocol. To obtain a standard curve dilutions of bovine serum albumin (BSA) protein standard were prepared ranging from 3.125 to 1000 μg/ml. Five microliters of standards and samples were pipetted into a clean, dry, microtiter plate followed by 25 μl of reagent A (alkaline copper tartrate solution) and 200 μl of reagent B (dilute Folin phenol reagent). The plate was gently agitated to mix the reagents and, after 15 min, absorbances were read at 650 nm using the plate-reader (Kinetic microplate reader; Molecular Devices, Sunnyvale, CA).

Analysis of Sulfa-Specific Cell Surface Protein Binding Using Flow Cytometry. MOLT-3 cells (2×10^6) or HEPA 1C1C7 cells (70% confluent; 60-mm dish) were incubated with concentrations of SMX (Sigma-Aldrich, St. Louis, MO), SMX-HA (Rieder et al., 1988), or SMX-NO (Rieder et al., 1995) from 0 to 800 μM in HEPES for time points from 0 to 30 min at 37°C. After incubation, cells were thoroughly washed three times with ice-cold PBS to remove any unbound drug. All subsequent incubations were carried out at 4°C. Cells were then incubated with blocking solution (5% goat serum in PBS) for 15 min followed by rabbit anti-SMX IgG antibody (1:500 in blocking solution) for 1 h. After the incubation, cells were washed three times with cold PBS and incubated with human absorbed biotin-conjugated goat anti-rabbit antiserum (1:500 in PBS) for 1 h followed by washing twice in cold PBS and incubation with streptavidin-FITC (1:1000 in PBS) for 15 min. The MOLT-3 cells were washed once with cold PBS and resuspended in 400 μl of PBS before being transferred to Falcon flow cytometry tubes (Falcon Plastics, Oxnard, CA) containing 100 μl of 8% formaldehyde. Hepa 1C1C7 cells were further incubated in 400 μl of 5 mM EDTA in PBS for 10 min to remove cells from the plate, transferred to Falcon flow cytometry tubes containing 100 μl of 8% formaldehyde and analyzed on the FACscan for cell surface immunofluorescence. For each flow cytometry analysis, the control cells were 1) untreated, unlabeled; 2) untreated, fully labeled; 3) treated, unlabeled; 4) treated, 2° antibody and label only. Sulfa-specific binding was defined by the percentage increase in immunofluorescence compared with the untreated, fully labeled control. A control gate for viable cells using propidium iodide was used for each experiment. Each sample was analyzed in duplicate.

Analysis of Sulfa-Specific Intracellular and Cell Surface Protein Binding Using Fluorescent Microscopy. HEPA 1C1C7 cells were plated directly on glass slides (tissue culture chamber/slides; Lab-Tek, Naperville, IL), allowed to grow to 50 to 70% confluence, and then incubated with 25 μM SMX, SMX-HA, or SMX-NO in HEPES for 1 h at 37°C. Cells were washed with cold PBS and fixed with a methanol/acetone (1:1, v/v) solution for 5 min at −20°C. After fixation, cells were washed with TBS (PBS + 0.05% Tween 20) and permeabilized with 0.05% Triton X-100 in PBS. Cells were then incubated in blocking solution (1% BSA, 5% goat serum, 0.05% Triton X-100 in PBS) for 30 min followed by liver acetone powder absorbed rabbit anti-SMX IgG antibody (1:500) for 1 h at room temperature. After the incubation, cells were washed in TBS and incubated with FITC-goat anti-rabbit antibody (1:200) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 min at room temperature. Cells were then washed with TBS and PBS before mounting with coverslips for microscopy. Fluorescence was visualized using a fluorescent microscope (IX50; Olympus, Tokyo, Japan) with 40× lens and Endow GFP (Chroma Technology Corp., Brattleboro, VT) filter set.

Analysis of Sulfa-Specific Intracellular and Cell Surface Protein Binding Using Confocal Microscopy. Hepa ICIC7 cells were cultured on coverslips in Petri dishes (60 mm) to 30% confluence and incubated with various concentrations of SMX, SMX-HA, or SMX-NO in HEPES for various time points (0–30 min) at 37°C. Cells were washed with cold PBS and fixed with a methanol/acetone (1:1, v/v) solution for 5 min at −20°C. To visualize cell surface binding alone, cells were washed three times with PBS and incubated in blocking solution (1% BSA, 5% goat serum in PBS) for 30 min followed by liver acetone powder-absorbed rabbit anti-SMX IgG antibody (1:500) for 1 h at room temperature. After the incubation, cells were washed three times in PBS and incubated with FITC-goat anti-rabbit antibody (1:200) for 30 min at room temperature. Cells were then washed with PBS before removing coverslips and mounting on slides for confocal microscopy. Staining for intracellular as well as cell surface binding was as described previously.

Confocal microscopy was performed on a Ziess LSM-510 laser scanning microscope using Zeiss 63 × 1.4 numerical aperture oil-immersion lenses (Carl Zeiss GmbH, Jena, Germany). FITC fluorescence was visualized with excitation at 488 nm and emission at 505 to 555 nm filter sets with argon lasers.

Identification of Haptenated Cellular Proteins Using Western Blot Analysis. MOLT-3 cells (5×10^5) or HEPA 1C1C7 cells (70% confluent, 60-mm dish) were incubated with varying concentrations of SMX, SMX-HA, or SMX-NO (0–100 μM) in HEPES for varying time points at 37°C. Cells were thoroughly washed three times in cold PBS and resuspended with lysis buffer (10% Triton X-100, 3 M NaCl, 1 M Tris, pH 7.5, 0.5 M EDTA, Complete mini protease cocktail inhibitor tablet) for 30 min on ice. The HEPA cells were harvested from the dish using 5 mM EDTA in PBS. Lysed cells were then microcentrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was collected and protein concentration was determined using the Bio-Rad protein estimation assay. Protein concentration was standardized to (400 μg/ml) and samples were diluted in 4× sample buffer (8% SDS, 8% 2-mercaptoethanol, 0.5 M Tris, pH 6.8, 40% glycerol, and 0.02% bromphenol blue) and boiled for 5 min to denature the proteins. Proteins were separated according to molecular weight using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad miniprotein II gel apparatus. Hapten inhibition experiments were performed to confirm the immunochemical recognition of the drug ligand (data not shown). Sample (8–10 μg) was loaded onto a 15-cm 10% discontinuous polyacrylamide separating gel (H_2O, 30% bis-acrylamide mix, 1.5 M Tris, pH 8.8, 10% SDS, 10% ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine) with a 5% stacking gel (H_2O, 30% bis-acrylamide mix, 0.5 M Tris, pH 6.8, 10% SDS, 10% ammonium persulfate, *N*, *N*, *N*′, *N*′-tetramethylethylenediamine) according to the procedure of Laemmli (1970). Gels were run in a chamber containing 1× running buffer (glycine, Tris, SDS) at 150 V for approximately 1 h or until the samples reached the bottom of the gel. The gel was then transferred onto a polyvinylidene difluoride membrane (Immobilion-P; Millipore Corporation, Bedford, MA) using the semidry electrophoretic transfer apparatus (Trans-Blot SD; Bio-Rad) for 43 min at 16 V. After protein transfer, the polyvinylidene difluoride membrane was blocked overnight at room temperature with 5% Carnation skim milk powder in TBST (3 M NaCl, 1 M Tris pH 8.0, 0.1% Tween 20). The membrane was then incubated with the rabbit anti-SMX IgG antibody (1:500 in TBST containing 0.5% skim milk powder) for 2 h followed by washing three times with TBST and incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:20,000 in TBST) (Jackson Immunoresearch Laboratories) for 1 h at room temperature. The membrane was then washed three times in TBST and the sulfa-specific haptenated proteins were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and developed on autoradiographic film (BioMax; Eastman Kodak, Rochester, NY) at suitable exposure times.

Analysis of the Effects of Antioxidants on Sulfa-Specific Haptenation Using Flow Cytometry and Western Blot Analysis. MOLT-3 cells (5×10^6) were incubated with varying concentrations of glutathione (Calbiochem, San Diego, CA), ascorbic acid (BDH Inc., Poole, Dorset, UK) and *N*-acetylcysteine (Roche Applied Science, Indianapolis, IN) in combination with 100 μM of either SMX-HA or SMX-NO for 1 h at 37°C. After incubation cells were stained and analyzed on the flow cytometer as described above or lysed and analyzed by Western blot analysis as described above.

Analysis of Sulfa-Specific Bound Protein Internalization. The internalization of sulfa-specific bound proteins over time was assessed by flow cytometry. Hepa 1C1C7 cells were cultured to 70% confluence in 60-mm dishes and incubated with either 50 μM SMX-HA or 25 μM SMX-NO in HEPES for 10 min at 37°C. The cells were then placed on ice, washed two times with ice-cold HEPES, and incubated for an additional 15 min in a blocking solution consisting of 5% goat serum in cold HEPES. The cell sulfa-specific haptenated proteins were specifically labeled on ice with a rabbit anti-SMX IgG antibody diluted 1:500 in blocking solution for 1 h and any unbound antibody was subsequently washed away by washing twice in cold-HEPES. The cells were warmed up to 37°C by washing twice in prewarmed HEPES and were incubated in HEPES at 37°C for the time periods indicated in the figures. The cells were immediately placed on ice and washed twice with cold HEPES. Any of the immunolabeled cell surface proteins remaining on the cell surface were then labeled with human absorbed biotin-conjugated goat anti-rabbit (1:1000) followed by streptavidin-FITC (1:1000) and analyzed by FACscan. The sulfa-specific protein internalization was defined as the percentage loss of immunolabeled surface proteins compared with cells kept at 4°C.

Analysis of Sulfa-Specific Bound Protein Internalization in the Presence of Inhibitors. The sulfa-specific protein internalization was assessed in the presence of 0.45 M sucrose, which is an inhibitor of clathrin-mediated internalization, and 50 μg/ml nystatin (ICN Biomedicals Inc., Costa Mesa, CA), which is an inhibitor of caveolae- mediated internalization and inhibits all membrane internalization. Hepa 1C1C7 cells were pretreated with either 0.45 M sucrose for 30 min or 50 μg/l nystatin for 3 h at 37°C. Cells were then exposed to 50 μM SMX-HA or 25 μM SMX-NO and analyzed for percentage internalization above.

Statistical Analysis. Dose-response and time course data were analyzed using Prism (GraphPad Software, San Diego, CA). Statistical significance was determined using paired two-tailed *t* test with $p < 0.05$. In the case of multiple determinations, analysis was performed by analysis of variance.

