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EFFECTS OF COREXIT DISPERSANTS ON CYTOTOXICITY PARAMETERS IN A CULTURED HUMAN BRONCHIAL AIRWAY CELLS, BEAS-2B

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Abstract

The objective of this study was to assess the cytotoxicity of COREXIT dispersants EC9500A, EC9527A, and EC9580A on human airway BEAS-2B epithelial cells. Cells were exposed to dispersants for 2 or 24 h at concentrations ranging from 0 to 300 ppm. COREXIT EC9527 at 100 ppm produced 50% viability loss as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 24 h. COREXIT 9527 at 200 ppm produced 50% cell death at 2 h and 100% at 24 h. At 300 ppm COREXIT 9527 induced 100% cell death at 2 or 24 h. In the case of COREXIT 9500A 50% cell viability was noted with 200 ppm at 2 or 24 h, with a significant decrease in cell survival to 2% at 300 ppm. In contrast, no marked change in cell viability was observed in cells treated at any COREXIT 9580A concentration examined. Western blot analysis showed an increase in expression of LC3B, a marker of autophagy, in cells treated for 2 h with 300 ppm COREXIT EC9527A as well as 100 or 300 ppm Corexit EC9500A. No marked effect on LC3B expression was observed for any COREXIT 9580A concentration. Apoptosis markers as measured by cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) were detectable only in cells incubated with 300 ppm COREXIT EC9527A. Although all three dispersants induced enhanced generation of reactive oxygen species (ROS) after 2-h treatment at 300 ppm, Western blot analysis revealed that 2-h incubation was not sufficient to induce a significant change in the protein expression of superoxide dismutases SOD1, SOD2, and SOD3. Data thus indicate exposure to certain dispersants may be harmful to human airway epithelial cells in a concentration-dependent manner.

In response to the *Deepwater Horizon* oil spill disaster, the largest scale application of oil dispersants in world history was utilized to reduce both visibility of spilled oil and consequent adverse effects on the ecosystem. Of the nearly 2 million gallons of COREXIT oil dispersants delivered into the water of the Gulf of Mexico, two dispersants predominantly applied were COREXIT EC9500A and EC9527A, both manufactured by Nalco. George-Ares and Clark (2000) compiled more than 100 data values on acute aquatic toxicity of crustaceans, molluscs, fish, and algae exposed to COREXIT dispersants 9500A and 9527A. Utilizing an effect concentration (EC_{50}), a concentration producing a specific effect in 50% test organisms, and a lethal concentration (LC_{50}), COREXIT 9527

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demonstrated a 24- to 96-h LC_{50} or EC_{50} values from 1.6 to >1000 ppm. In the case of COREXIT 9500 the LC_{50} and EC_{50} values ranged from 0.7 to >400 ppm. George-Ares and Clark (2000) categorized the acute aquatic toxicity to be considered low to moderate for these dispersants. Judson et al. (2010) examined the cytotoxic effects of COREXIT 9500A dispersant in aquatic species and cultured mammalian cells and found a median LC50 value of approximately 100 ppm. A comprehensive examination on the effects of COREXIT 9500A on the immune, cardiovascular, and pulmonary systems in rats was conducted by the National Institute for Occupational Safety and Health (NIOSH; Anderson et al., 2011; Krajnak et al., 2011; Roberts et al., 2011; Sriram et al., 2011). COREXIT 9500A did not induce marked alterations in peripheral and pulmonary immune parameters but transiently increased breathing difficulties (Anderson et al., 2011; Roberts et al., 2011). There were also transient changes in cardiovascular and peripheral vascular functions (Krajnak et al., 2011), while Sriram et al. (2011) noted disruptions in olfactory signal transduction and axonal functions.

Type I and type II programmed cell death are termed apoptosis and autophagy, respectively, and both involve degradation of cell components in lysosomes. The difference between the two cell death pathways is that apoptotic cells are engulfed by phagocytes for degradation in the engulfing cells, whereas autophagic cells “eat” their own cell components in their own lysosomes (Cecconi and Levine, 2008). Cells undergoing apoptosis or autophagy maintain intact cell membranes, without releasing cellular contents that may further damage the cell (Fink and Cookson, 2005). Both types of cell death are regulated pathways designed to remove unwanted or damaged cells. However, autophagy is also a cell survival mechanism under certain circumstances (Ryter et al., 2010) and has the potential to prolong cell survival by inhibiting apoptosis (Yuan and Kroemer, 2010). Recently, Bandele et al. (2012) reported that COREXIT 9527A and 9500A induced cytotoxicity and oxidative stress in a cultured human hepatocyte cell line. Further, Wang et al. (2012) demonstrated that exposure to mixtures of oil dispersants resulted in adverse effects in A549 cells with consequent mortality, which occurred more frequently than seen with oil alone. Thus, it appears that exposure to oil dispersant exerts a cytotoxic effect in cultured cell lines.

