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EXPERIMENTAL

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Diprivan attenuates the cytotoxicity of nitric oxide in cultured human bronchial epithelial cells

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Abstract *Objective:* Excess nitric oxide (NO) and its reactive derivatives cause oxidative reactions that lead to cell death. Propofol, an intravenous anesthetic, exhibits antioxidant properties. Diprivan is a widely used commercial preparation of propofol that is emulsified in 10% intralipids. We sought to test the hypothesis that clinically encountered concentrations of Diprivan attenuate the toxicity of NO in a cell culture model. *Design:* Prospective, randomized, controlled trial.
Setting: University research laboratory. *Subjects:* Cultured human bronchial epithelial (IB-3) cells.
Interventions: Human bronchial epithelial cell cultures were randomly assigned to one of the following six groups: no additives (negative control), NO alone (positive control), NO with either 1 µM, 10 µM or 100 µM Diprivan, and 100 µM Diprivan alone (Diprivan control). S-nitroso-N-acetylpenicillamine (SNAP) was used to generate NO.
Measurements and results: Hemacytometry with trypan blue staining was used to measure cell survival. To assess direct NO toxicity, immunoblot assays for nitrotyrosine-containing proteins in cell homogenates were performed. Exogenous NO significantly decreased live cell numbers and increased intracellular nitrotyrosine-containing protein concentrations ($p<0.001$). Diprivan significantly attenuated these changes in a concentration-independent manner ($p<0.001$). At concentrations as low as 1 µM, Diprivan exhibited cytoprotective effects.
Conclusions: Diprivan effectively attenuates the cytotoxicity of excessive NO exposure in IB-3 cells at concentrations that are clinically attainable.

Keywords Nitric oxide · Propofol · Diprivan · Cultured human bronchial epithelial cells · Nitrotyrosine

Introduction

Nitric oxide (NO) is a bioactive free radical that can react with molecular oxygen to form nitrogen dioxide (NO_2) [1] or with superoxide to form a highly reactive free radical, peroxynitrite [2]. Excess NO and its reactive derivatives cause DNA coding errors [3] and interfere with mitochondrial respiration [4, 5]. In addition, they can disrupt cell membranes through peroxidation of lipids [6] and nitration of tyrosine residues [7]. These disturbances are enhanced by hyperoxic conditions [8] and can lead to cell death [5, 9].

Antioxidants (such as β-carotene, vitamin E and vitamin C) have been shown to prevent cell death caused by NO and its reactive derivatives [10, 11, 12]. Propofol (2,6-diisopropylphenol), a widely used intravenous anesthetic agent, is structurally similar to vitamin E and has been shown to possess similar antioxidant properties [13, 14, 15]. In vitro studies have shown that propofol scavenges NO derivatives such as peroxynitrite [16]. Diprivan, a commercially prepared emulsion of propofol in 10% intralipid, is a widely used sedative and anesthetic. Although both propofol and intralipids are known to exhibit antioxidant properties, the antioxidant properties of propofol have been shown to be substantially greater than those of intralipids [17, 18].

Previous studies have shown, using Diprivan, that peak plasma concentrations of propofol are ordinarily 40–60 μM with the induction of anesthesia and 10–25 μM with sedation and during the maintenance of anesthesia [19]. We therefore decided to test the hypothesis that these concentrations of propofol, administered as Diprivan, increase cell survivorship during exposure to NO. We also sought to determine whether the cytoprotective effects are associated with attenuated cellular damage.

Materials and methods

Cell culture and cytotoxicity assays

Immortalized human bronchial epithelial (IB-3) cells were grown in LHC-8 culture medium (Biosource, Camarillo, Calif.) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin (Life Technologies, Gaithersburg, Md.). The number of passages of the IB-3 cells was between 8 and 12. The cells were grown in 35mm culture dishes and maintained at 37°C in 95% room air/5% CO_2 in a humidified chamber. Cells were seeded at an initial concentration of 5×10^5 cells/ml and left to grow overnight.

The next day, the cell cultures were randomly assigned to one of the following six groups: no additives (negative control), NO alone (positive control), 100 μM Diprivan alone (Diprivan control), NO with either 1 μM, 10 μM or 100 μM Diprivan. A commonly used NO donor, S-nitroso-N-acetylpenicillamine (SNAP) (Sigma-Aldrich, St. Louis, Mo.), was used to generate NO. Cells were treated using 2 mM of SNAP in accordance with those methods published by Narula et al. [8]. They previously showed that adding SNAP to culture media at an initial concentration of 2 mM caused the NO concentrations to peak 2 h later at around 4.4 μM and then gradually decrease over the next 15 h. A commercially prepared emulsion of propofol in 10% intralipids (Diprivan; Astra-Zeneca Pharmaceuticals, Wilmington, Del.) was used for testing. To promote the cytotoxic effects of the NO donor, ensure the availability of propofol and avoid cytotoxicity from metabolic waste products, we refreshed the media and additives daily.

Separate cell cultures were grown for each assay. Each result shown represents the mean obtained from three different cultures. The numbers of live cells were distinguished by the exclusion of trypan blue dye (Life Technologies) and counted with a hemacytometer.

Immunoblot analysis

Concentrations of nitrotyrosine-containing proteins were evaluated by immunoblot assays. Cell cultures were harvested for analysis on day 3. The cells were washed 3 times with phosphate-buffered saline (PBS) (Life Technologies) and lysed in 300 μl of lysis buffer [10 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride, pH 6.8, 10 μg/ml of aprotinin and 1 mM phenylmethylsulfonyl fluoride] per dish. The protein concentration of each sample was measured using a BCA protein assay kit (Pierce Chemical, Rockford, Ill.). An equal volume of 2 times sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) was added to all samples and boiled for 3–5 min.