Results

Analysis of Haptenation by SMX, SMX-HA, and SMX-NO. To determine whether SMX or its reactive metabolites

bind to cellular proteins, flow cytometry, fluorescent microscopy, and confocal microscopy techniques were employed. SMX, SMX-HA, or SMX-NO was incubated with MOLT-3 cells and cells were subsequently examined for binding to membrane proteins. Results (Fig. 1A) demonstrated that the parent compound SMX did not bind to membrane proteins to any significant degree and, even at high concentrations (800 μM), mean binding was less than 9.9 $\pm$ 4.6%. SMX-HA bound to 46 $\pm$ 4.7% of membrane proteins at 25 μM and increased to a maximum binding of 78.7 $\pm$ 3.0% at 800 μM concentration (Fig. 1B). SMX-NO however, showed 74.7 $\pm$ 2.6% binding at 25 μM with maximum binding of 80.7 $\pm$ 4.7% at 50 μM (Fig. 1C). At higher concentrations ($>$ 200 μM), binding was actually shown to decrease.

The time required for sulfa-specific binding to occur was determined by incubating SMX-HA and SMX-NO with Hepa cells for varying time points ranging from 2.5 to 60 min. The parent drug SMX was not tested over time as previous experiments (Fig. 1A) demonstrated that significant binding did not occur. Incubation with both SMX-HA and SMX-NO (Fig. 2) revealed that sulfa-specific binding occurs within the first 5 min, varying slightly depending on the metabolite and concentration used. Maximum binding was obtained with 50 μM of SMX-HA and 25 μM of SMX-NO (84.59 $\pm$ 1.37% and 85.49 $\pm$ 1.11%, respectively); therefore, these optimal concentrations were used for subsequent binding experiments.

The time course experiment was repeated at 4°C to determine whether a decrease in temperature would affect sulfa-specific binding. Maximum binding was not significantly different when the cells were incubated at 4°C with both SMX-HA and SMX-NO (Fig. 3). However, it seems that the initial rate of binding is slowed down at 4°C but reaches plateau at the same amount of maximum binding by 30 min.

Identification of Haptenated Cellular Proteins. Flow cytometry and microscopy experiments described previously have demonstrated that sulfa-specific binding to cellular proteins occurs with the reactive metabolites SMX-HA and SMX-NO but not the parent compound SMX. To establish the specific proteins were being haptenated by the sulfa metabolites, MOLT-3 cells were incubated with DMSO, SMX, SMX-HA, or SMX-NO and their cell lysates were run on a 10% SDS-PAGE; Western blot analysis was performed using a sulfa-specific polyclonal antibody. A protein band at approximately 64 kDa was detected in all lanes, including the DMSO control, indicating that it is a nonspecific protein band (Fig. 4). Incubation of cells with SMX-HA and SMX-NO revealed more than 20 protein bands (Fig. 4); however, the intensity and the total number of protein bands detected was greater with SMX-NO. Particularly strong bands can be seen at ~55, 80, and 150 kDa in both SMX-HA and SMX-NO lanes when the other protein bands are of approximately equal intensity.

To determine whether specific proteins were bound at different concentrations of the sulfa metabolites, MOLT-3 cells were incubated with SMX-HA and SMX-NO at concentrations ranging from 12.5 to 400 μM and probed for sulfa-specific binding using Western blot analysis. SMX-HA protein binding to multiple proteins can be detected beginning at 25 μM (Fig. 5A) and 12.5 μM for SMX-NO (Fig. 5B). Intensity of the protein bands increases as concentration of the sulfa metabolites increases. However, specific proteins bound

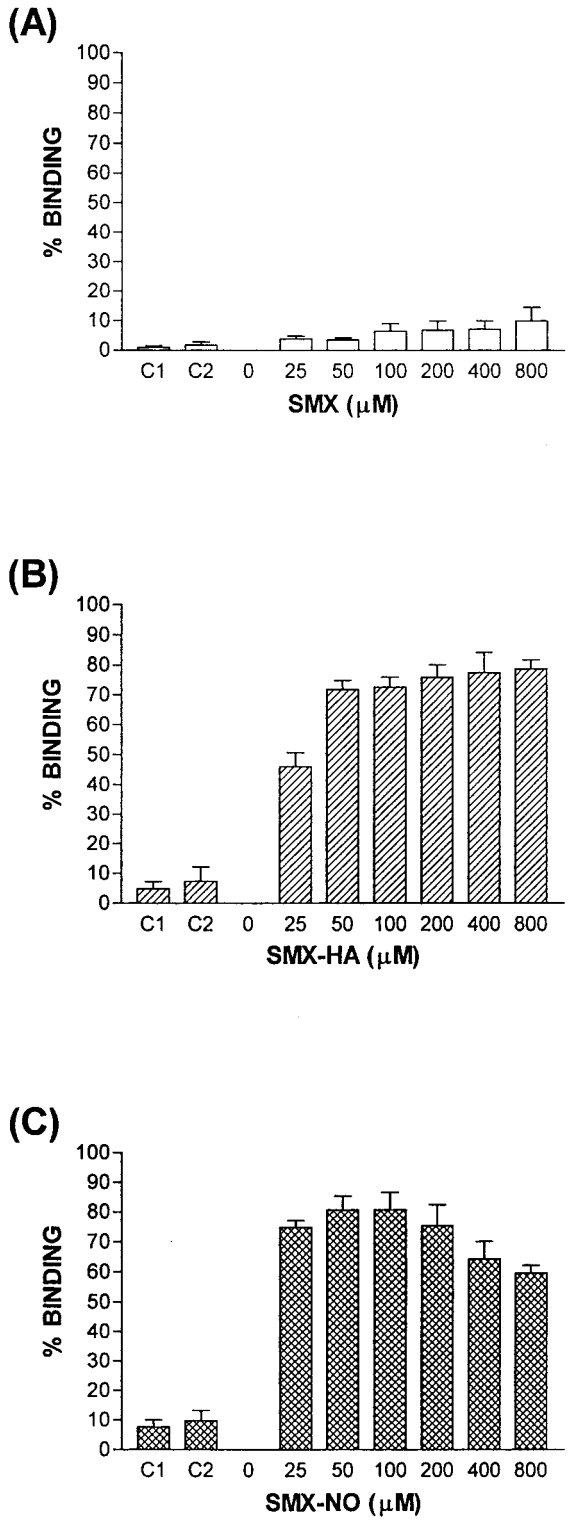
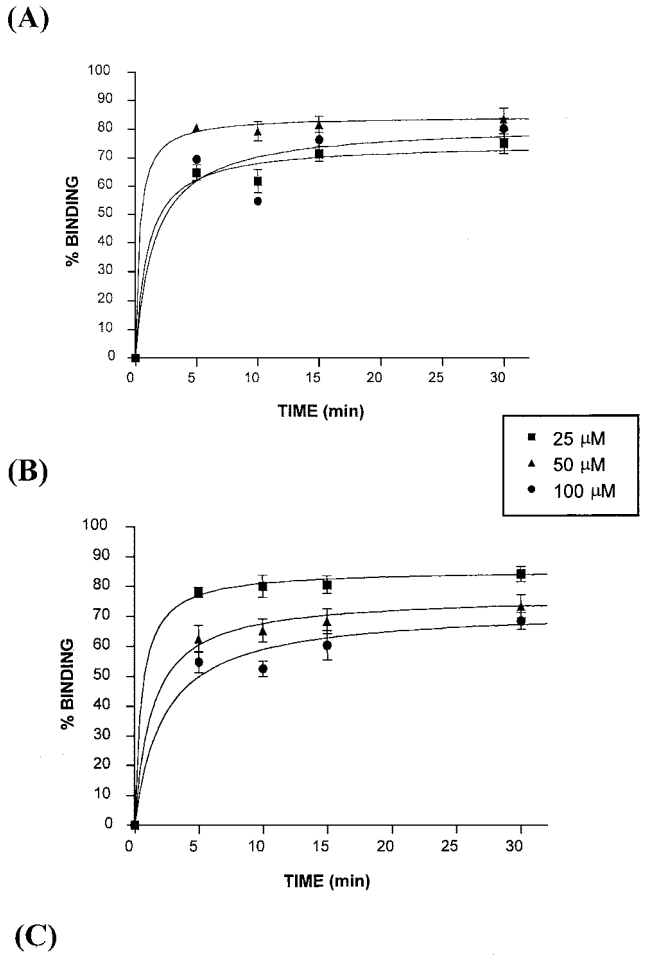


Fig. 1. Dose response of extracellular protein binding by SMX, SMX-HA, and SMX-NO in MOLT-3 cells using flow cytometry. MOLT-3 cells were treated with various concentrations of SMX (A), SMX -HA (B), or SMX-NO (C) and analyzed for cell surface binding using flow cytometry. Controls were fully treated and unstained (C1) or fully treated and stained only with the secondary antibody (C2) to account for nonspecific binding. Binding was calculated as a percentage increase in the mean fluorescence of the immunolabeled, fully treated cells compared with immuno-labeled untreated control cells. There is a significant increase in binding associated with SMX-HA and SMX-NO compared with SMX ($p >$ 0.05). Data show the mean $\pm$ S.E.M. of three independent experiments.

seem to be the same; the 55- and 150-kDa bands are especially apparent at the lower concentrations (Fig. 5).

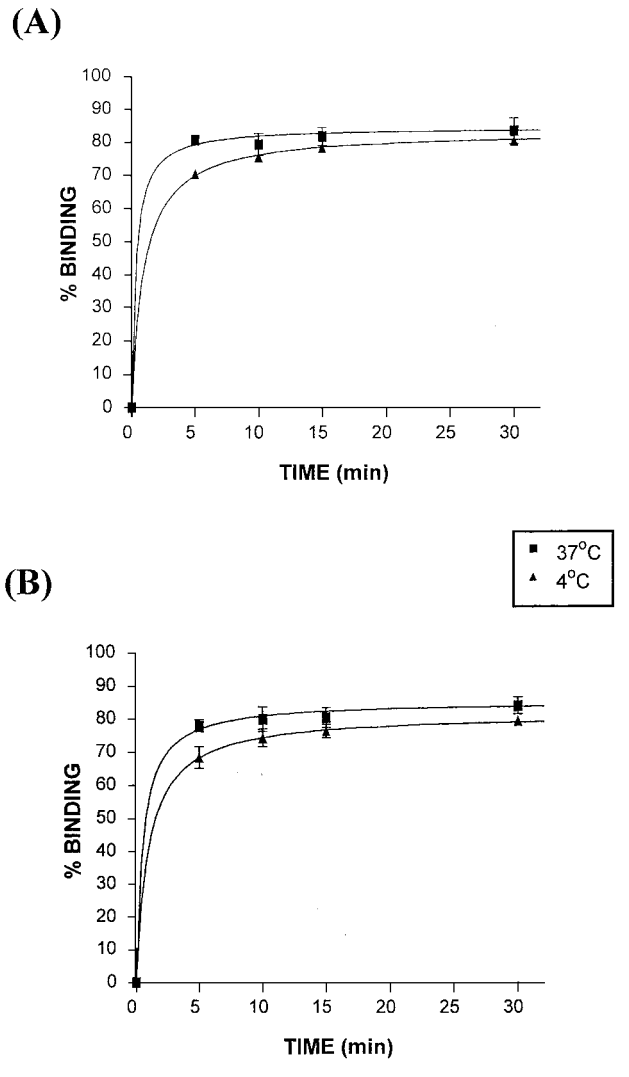
HEPA 1C1C7 cells were also examined by Western blot for sulfa-specific protein binding and demonstrated a pattern of more than 20 protein bands in both SMX-HA and SMX-NO, similar to that seen with MOLT-3 cells (Fig. 6). Using the liver-absorbed anti-SMX antibody, no nonspecific protein bands were seen with the DMSO control and SMX-incubated cells. The intense 55 kDa band was also apparent in Hepa cells along with strong 70-, 72-, 90-, 95-, and 120-kDa protein bands that were not seen in the MOLT-3 cells (Fig. 6).