Currently, there is a lack of knowledge regarding the cell death pathways as evidenced from the observed cytotoxic effects induced by COREXIT dispersants. This study was thus designed to investigate the cell death pathways in cultured airway epithelial cells exposed to several COREXIT dispersants including 9527A, 9500A, and 9580A.

MATERIALS AND METHODS

Chemicals

Commercially available COREXIT 9500A, 9527A, and 9580A dispersants as liquid solutions were provided by a contract between Nalco/Exxon Energy Chemicals, L.P. (Sugar Land, TX) and Tulane University (New Orleans, LA). Fibronectin, collagen, Dulbecco’s modified Eagle’s medium with nutrient mixture (DMEM/F-12), and penicillin/streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Cell Signaling Technology (Beverly, MA).

Epithelial Cell Culture

The human bronchial cell line (BEAS-2B) was purchased from the American Type Culture Collection (ATCC, Wiltshire, USA). Cells were cultured as described previously (Wang et al., 2010; Shi et al., 2010a, 2010b) and maintained at 37°C in a 100% humidified atmosphere of 5% CO_2. Subcultures for the experiments were established the day prior to treatment.

Treatment

COREXIT9527A, 9500A, and 9580A dispersants were prepared in medium at concentrations ranging from 0, 10, 100, 200, or 300 ppm (volume to volume). When 80–90% confluence was reached, BEAS-2B cells were incubated with the indicated concentrations of dispersants for 2 or 24 h for the cell viability study. Since 95–100% mortality was found for COREXIT 9527 and 9580 at 24 h, subsequent studies with respect to autophagy and apoptosis were conducted at dispersant concentrations of 0, 100, or 300 ppm for only 2 h of incubation.

MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Sigma-Aldrich, St. Louis, MO, and was used to determine cytotoxicity of COREXIT dispersants. All procedures were performed according to the manufacturer's instructions, as described in previously by Yadav et al. (2010a, 2010b)

Western Blot Analysis

Western blotting was conducted as described by Shi et al. (2010a, 2010b). Isolated proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and immunoblot analysis was performed according to the manufacturer's instructions (Cell Signaling Technology, Boston, MA). The proteins were visualized by using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) after incubation with the appropriate secondary antibodies for 1 h at room temperature. The following primary antibodies were used: anti-myosin light chain-3 (LC3), anti-cleaved caspase-3, anti-cleaved PARP, and anti-GAPDH, all obtained from Cell Signaling (Cell Signaling Technology, Beverly, MA). Anti-superoxide dismutase (SOD) 1, anti-SOD2, and anti-SOD3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Reactive Oxygen Species Assay

To measure ROS generation, a nonfluorescent compound, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), was employed. Briefly, cells were treated with different concentrations of the dispersants for 2 h. After washing, the cells were incubated with 40 μ*M* DCFH-DA for 45 min in the dark as previously described (Shi et al., 2010a). At the end of incubation with DCFH-DA, cells were washed with phosphate-buffered saline (PBS), lysed with 1 *N* NaOH, and aliquots were transferred to a black well plate (BD Falcon). The fluorescence intensity was measured using a multidetection microplate reader (FLUOstar Optima Microplate Reader, BMG LABTECH) with excitation and emission wavelengths of 485 nm and 520 nm, respectively. Three independent experiments were conducted.

Statistical Analysis

All experiments were repeated at least three times, with each data point performed in triplicate. Data were expressed as mean ± SE (standard error). Intergroup differences were tested by analysis of variance (ANOVA) followed by Tukey's test. The criterion for statistical significance was $p < .05$. The software used was Excel (Microsoft) and Prism (GraphPad).