Results from flow cytometry data described above, indicated that protein binding is occurring within 5 min of incubation with the reactive metabolites. Therefore, Western blot analysis



Concentration	SMX-HA Max Binding (%)	SMX-NO Max Binding (%)
25 μM	75.08 ± 2.94	85.49 ± 1.11
50 μM	84.59 ± 1.37	76.68 ± 1.86
100 μM	81.61 ± 7.65	72.16 ± 4.09

Fig. 2. Time course of extracellular protein binding by SMX, SMX-HA, and SMX-NO in HEPA 1C1C7 cells using flow cytometry. HEPA 1C1C7 cells were treated with 25, 50, and 100 μM concentrations of SMX-HA (A) or SMX-NO (B) for 5, 10, 15, and 30 min at 37°C. Cells were analyzed for cell surface binding using flow cytometry as described under *Materials and Methods*. C, summary table of the maximum percentage binding at each concentration. Binding was calculated as a percentage increase in the mean fluorescence of the treated cells compared with immunolabeled untreated control cells. There is a significant time-related increase in binding up to time 10 min ($p > 0.05$). Data show the mean ± S.E.M. of three independent experiments.



Temperature	SMX-HA Max Binding (%)	SMX-NO Max Binding (%)
37°C	82.83 ± 1.37	84.03 ± 1.11
4°C	82.65 ± 0.59	81.90 ± 0.99

Fig. 3. Effect of temperature on extracellular protein binding by SMX, SMX-HA, and SMX-NO in HEPA 1C1C7 cells using flow cytometry. HEPA 1C1C7 cells were incubated at 37°C and 4C with either 50 μM SMX-HA (A) or 25 μM SMX-NO (B) for 5, 10, 15, and 30 min, respectively. Cells were analyzed for cell surface binding using flow cytometry. C, summary table of the maximum percentage binding at each concentration. Binding was calculated as percentage increase in the mean fluorescence of the treated cells compared with immunolabeled untreated control cells. There is significantly more binding at 37°C versus 4°C for times under 10 min ($p > 0.05$). Data show the mean ± S.E.M. of three independent experiments.

was repeated at 5 min of incubation with SMX-HA and SMX-NO to determine whether specific proteins were bound earlier on in the incubation (Fig. 7). Figure 7 shows that the 70-, 72-, and 95-kDa bands are visible at the same intensity at 5 min of incubation as they are at 60 min (Fig. 6), whereas the 55-, 90-, and 120-kDa bands are significantly lighter in intensity at 5 min. Protein haptenation occurs within 5 min but to fewer proteins compared with 60-min incubation.

Confocal Studies of Cellular Protein Binding. Flow cytometry experiments (Figs. 1–3) illustrated sulfa-specific binding patterns to cell membrane proteins but did not give any information about what was occurring inside the cell. The very faint amounts of green seen associated with SMX (Fig. 8B) are the same as the DMSO control (Fig. 8A), suggesting background fluorescence only and not SMX-specific binding. However, in the case of the cells incubated with SMX-HA (Fig. 8C) and SMX-NO (Fig. 8D), there is a great deal of fluorescence, indicating sulfa-specific binding. Interestingly, binding seems to be localized to specific punctata and is not simply diffused throughout the cell.

To further visualize sulfa-specific binding patterns, confocal microscopy was used, initially to examine cell membrane binding by SMX, SMX-HA, and SMX-NO at 5 and 60 min. Sulfa-specific binding to membrane proteins was not seen when cells were incubated with either DMSO (Fig. 9A) or the parent compound SMX (Fig. 9B). However, incubation with both SMX-HA (Fig. 9C) and SMX-NO (Fig. 9D) revealed similar sulfa-specific binding within 5 min of incubation. The binding pattern is seen to be a ring around the membrane, with greater fluorescent intensities at specific areas. When cells were permeabilized and examined for intracellular as well as extracellular binding confocal microscopy, much of the binding can be visualized inside the cell in vesicles near the membrane within 5 min of incubation with SMX-HA and SMX-NO (Fig. 10, A and B). By 60 min, binding can be visualized throughout the cell (excluding the nucleus); it seems to be concentrated in discrete areas and not simply diffused throughout the cell. It also seems that by 60 min, most of the fluorescence is inside the cell compared with being localized to the cell membrane, indicating some sort of internalization mechanism.

To prevent internalization, cells were incubated with SMX-HA and SMX-NO for 60 min on ice and then examined intra- and extracellularly for sulfa-specific binding (Fig. 11).

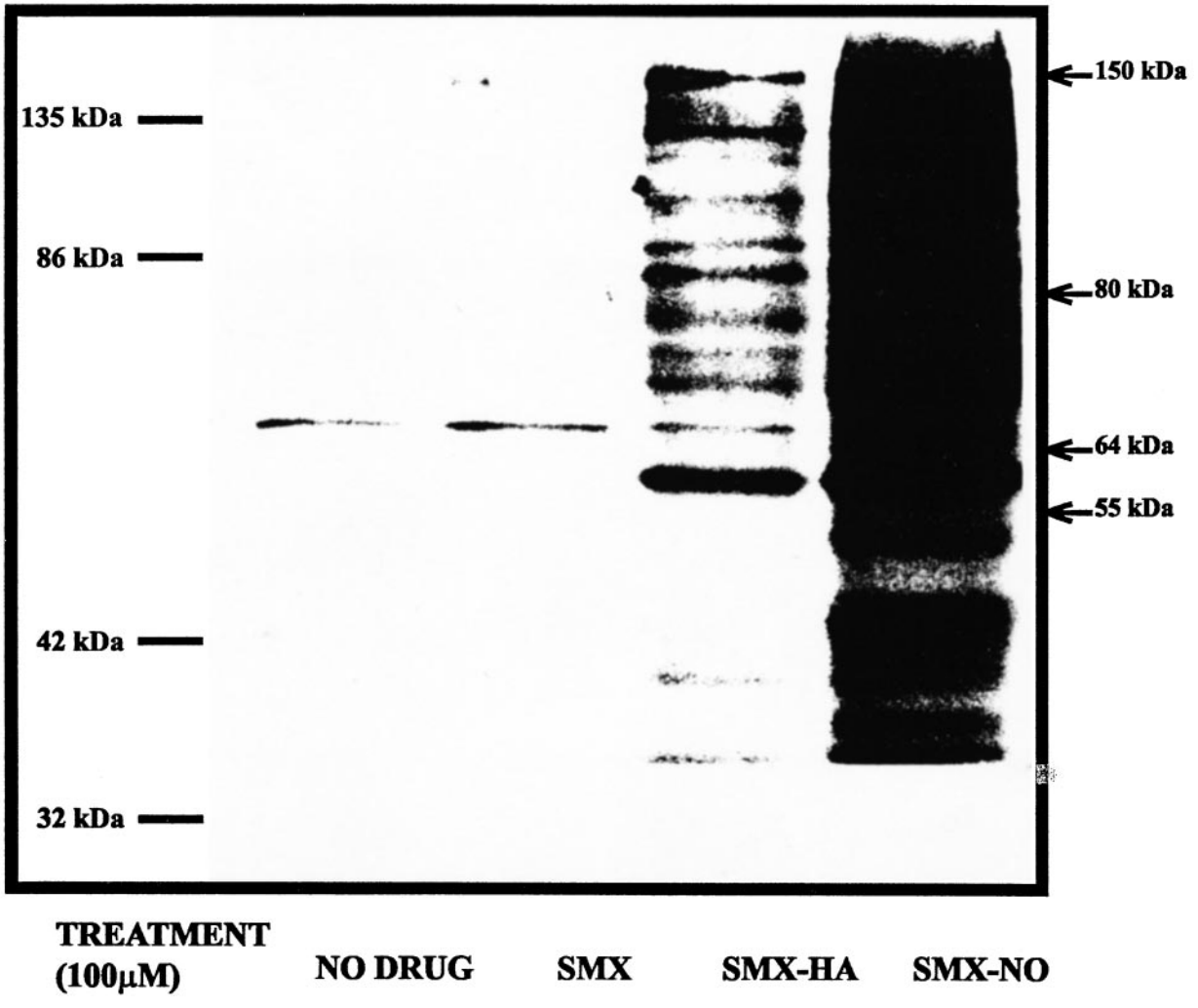


Fig. 4. Western blot analysis of protein binding by SMX, SMX-HA, and SMX-NO in MOLT-3 cells. MOLT-3 cells were incubated with 100 μM SMX, SMX-HA, or SMX-NO for 60 min at 37°C. "No drug" represents incubation with vehicle DMSO only. Whole-cell lysates (10 μg) were run on 10% SDS-PAGE and examined for sulfa-specific binding using Western blot analysis.

Under these conditions, there still seems to be sulfa-specific binding intracellularly but to a much lesser extent than at 37°C. The 60-min incubation on ice (Fig. 11) looks similar to the 5-min incubation at 37°C condition (Fig. 10). However, it does seem that the concentrated sulfa-bound areas are localized to the cell membrane when incubated on ice.

Analysis of the Effects of Antioxidants on Sulfa-Specific Binding. To examine the effects of antioxidants on

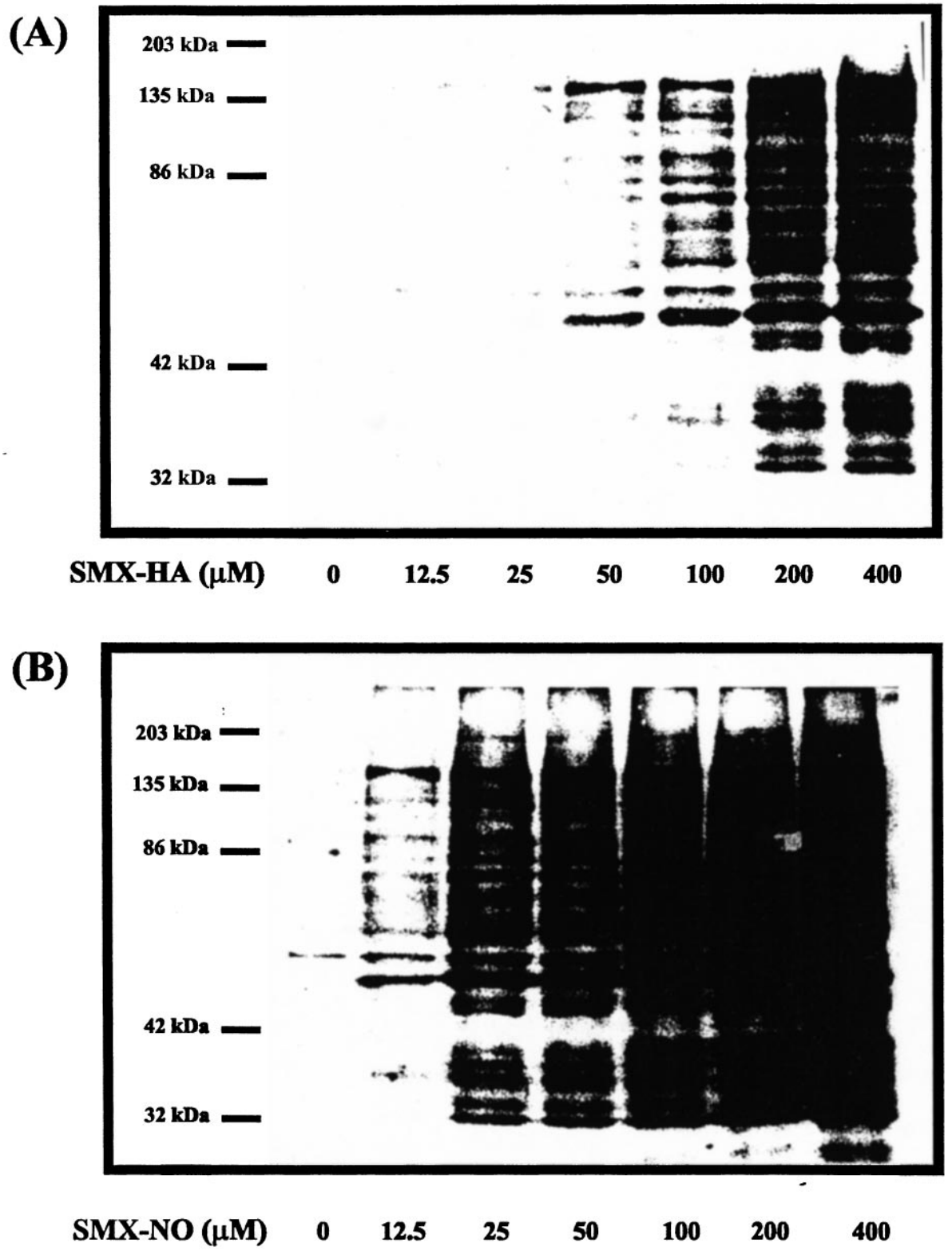


Fig. 5. Dose response of protein binding by SMX, SMX-HA, and SMX-NO in MOLT-3 cells using Western blot analysis. MOLT-3 cells were treated with either 12.5, 25, 50, 100, 200, and 400 μM of either SMX-HA (A) or SMX-NO (B) for 60 min at 37°C. "No drug" represents incubation with vehicle DMSO only. Whole-cell lysates (10 μg) were run on 10% SDS-PAGE and examined for sulfa-specific binding using Western blot analysis.

sulfa-specific binding, MOLT-3 cells were coincubated with either glutathione, *N*-acetylcysteine, or ascorbic acid, along with SMX-HA or SMX-NO. Results (Fig. 12) indicate that 1.0 mM glutathione was able to decrease SMX-HA–specific binding by 71.7 ± 8.2% and SMX-NO–specific binding by 45.8 ± 5.4% (Fig. 12A). As well, 1.0 mM *N*-Acetylcysteine decreased SMX-HA and SMX-NO binding by 70.4 ± 1.9 and 29.5 ± 3.1%, respectively (Fig. 12B). The greatest decrease in binding was seen by 1.0 mM ascorbic acid, which decreased SMX-HA binding by 80.5 ± 1.0% and SMX-NO binding by 41.9 ± 5.4% (Fig. 12C). The results of these studies as well as the effects of these antioxidants at lower concentrations are summarized in Fig. 12D. Western blot analysis was then carried out to determine whether the above antioxidants decreased binding of specific cellular proteins. The results of Western blot analysis revealed a decrease in protein binding with the antioxidants corresponding to the flow cytometry data with both the SMX-HA and the SMX-NO (Fig. 12E). The Western blot shown in Fig. 13E is that of glutathione; however, the blots for *N*-acetylcysteine and ascorbic acid were identical. There were no specific proteins that were inhibited by the antioxidants; rather, a generalized decrease in all sulfa-bound proteins was seen.

Analysis of Haptenated Cellular Protein Internalization. Confocal analysis of sulfa-specific protein haptenation illustrated that binding was occurring on the cell membrane as well as inside the cell. Therefore, we used flow cytometry to examine internalization of cellular proteins from the cell membrane into the cell. Results (Fig. 13) indicate that 60.12 ± 6.05% of cell membrane proteins bound by SMX-HA are internalized within 30 min. Correspondingly, 53.99 ± 4.30% of cell membrane proteins bound by SMX-NO are internalized within 30 min.

To determine the mechanism of internalization, HEPA cells were preincubated with inhibitors of internalization before being exposed to SMX-HA and SMX-NO. The inhibitors used were incubation at 4°C (which inhibits overall cell membrane internalization), sucrose (which inhibits clathrin-mediated internalization), and nystatin (which is an inhibitor of caveolae-mediated internalization). Incubating cells at 4°C with the reactive sulfa metabolites decreased maximum internalization of sulfa-bound proteins from 60.12 ± 6.05 to 5.68 + 0.91% ($p < 0.05$) with SMX-HA and from 53.99 ± 4.30 to 6.04 + 1.46% ($p < 0.05$) with SMX-NO (Fig. 14). Sucrose decreased maximum internalization of sulfa-bound proteins to 45.52 ± 6.68% with SMX-HA and to 45.16 ± 3.35% (Fig.

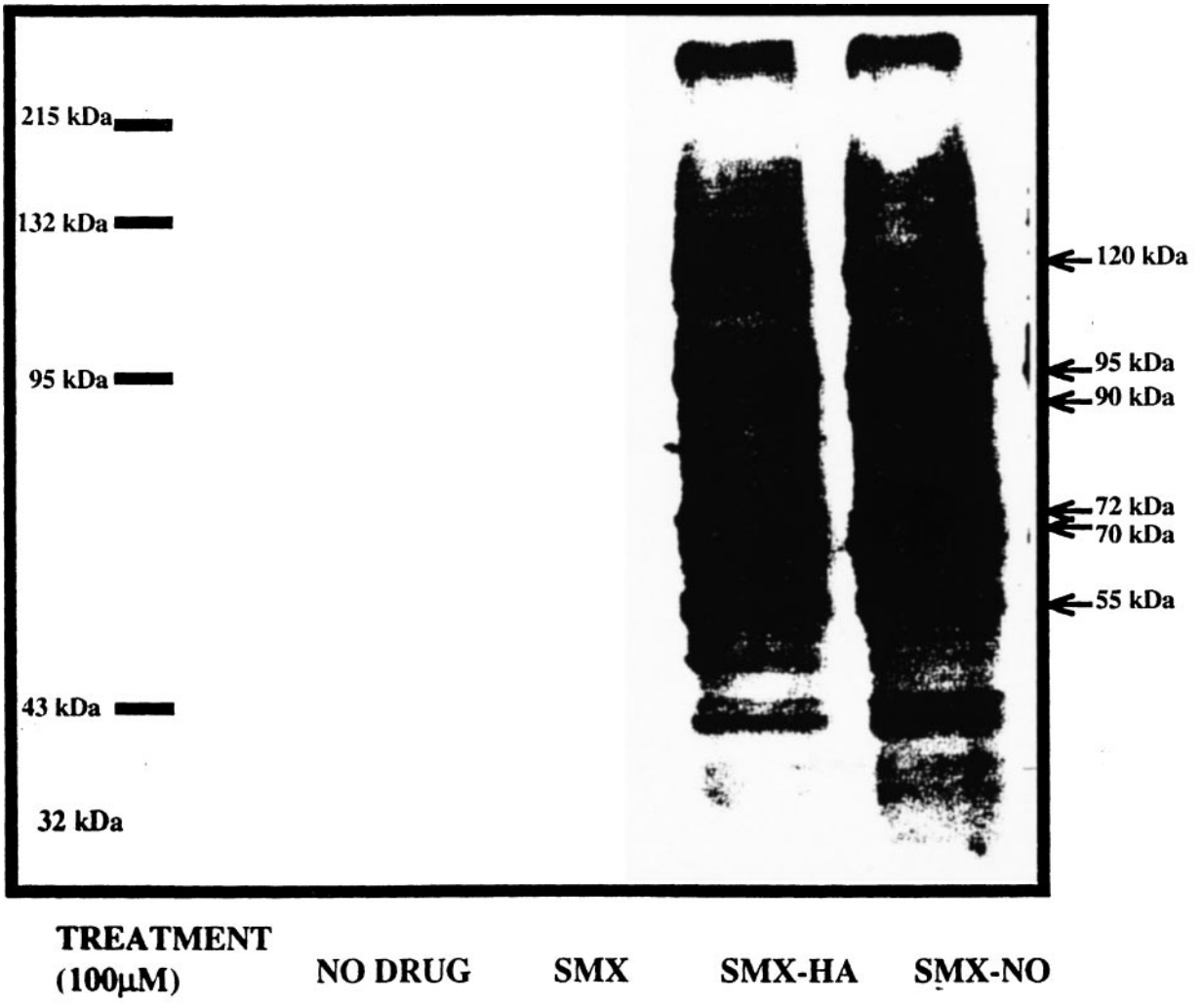


Fig. 6. Western blot analysis of protein binding by SMX, SMX-HA, and SMX-NO in HEPA 1C1C7 cells. HEPA 1C1C7 cells were treated with 100 μM SMX, SMX-HA, or SMX-NO for 60 min at 37°C. "No drug" represents incubation with vehicle DMSO only. Whole-cell lysates (10 μg) were run on 10% SDS-PAGE and examined for sulfa-specific binding using Western blot analysis.