RESULTS

Influence of Dispersant Exposure on Cell Viability in Cultured BEAS-2B Cells

Loss of cell viability in BEAS-2B cells following exposure to three dispersants was assessed by MTT assay. Results of BEAS-2B cells treated with various concentrations of COREXIT 9527A, 9500A, and 9580A for 2 or 24 h are presented in Figure 1. No marked changes were

noted in cell viability when cells were incubated with 10 ppm COREXIT 9527A, 9500A, or 9580A for 2 or 24 h (Figure 1a). Treatment with either COREXIT 9527A induced significant cytotoxicity from 50% cell loss at 100 following a 24-h exposure. At 200 ppm, COREXIT 9527A produced 50% mortality at 2 h, and 100% mortality occurred at 24 h. At 300 ppm, COREXIT 9527A induced approximately 100% cell death at 2 and 24 h following incubation. In the case of COREXIT 9500A, incubation for 2 or 24 h at 200 ppm significantly decreased cell survival to 50% (Figure 1b), which was further reduced to approximately 90% at 24 h with 300 ppm. In contrast, treatment with COREXIT 9580A produced no marked changes in cell mortality for any of the concentrations examined (Figure 1c). The MTT results suggested a cytotoxicity ranking of COREXIT 9527A > 9500A > 9580A in a concentration-dependent manner.

Dispersant Exposure-Induced Autophagy

The LC3B protein plays a critical role in autophagy and may be used as a general marker for this process (Tanida, 2011). The concentrations selected based upon Figure 1 are representative of 0 (control), 100 (no observed effect), and 300 (>95% cytotoxicity) ppm. Treatment with 300 ppm COREXIT 9527A or 9500A significantly increased in LC3B expression approximately threefold (Figure 2). Cells incubated with the lower 100 ppm concentration of COREXIT 9500A were also found to display markedly approximately threefold elevation in LC3B protein expression. However, no detectable changes in LC3B protein were observed in BEAS-2B cells incubated with COREXIT 9580A in accordance with a lack of effect on cell viability. In comparison with the 2-h treatment survival results in Figure 1, the measurement of autophagy by LC3B protein expression induction appeared more sensitive, as this method was able to detect changes in cells incubated at lower concentrations of COREXIT 9500A.

Induction of Apoptosis Markers PARP and Caspase-3

Since cell viability loss was demonstrated in cells treated with COREXIT 9527A or 9500A, it was of interest to examine whether apoptosis as determined by cleaved caspase-3 and PARP protein expressions might be induced in human BEAS-2B exposed to these dispersants. Our results showed no marked detectable induction of apoptosis except for significantly elevated levels of approximately 2.5-fold cleaved caspase-3 and approximately threefold cleaved PARP at 300 ppm COREXIT 9527A (Figure 3). No marked apoptosis was detected with cells treated with either COREXIT 9500A or 9580A.

Reactive Oxygen Species (ROS) Generation

To determine whether COREXIT dispersants induced oxidative stress, intracellular reactive oxygen species (ROS) was measured as a biomarker. For all three dispersants, intracellular ROS increased significantly in cells treated for 2 h at 300 ppm (Figure 4). ROS generation appeared to be a less sensitive biomarker than autophagy LC3B in cells for COREXIT 9500A but more sensitive in the case 9580A.

Superoxide Dismutases 1, 2, and 3

In mammalian cells, increased ROS is commonly associated with induction of the superoxide dismutase multigene family: SOD1, SOD2, and/or SOD3 (Zelko et al., 2002). Western blot analysis of the three SOD proteins showed no marked changes in cells treated with any of the COREXIT dispersants (Figure 5).

DISCUSSION

Several parameters were employed to evaluate the in vitro adverse effects of COREXIT dispersants, including ROS generation and cell death pathways involving lysosome degradation. ROS generation increased in BEAS-2B cells exposed to all three dispersants tested, whereas the expression of SOD1, SOD2, and SOD3 did not markedly change after 2 hr treatment (Figures 4 and 5). This is not surprising, considering that poststress induction of SOD expression may require more than 2 h to yield a detectable rise in expression of these proteins (Zelko et al., 2002). Superoxide is the so-called primary ROS, and its degradation by SODs into other oxygen species is a crucial step in antioxidant mechanisms. The concomitant increase of ROS levels following a 2-h COREXIT exposure and the lack of a marked change in SOD protein expression suggest that the exposed cells may have undergone a period of intense uncontrolled damage. This is supported by our observations that 2-h exposures to 300 ppm COREXIT 9500A and 9527A displayed the highest induction of ROS levels and was correlated with the least cellular survival (Figures 1 a and 1b).