14). The overall effect of sucrose was minimal, suggesting that clathrin-mediated internalization is not a major mechanism by which sulfa-bound proteins are internalized. Alternatively, nystatin was found to decrease the initial rate of binding up to 15 min; over the entire time-course, nystatin was found to display an overall significant inhibition in sulfa-bound protein internalization (Fig. 14) ($p < 0.05$). From these results, it seems that early internalization of haptenated cellular proteins (< 15 min) may occur through a caveolae-dependent mechanism.

Discussion

Sulfonamides are common antimicrobial agents. Unfortunately, sulfa therapy is often associated with adverse drug reactions (Rieder et al., 1989; Pirmohamed and Park, 1999). The most severe adverse events are hypersensitivity reactions. The mechanisms by which sulfonamides produce hypersensitivity adverse reactions are unclear; however, the clinical manifestations suggest involvement of the immune system (Hess and Rieder, 1997; Naisbitt et al., 1999). Bioactivation of sulfonamides to reactive metabolites seems to play a major role in the initial steps leading to these reactions (Shear et al., 1986; Rieder et al., 1989). Studies have shown that in vivo and in vitro metabolic production of electrophilic reactive metabolites may lead to binding to essential cellular proteins, hapten formation, and activation of immune responses (Meekins et al., 1994; Cribb et al., 1995, 1996). Anti-SMX antibodies, as well as specific T cell responses, have been detected in patients experiencing hypersensitivity reactions to sulfonamides but also in patients who tolerate therapy (Daftarian et al., 1995; Mauri-Hellweg et al., 1995; Gill et al., 1997). There is very little knowledge of the immune response to sulfa haptenated proteins; understanding this response would provide insights into the biology of xenobiotic-immune system interactions leading to idiosyncratic adverse drug reactions.

Sulfa-Specific Haptenation to Cellular Proteins. The reactive metabolites SMX-HA and SMX-NO, but not SMX, bound to cellular proteins at concentrations of 25 μM and above. Maximum plasma concentrations of SMX can reach 1.5 to 2 mM in patients on high-dose cotrimoxazole; concentrations of SMX-HA can be 10 to 20% of the dose and SMX-NO is less than 2% (Cribb et al., 1995). The concentrations at which haptenation occurs in vitro are within the therapeutic range achieved in vivo. This binding occurred within 5 min of incubation. Because the nitroso is inherently more reactive (Cribb et al., 1995), it is not surprising that maximum binding of the nitroso occurs at lower concentrations and more quickly than with SMX-HA.

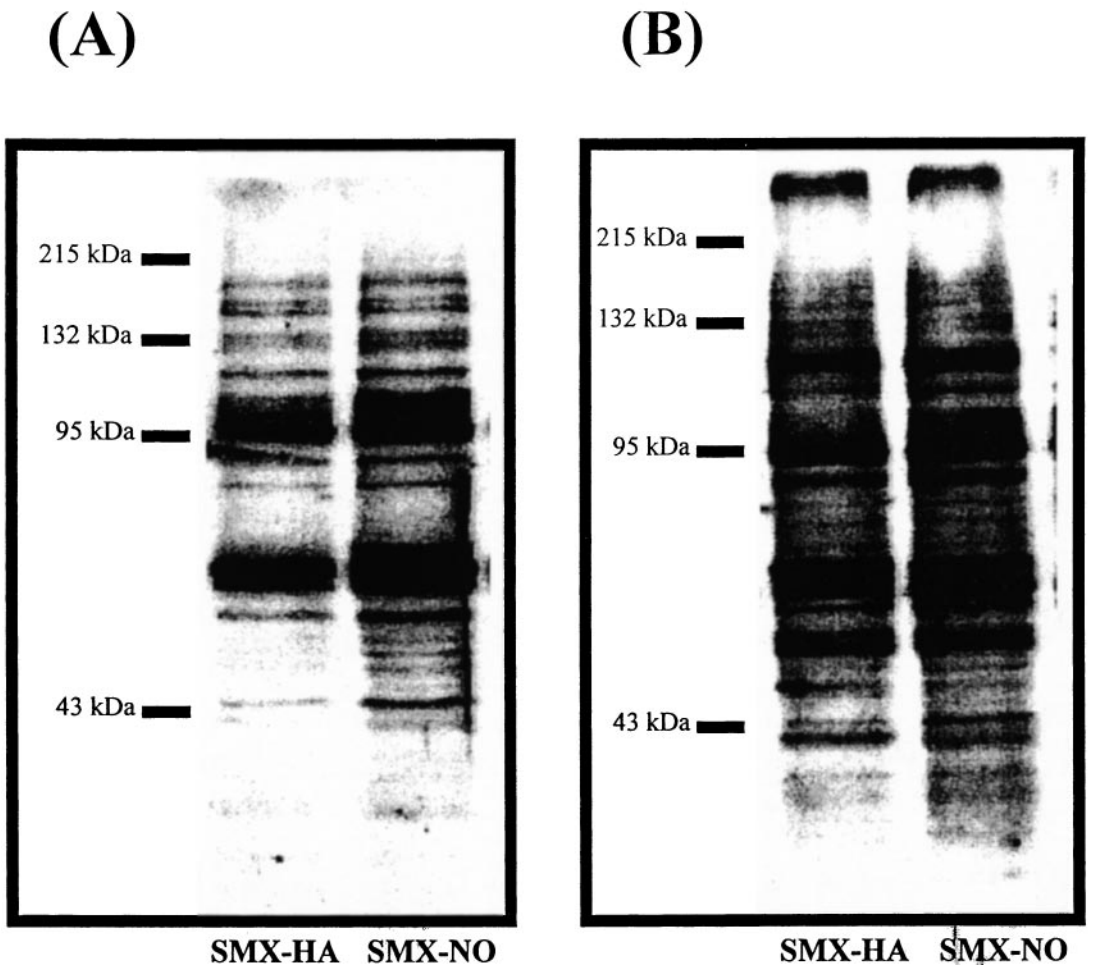


Fig. 7. Protein binding by SMX-HA and SMX-NO in HEPA 1C1C7 cells at different time points using Western blot analysis. HEPA 1C1C7 cells were treated with 50 μM of SMX-HA or SMX-NO at 37°C at for 5 min (A) or 60 min (B). Whole-cell lysates (10 μg) were run on 10% SDS-PAGE and examined for sulfa-specific binding using Western blot analysis.

These results agree with previous in vitro cytotoxicity assays showing toxicity for the hydroxylamine and nitroso derivatives of SMX over a concentration range of 100 to 400 μM and inhibition of lymphocyte proliferation at concentrations from 50 to 200 μM. The concentrations at which toxicity is demonstrated are much greater than those at which significant haptenation occurs (Rieder et al., 1989). It can be assumed that haptenation occurring at the lower concentrations ($<$ 200 μM) is affecting viable cells. Naisbitt et al. (1999) reported that haptenation can occur without loss of cell membrane integrity, depletion of GSH, or detectable perturbation of redox-sensitive factors. Our results demonstrate that SMX-HA and SMX-NO haptenate cells without loss of viability, allowing formation of viable cell-drug conjugates. Thus, haptenation alone does not cause enough direct cellular damage to lead to toxicity. Other mechanisms, such as formation of viable cell-drug conjugates that function as antigenic stimuli to activate the immune system must play a role in vivo.

Identification of Sulfa-Specific Haptenated Cellular Proteins. Specific proteins haptenated by SMX-HA and SMX-NO in vitro were detected using Western blot analyses. Sulfa-specific haptenation revealed a binding pattern of more than 20 bands with both the SMX-HA and SMX-NO metabolites in both cell lines. The intensity and the total number of haptenated protein bands detected was greater with SMX-NO, probably because of its greater reactivity.

A number of particularly strong bands can be visualized in both cell types; however, only the 55-kDa band is clearly common. It is likely that there are other common protein bands, but better separation is needed for a clear correlation. It is not known whether these intense protein bands are specific high-affinity targets for protein haptenation or simply a reflection of relative abundance in the cell. Detection of haptenated proteins over time shows that selective proteins are haptenated to the same extent at 5 min as they are at 60 min of incubation with the metabolite. Alternately, other