Data showed that increased ROS levels were seen with all three dispersants, including 9500A, which failed to induce apoptosis, and 9580A, which failed to induce autophagy and apoptosis at concentrations examined. It is noteworthy that ROS induction is associated with enhanced DNA damage (Storr et al., 2013). Thus, the lack of elimination of cells with increased ROS levels by apoptosis or autophagy may lead to accumulation of cells surviving with mutated genomes. Consequently, this may become a major concern if exposure also impairs DNA repair functions.

Autophagy is a general term for the degradation of cytoplasmic components within lysosomes (Mizushima and Klionsky, 2007). Although there are three types of autophagy, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy, in this study “autophagy” refers to macroautophagy, which involves autophagic cell death. Using the LC3B marker, induction of autophagy was detected following COREXIT 9527A and 9500A exposure. Autophagy induced by COREXIT 9500 A occurred at lower concentrations (100 ppm; Figure 2) than cell death (cytotoxicity, Figure 1), suggesting that autophagy appears to be a more sensitive biomarker of dispersant effect.

However, at higher COREXIT 9500A concentrations, significant cell viability loss occurred in addition to autophagy, indicating that these biomarkers were not distinguishable with respect to the actions of the dispersant on cell survival.

In this study, apoptosis was detected only in cells treated with the highest concentration of COREXIT 9527A dispersant. The increase of cleaved PARP expression, a caspase-dependent process, in cells is indicative of DNA damage (Burkle et al, 2005), since the main role of PARP is to detect and signal single-strand DNA breaks (SSB) for the enzymatic machinery to undertake SSB repair (Lazebnik et al, 1994) In cells treated with COREXIT 9500A and 9580A apoptosis was not found. Although the basis for this observation is not known, it is possible that the chemical composition of COREXIT 9527A, which contains the genotoxin 2-butoxyethanol, may have contributed to the observed effects.

This study focused on cell death pathways involving degradation of cell content within lysosomes. Clearly, cell viability loss induced by COREXIT 9527A and 9500A may likely involve a combination of different cell death pathways, including programmed necrosis, accidental necrosis, or other pathways (Yuan and Kroemer, 2010). COREXIT 9527A may act as a potentially harmful dispersant to human airway epithelial cells as evidenced by induction of autophagy, elevated ROS levels, and apoptosis. It is of interest that Bandele et al. (2012) also demonstrated an increase in ROS levels following exposure of hepatocytes to COREXIT 9527A.

There are limitations to this study that need to be considered. As it was not possible to monitor the concentration of dispersants used in response to the *Deepwater Horizon* disaster, the actual environmental concentrations of dispersant remain nonquantifiable. Based upon our data, it would seem that a minimal level of exposure to COREXIT 9527A needs to be established in the future with consideration for possible complete elimination of dispersant use. Similarly, exposure to COREXIT 9500A also induced most of the markers evaluated except for apoptosis at the 300 ppm concentration. As COREXIT 9580 A exposure did not induce apoptosis or markedly affect cytotoxicity, it is proposed that COREXIT 9580 A may be a better choice for oil spill response in terms of potential respiratory health risk.

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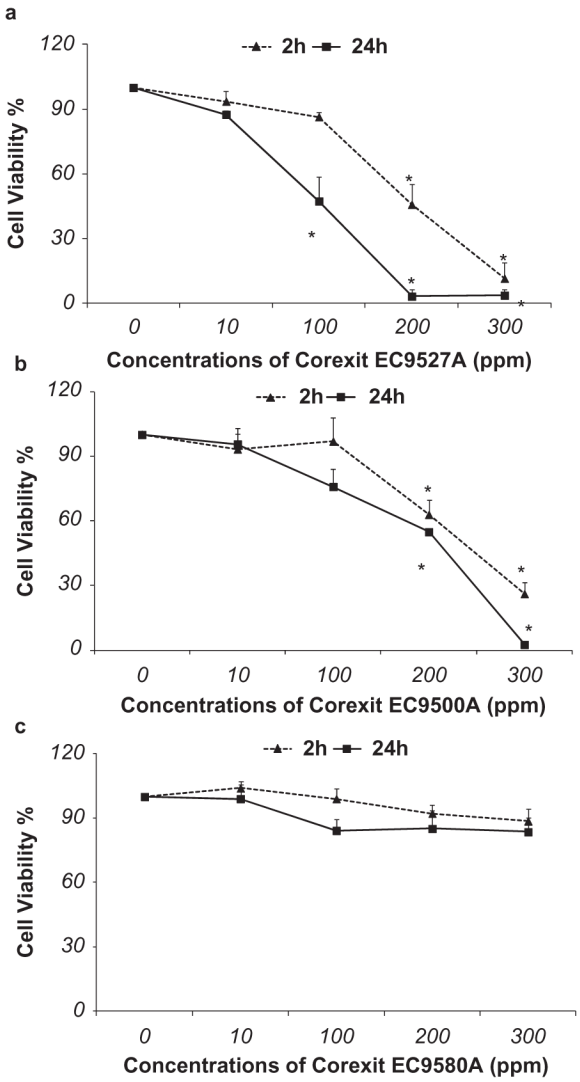