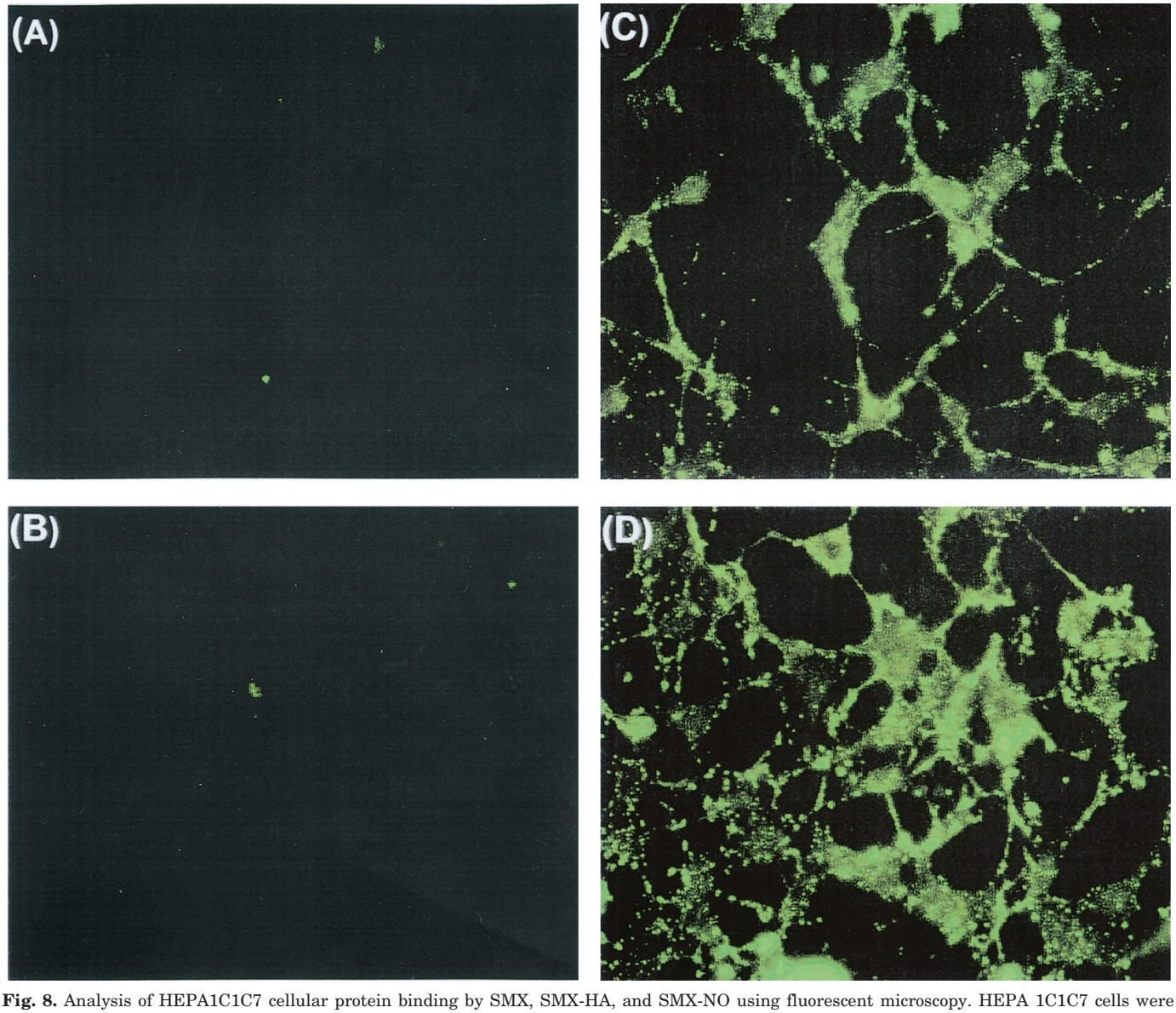


Fig. 8. Analysis of HEPA1C1C7 cellular protein binding by SMX, SMX-HA, and SMX-NO using fluorescent microscopy. HEPA 1C1C7 cells were incubated vehicle DMSO alone (A) or 25 μM SMX (B), SMX-HA (C), or SMX-NO (D) for 60 min at 37C. Cells were fixed, permeabilized, stained for sulfa-specific binding, and analyzed on the fluorescence microscope as described under *Materials and Methods*.

proteins show increased haptenation over time. This pattern indicates some degree of selectivity. Initial binding of reactive metabolites to cellular proteins may be a specific selective process. However, over time and with increased concentrations, binding to cellular proteins in vitro may become more general and nondiscriminate. It should be noted that there are a large number of candidate proteins in these bands (Lefkovitz et al., 2000).

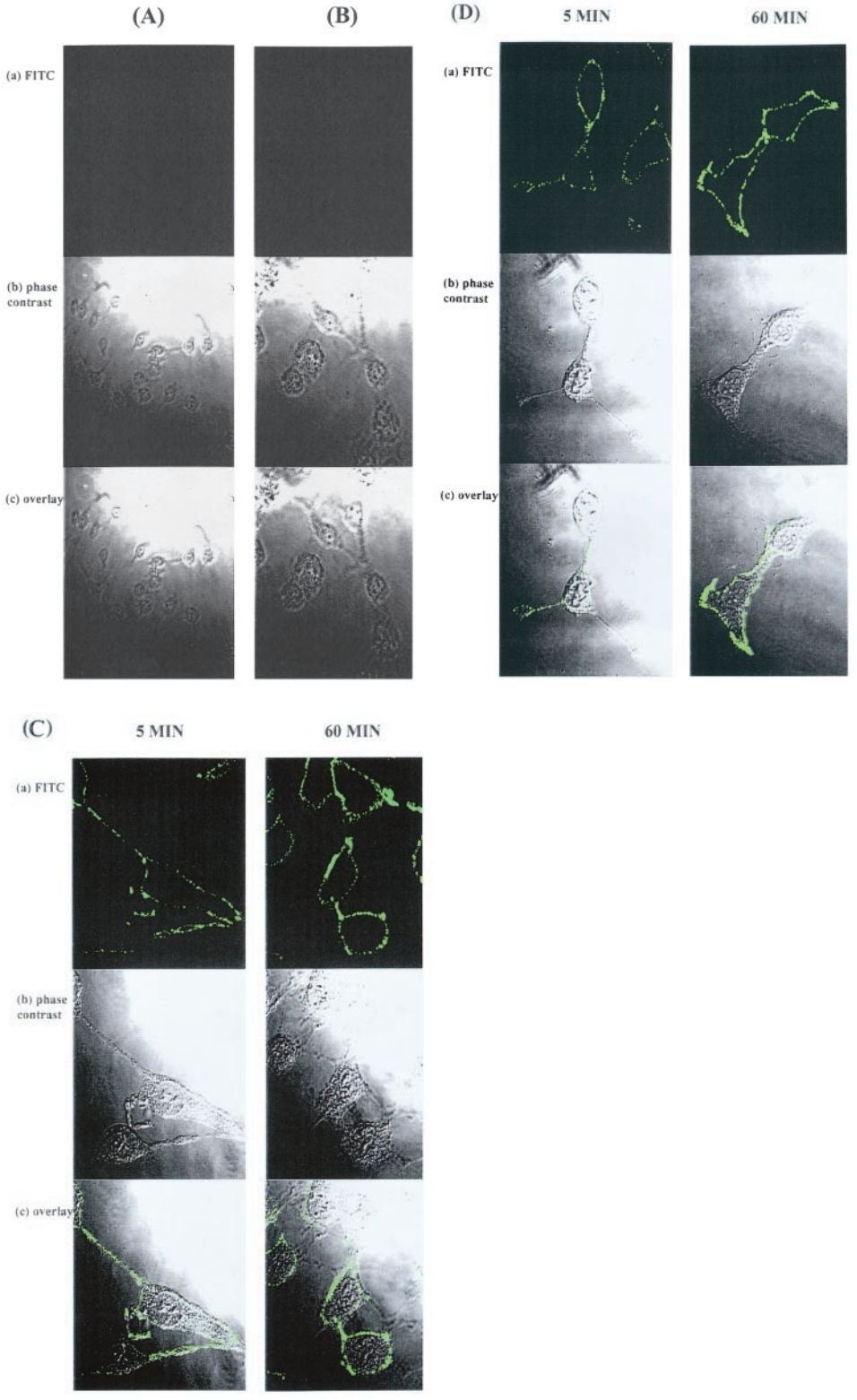


Fig. 9. Analysis of HEPA1C1C7 cell membrane protein binding by SMX, SMX-HA, and SMX-NO using confocal microscopy. HEPA 1C1C7 cells were incubated vehicle DMSO alone for 60 min (A), 50 μM SMX for 60 min (B), 50 μM SMX-HA for 5 and 60 min (C), or 50 μM SMX-NO for 5 and 60 min (D). Cells were immunostained for sulfa-specific binding, fixed, and analyzed on the confocal microscope as described under *Materials and Methods*. Each depicts FITC staining alone, phase contrast of cells alone, and an overlay of FITC staining with the phase contrast.

Effect of Antioxidants on Sulfa-Specific Protein Haptenation. Addition of antioxidants to cells incubated with reactive metabolites of SMX attenuated sulfa-specific binding with both the hydroxylamine and nitroso metabolites, although attenuation was greater with SMX-HA. Antioxidants such as GSH may be responsible for reducing reactive metabolites to inactive compounds and higher levels in the liver may explain why SMX-mediated hepatotoxicity is rarely observed clinically (Cribb et al., 1996).

Extensive reduction of both SMX-HA and SMX-NO have been reported in in vivo models (Gill et al., 1997). SMX-NO undergoes reduction in plasma, with concomitant depletion of thiols in vitro (Naisbitt et al., 1999). Spontaneous reaction of thiols with hydroxylamine and nitroso derivatives is dependent on thiol concentrations, pH, and the aromatic ring substituents of the drug (Ellis et al., 1992). The reaction is initiated by nucleophilic attack by GSH on the nitroso moiety, resulting in formation of an unstable semimercaptal intermediate. This intermediate can yield a stable sulfonamide conjugate, a hydroxylamine, or the parent compound (Cribb et al., 1991; Ellis et al., 1992; Naisbitt et al., 1999). This may provide a mechanism for detoxication of SMX-NO. Both *N*-acetylcysteine and GSH markedly decrease cytotoxic effects of SMX-HA (Rieder et al., 1988, 1995; Carr et al., 1993). Attenuation of binding is less with the SMX-NO metabolite, possibly because detoxication first requires formation of a semimercaptal before reduction to SMX-HA or the parent amine. Glutathione decreases covalent binding of SMX-HA without formation of stable GSH conjugate (Cribb et al., 1996); therefore, reduction of the hydroxylamine would

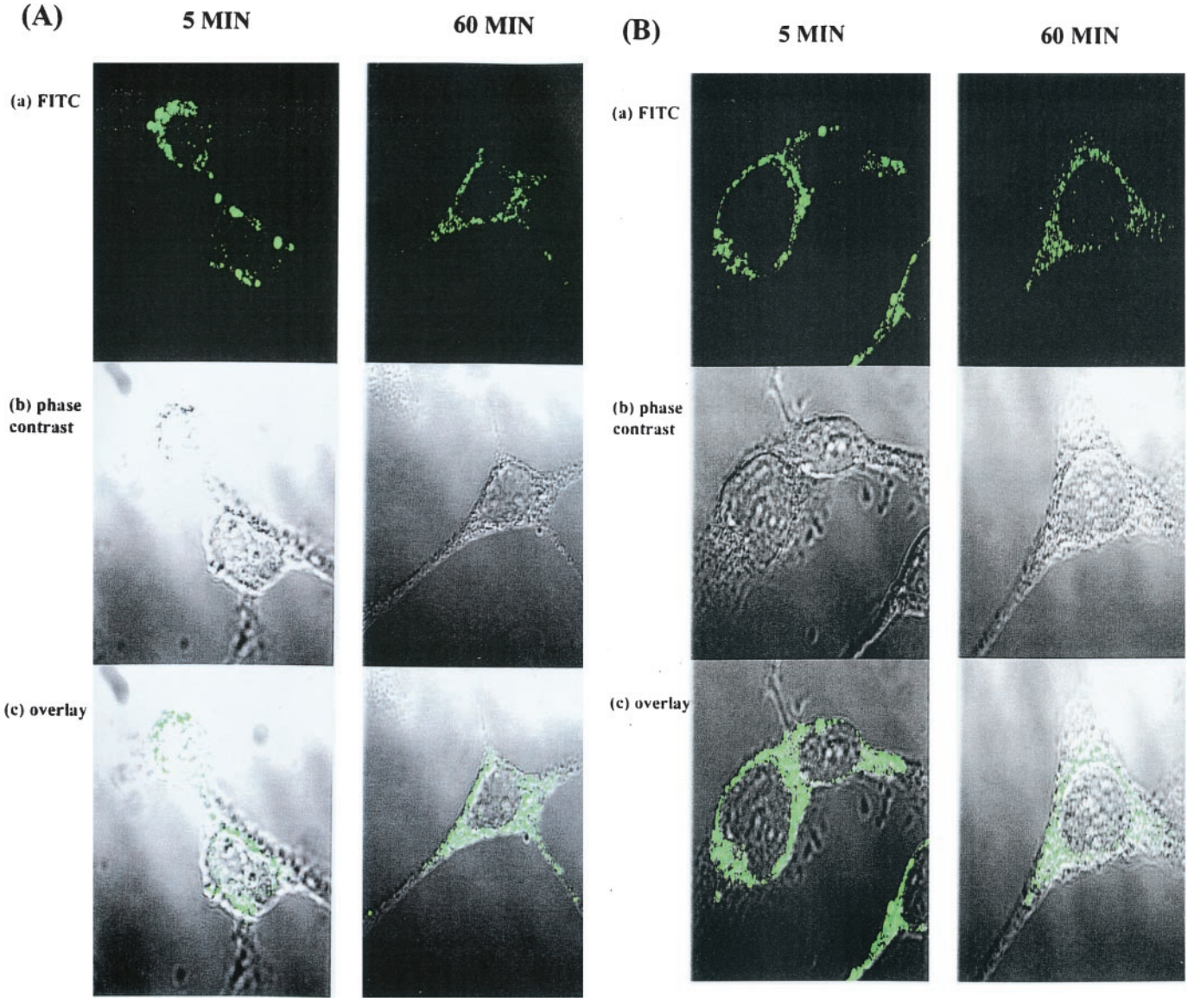


Fig. 10. Analysis of HEPA1C1C7 intracellular and extracellular protein binding by SMX, SMX-HA, and SMX-NO using confocal microscopy. HEPA 1C1C7 cells were incubated at 37°C with 50 μM SMX-HA for 5 and 60 min (A) or 25 μM SMX-NO for 5 and 60 min (B). DMSO and SMX control pictures were identical to those seen in Fig. 9, A and B. Cells were fixed, permeabilized, immunostained, and analyzed on the confocal microscope for sulfa-specific binding as described under *Materials and Methods*. Each depicts FITC staining alone, phase contrast of cells alone, and an overlay of FITC staining with the phase contrast.

be a more rapid process. Alterations in GSH concentration of GSH have been observed in patients with AIDS and may be a factor in their increased susceptibility to adverse reactions.

Internalization of Haptenated Cellular Proteins. Sulfa-specific haptenation can be visualized on the cell membrane as well as intracellularly. Binding pattern occurred at specific sites on the membrane within 5 min of incubation. The confocal images show specific and not indiscriminate areas at which binding is localized on the cell membrane. Looking intracellularly within 5 min of exposure to metabolite, haptenated cellular proteins seem to be in vesicles near the plasma membrane, but later distribute throughout the cell (excluding the nucleus). This suggests a mechanism of internalization of haptenated proteins from the cell membrane into the cell.

Virtually all eukaryotic cells continually ingest bits of plasma membrane as small pinocytic (endocytic) vesicles that later return to the cell surface. The endocytic part of the cycle usually begins at specialized regions of the plasma membrane called clathrin-coated pits. The lifetime of clathrin

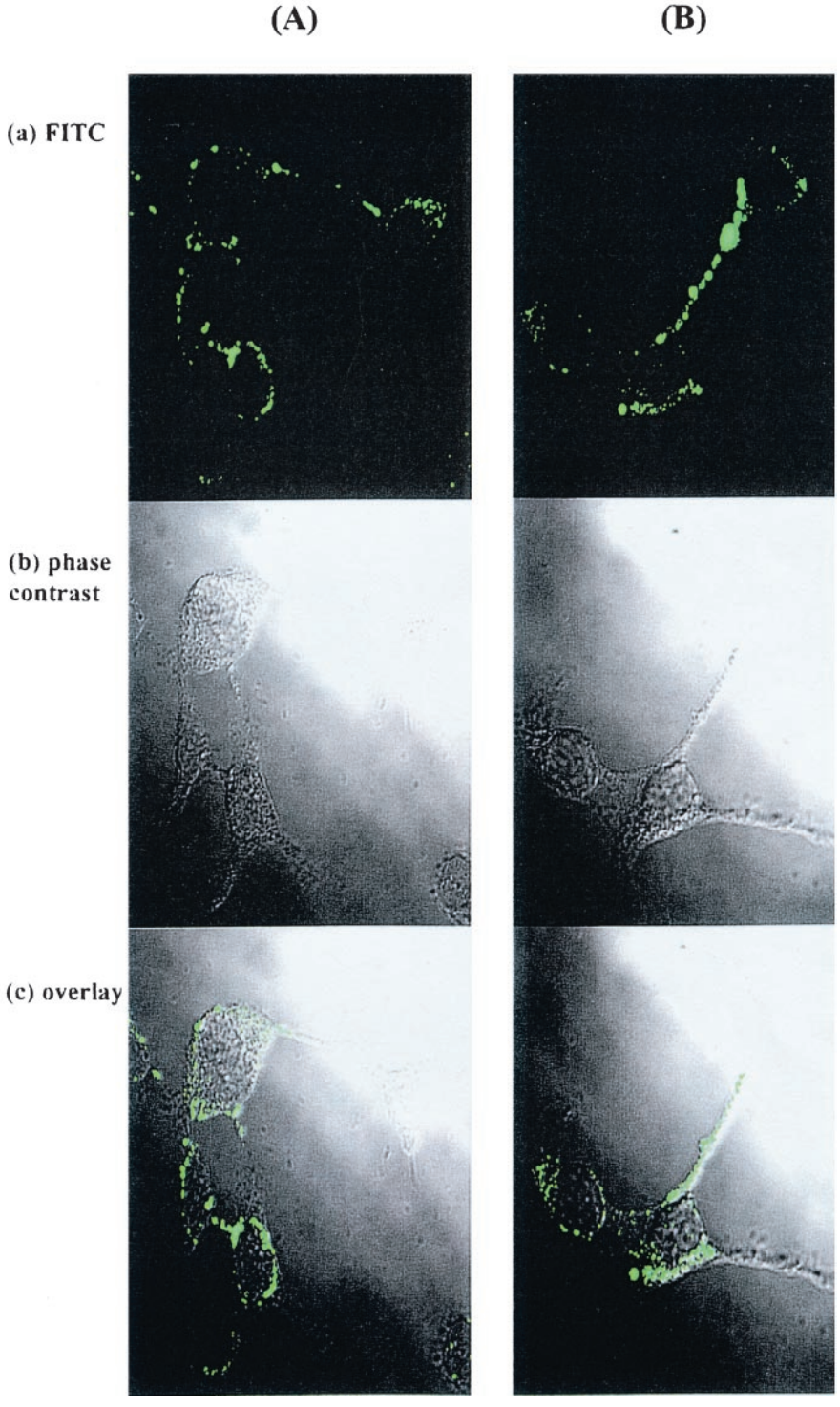
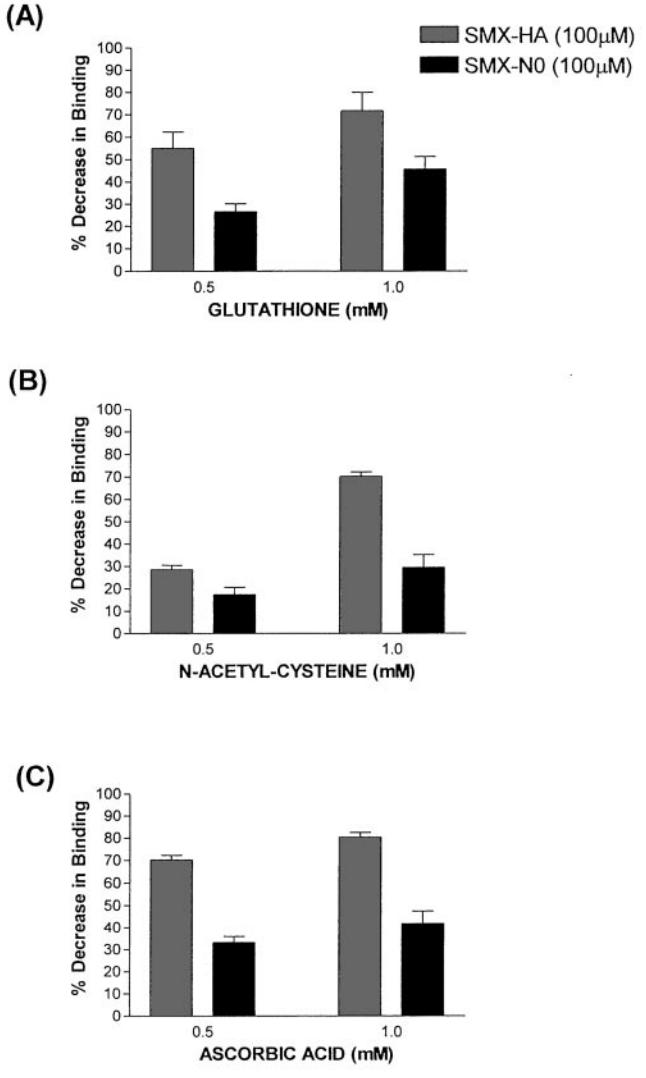


Fig. 11. Analysis of HEPA1C1C7 intracellular and extracellular protein binding by SMX, SMX-HA and SMX-NO at 4C using confocal microscopy. HEPA 1C1C7 cells were incubated at 4°C with 50 μM SMX-HA (A) or 25 μM SMX-NO (B). Cells were fixed, permeabilized, immunostained, and analyzed on the confocal microscope for sulfa-specific binding as described under *Materials and Methods*. Each depicts FITC staining alone, phase contrast of cells alone, and an overlay of FITC staining with the phase contrast.

coated pits is short; within a minute of being formed, they are invaginated into the cell and pinched off to form clathrin-coated vesicles, which within seconds shed their coats and fuse with early endosomes. In most animal cells, clathrin-coated pits and vesicles provide an efficient pathway known as receptor-mediated endocytosis for taking up specific macromolecules bound to cell surface receptors or transmembrane proteins. The plasma membrane of most cells also has distinct invaginations called caveolae, thought to bud off to form calveolin-coated vesicles that provide another mechanism for endocytosis. Inhibiting clathrin-mediated endocytosis did not significantly decrease internalization of sulfa-haptenated proteins from the cell membrane. Inhibition of caveolae-mediated endocytosis decreased the time of internalization but not the overall amount or protein that was internalized. This indicates that caveolae-mediated endocytosis may be important in early internalization of haptenated proteins but does not rule out other mechanism of internalization.

Two sets of endosomes can be readily distinguished in labeling experiments: early endosomes, just beneath the plasma membrane within 1 to 2 min of incubation, and late endosomes, which accumulate closer to the Golgi apparatus and nucleus within 15 min. The intracellular haptens seen at 5 min near the plasma membrane may be in the early endosomes, whereas by 60 min, they are in late endosomes. Endogenous antigens (such as drugs and metabolites) can be endocytosed by antigen-presenting cells, routed to endosomes, and associated with MHC class II to elicit a specific T-cell response.