FIGURE 1.

Effects of COREXIT dispersants on cell viability in normal human lung cells. The effect of dispersants was determined on cell viability of human lung BEAS-2 cells using the MTT assay after exposure to different concentrations of COREXIT EC9527A (a), EC9500A (b), and EC9580A (c) for 2 or 24 h. The results represent three independent experiments using triplicates for each concentration point. Asterisk indicates significant difference from respective control, $p < .05$.

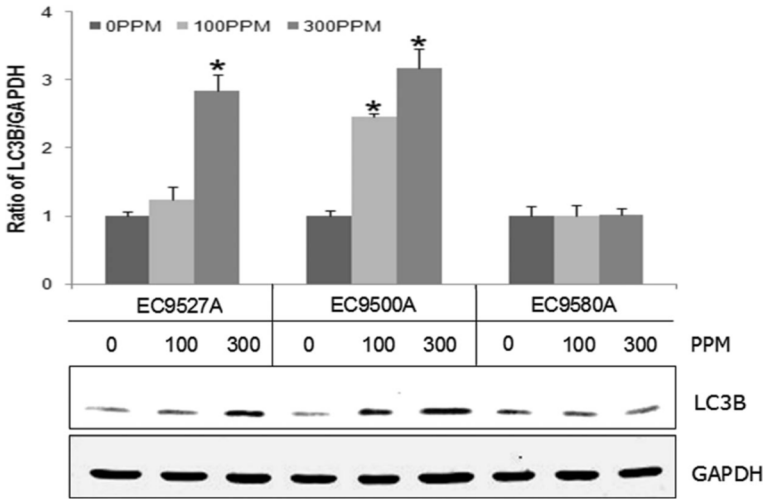

FIGURE 2.

Influence of dispersants on autophagy marker LC3B in human BEAS-2B cells. Western blot analysis of the autophagy indicator LC3B was performed on cell lysates following 2-h incubation with Corexit 9527, E9500A, or 9580A. Three independent experiments were conducted (a representative blot is shown). Asterisk indicates significant difference from control, $p < .05$.