We have demonstrated that the reactive metabolites SMX-HA and SMX-NO bind covalently to cellular proteins at



(D)

Treatment (100 μM)	GSH 0.5 mM	GSH 1.0 mM	NAC 0.5 mM	NAC 1.0 mM	AA 0.5 mM	AA 1.0 mM
SMX-HA	55.0 ± 7.3	71.7 ± 8.2	28.5 ± 2.1	70.4 ± 1.9	70.2 ± 2.1	80.5 ± 1.9
SMX-NO	26.6 ± 3.6	45.8 ± 5.4	17.6 ± 3.1	29.5 ± 5.7	33.3 ± 2.8	41.9 ± 5.7

(E)

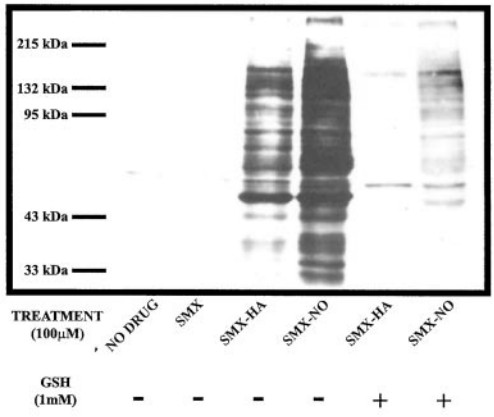
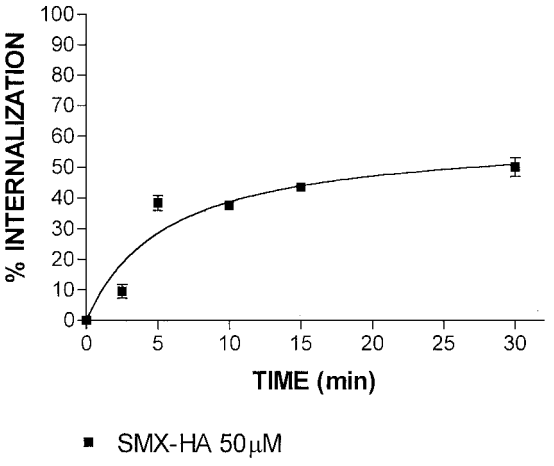


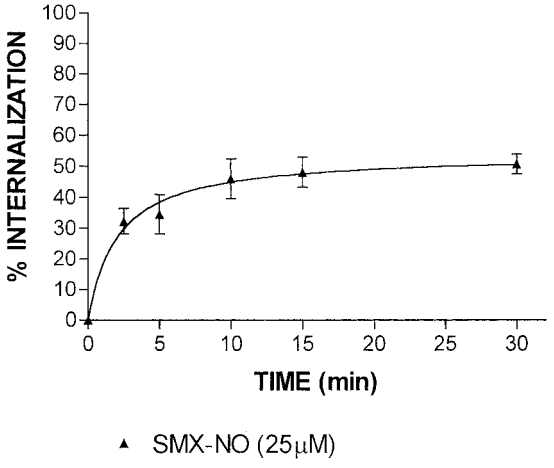
Fig. 12. Effect of antioxidants on extracellular protein binding by SMX, SMX-HA, and SMX-NO in MOLT-3 cells. A to D, MOLT-3 cells were incubated for 60 min at 37°C with 100 μM SMX-HA or SMX-NO in the absence or presence of GSH (A), *N*-acetyl-cysteine (NAC) (B), or ascorbic acid (AA) (C) at 0.5 and 1.0 mM concentrations. Cells were analyzed for cell surface binding in the absence and presence of these antioxidants using flow cytometry. Results are displayed as a percentage decrease in mean fluorescence in the presence and absence of antioxidants compared with cells treated with the metabolites alone. There was a significant decrease in binding associated with increasing antioxidant concentrations for SMX-HA for all three antioxidants ($p < 0.05$). D, summary table of the percentage decreases in binding at each condition (% decrease in binding ± S.E.). Data shows the mean ± S.E.M. of three independent experiments. E, MOLT-3 cells were incubated for 60 min at 37°C with 100 μM SMX, SMX-HA, or SMX-NO in the absence or presence of 1.0 mM GSH. Whole-cell lysates were run on 10% SDS-PAGE and examined for sulfa-specific binding using Western blot analysis. Similar results were obtained when MOLT-3 cells were incubated in the presence of NAC or AA (data not shown).

concentrations found in plasma during sulfonamide therapy. Sulfa-specific binding seems to be somewhat discriminate; specific proteins were detected early after incubation with metabolites. Antioxidants such as GSH, *N*-acetylcysteine, and ascorbic acid were able to attenuate sulfa-specific binding. This provides further support for their key role as a defense mechanism against sulfa toxicity and may account for interpatient variability in toxic response. Internalization of sulfa-bound proteins was also demonstrated, indicating specific mechanisms of internalization and processing of these haptenated structures.

(A)



(B)

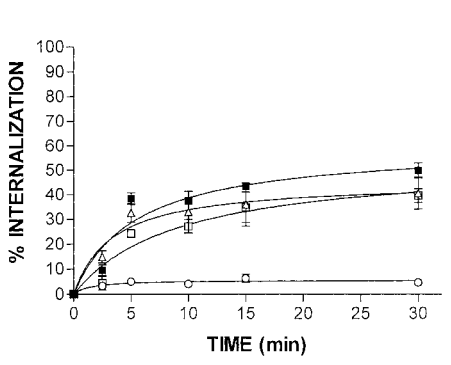


(C)

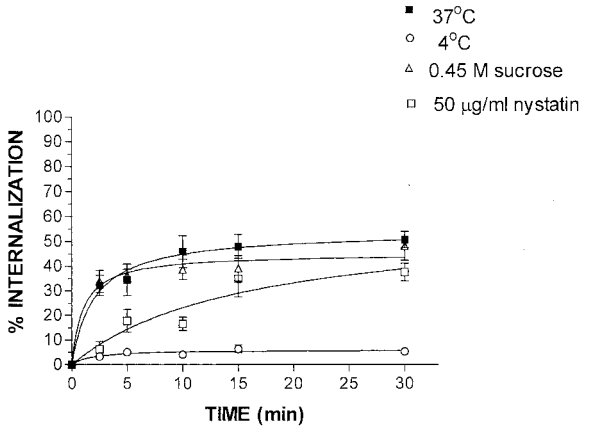
Treatment	Max Internalization (% of total)
50 μM SMX-HA	60.12 + 6.05
25 μM SMX-NO	53.99 + 4.30

Fig. 13. Time course of disappearance of extracellular proteins haptenated by SMX, SMX-HA, and SMX-NO from the cell surface using flow cytometry. HEPA 1C1C7 cells were incubated at 10 min at 37°C with either 50 μM SMX-HA (A) or 25 μM SMX-NO (B) and internalization of proteins bound by these metabolites was determined at 0, 2.5, 5, 10, 15, and 30 min using flow cytometry as described under *Materials and Methods*. Internalization was defined as the percentage loss of antibody labeled cellular proteins (mean fluorescence) over the various time periods. There is a significant time-related disappearance up to 15 min ($p > 0.05$). C, summary table of the maximum internalization for each experimental condition. Data represent the mean ± S.E.M. of three independent experiments.

(A)



(B)



(C)

Maximum Internalization (% of total)				
Treatment	37°C	4°C	± 0.45M sucrose	± 0.05 μg/ml nystatin
SMX-HA (50μM)	60.12 ± 6.05	5.68 ± 0.91	45.52 ± 6.68	53.68 ± 9.196
SMX-NO (25μM)	53.99 ± 4.30	6.04 ± 1.46	45.16 ± 1.02	59.25 ± 8.49

Fig. 14. Effect of inhibitors of disappearance of extracellular proteins haptenated by SMX-HA and SMX-NO from the cell membrane using flow cytometry. HEPA 1C1C7 cells were preincubated with 0.45 M sucrose or 50 mg/ml Nystatin for 30 min and 3 h, respectively, at 37°C. Cells were then incubated at 10 min at 37°C with either 50 μM SMX-HA (A) or 25 μM SMX (B) and internalization of proteins bound by these metabolites was determined at 0, 2.5, 5, 10, 15, and 30 min using flow cytometry as described under *Materials and Methods*. Cells were also maintained at 4°C under all treatment conditions and analyzed for internalization. Internalization was defined as the percentage loss of antibody-labeled cellular proteins (mean fluorescence) over the various time periods. There is no significant difference in the percentage disappearance associated with incubation with sucrose; in contrast, there is a significant decrease in disappearance associated with incubation with nystatin up to 15 min ($p > 0.05$). C, summary table of the maximum internalization for each experimental condition. Data represent the mean ± S.E.M. of three independent experiments.

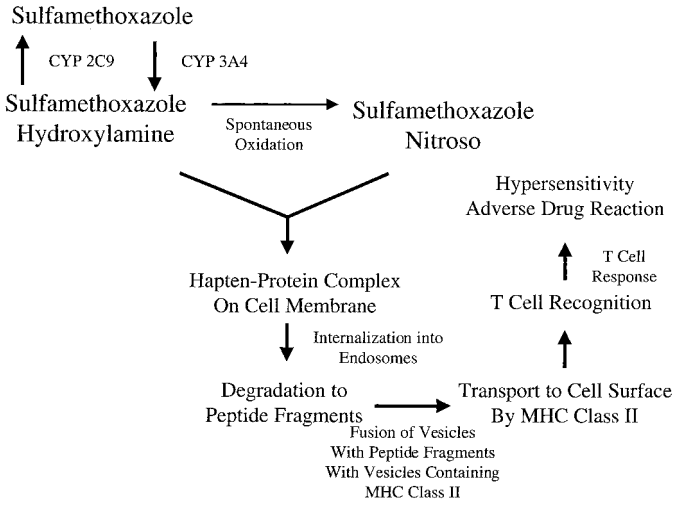


Fig. 15. Proposed pathogenesis of hypersensitivity reactions to sulfamethoxazole. Sulfamethoxazole can be acetylated but also is metabolized to its reactive metabolites SMX-HA and SMX-NO. These reactive metabolites can covalently bind to cellular proteins, forming immunogenic hapten-protein complexes. These can then be internalized, processed, and presented for recognition by cells of the immune system, leading to drug hypersensitivity.

Understanding specific protein target(s) for sulfonamides will be important in understanding how induction of an immune response can be clinically manifested as an adverse drug reaction to sulfonamide therapy (Fig. 15). Further characterization of the mechanisms of haptenation, as well as understanding hapten processing, is essential to gain a better understanding of the pathobiology of sulfonamide hypersensitivity reactions.

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