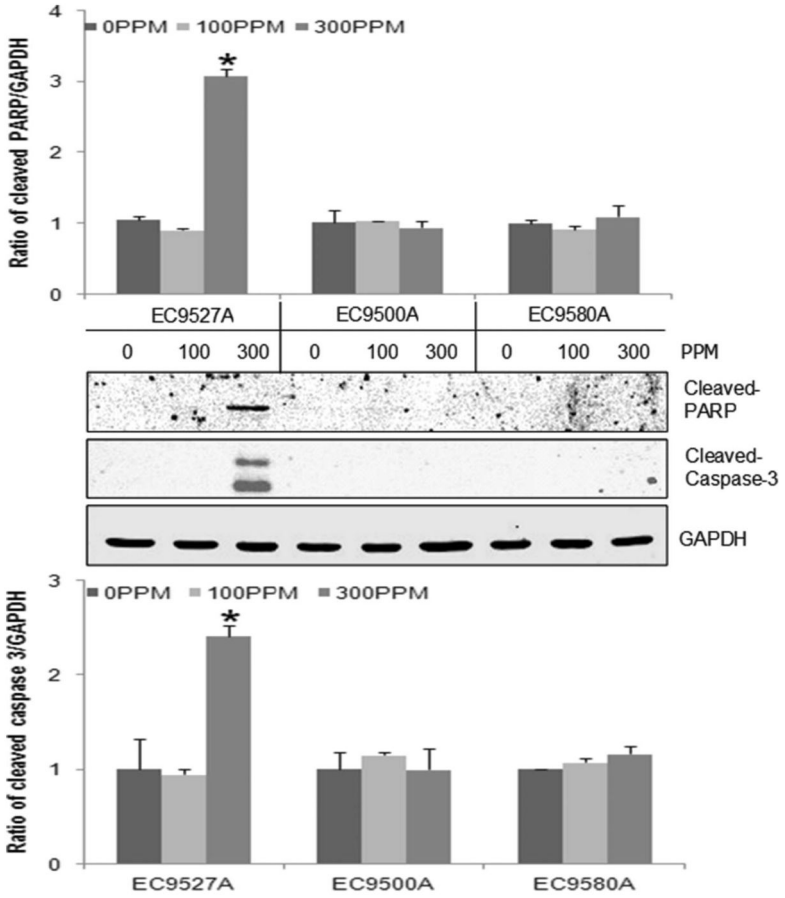

FIGURE 3.

Apoptosis markers in human BEAS-2B cells following treatment with Corexit dispersants. Western blot analysis was performed on cell extracts from BEAS-2B cells exposed to 0, 100, or 300 ppm COREXIT for 2 h. Antibodies specific to cleaved PARP and cleaved caspase-3 were used as indicators of apoptosis, and GAPDH was used as a loading control. Three independent experiments were conducted (a representative blot is shown). Asterisk indicates significant difference from control, $p < .05$.

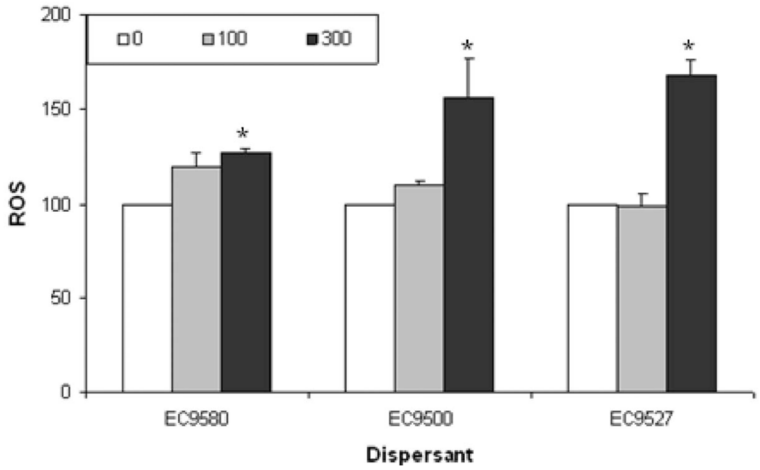

FIGURE 4.

COREXIT dispersants effects on intracellular reactive oxygen species (ROS). ROS was measured by the fluorescence of DCFH-DA and plotted as percent ROS from untreated cells. The results represent three independent experiments using triplicates for each concentration point. Asterisk indicates significant difference from control, $p < .05$.

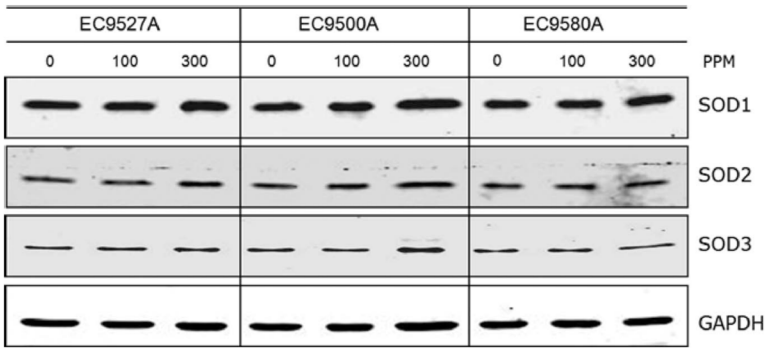

FIGURE 5.

Superoxide dismutase levels following treatment with COREXIT dispersants. Western blot analysis was performed on cell extracts from BEAS-2B cells exposed to 0, 100, or 300 ppm dispersants for 2 h. Antibodies specific to superoxide dismutase proteins SOD1, SOD2, and SOD3 were used, with GAPDH was used as a loading control. Three independent experiments were conducted (a representative blot is shown).