An equal amount of protein (65 μg) from the cell homogenates was loaded into wells of 7.5% Tris-glycine polyacrylamide gels (Bio-Rad) and separated by electrophoresis at 50 V constant current for 180 min using a Mini-Protean electrophoresis system (Bio-Rad). Lysate from peroxynitrite-treated proteins was used as a positive control (Upstate Biotechnology, Lake Placid, N.Y.). The positive control was provided by a combination of three proteins: bovine superoxide dismutase (SOD, approximately 16 kDa), bovine serum albumin (BSA, approximately 66 kDa), and rabbit mu myosin (approximately 215 kDa) that were nitrated using peroxynitrite (Upstate Biotech). The proteins were then transferred from the gel to nitrocellulose membranes (Bio-Rad) at 100 V constant current for 60 min in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.05% SDS). Staining of the gel with ponceau S after protein transfer was performed to ensure equal and efficient protein transfer.

The nitrocellulose membranes were immediately placed into blocking buffer (5% non-fat dry milk, 10 mM Tris pH 7.5, 100 mM sodium chloride, 0.1% Tween-20) and blocked at room temperature for 60 min. After blocking, the membranes were incubated overnight at 4°C in primary antibody solution (polyclonal anti-nitrotyrosine IgG antibody, 1:1000 dilution, Upstate Biotechnology). Horseradish peroxidase conjugated sheep anti-rabbit IgG antibody (1:1500 dilution in blocking buffer; Amersham Pharmacia Biotech, Piscataway, N.J.) was used as a secondary antibody. Bound antibody was detected by chemiluminescence (ECL plus kit; Amersham). Densitometric techniques were performed to quantify the protein band densities using NIH software (Scion, Frederic, Md.).

Statistical analysis

An analysis of variance (ANOVA) was used to test for an effect of the media additives on the live cell counts. Likewise, an effect of the additives on the immunoblot densities was evaluated by an ANOVA. Multi-group comparisons were made using the Student-Newman-Keuls test. An alpha of 0.05 was considered significant.

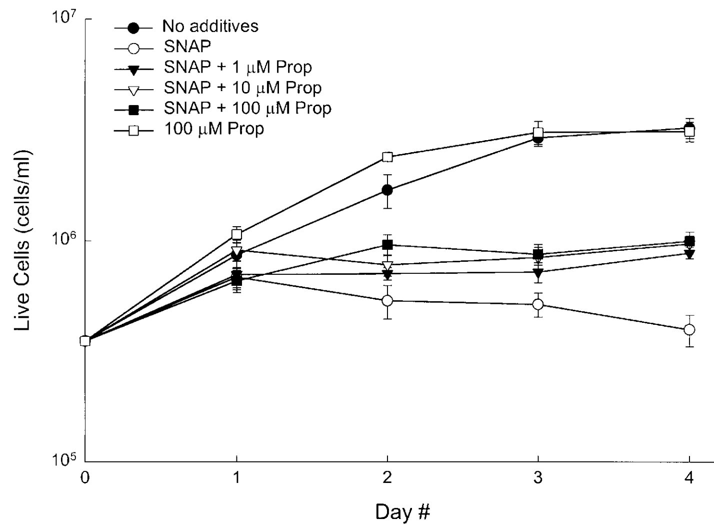
Results

Cytotoxicity assay

The initial live cell numbers in the six groups were similar (Fig. 1). The live cell numbers in both the negative control group and Diprivan control group increased daily. In the negative control group, live cell numbers on day 4 were about ninefold higher than those on day 0. No difference between the negative control group and the Diprivan control group was detected.

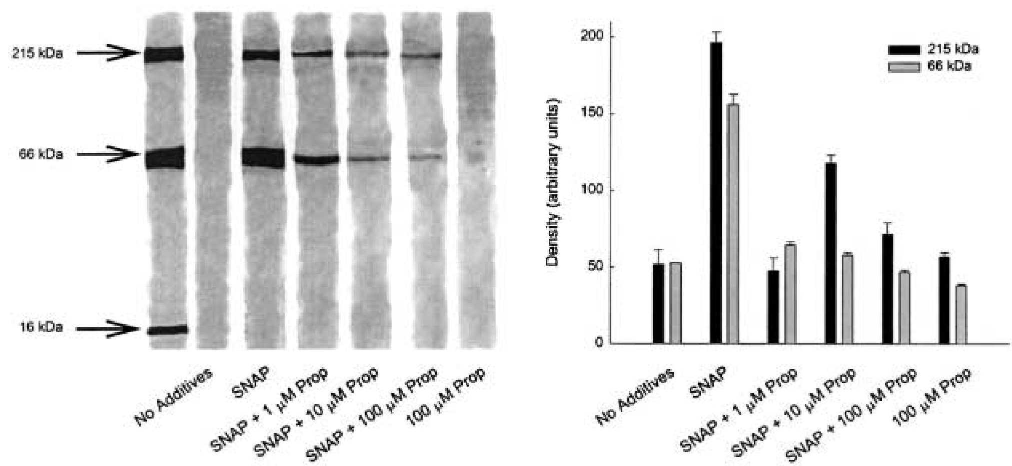
After being exposed to NO for 2 days, the live cell numbers in all NO exposed groups were notably lower than those in the negative control group and the Diprivan control group ($p<0.001$). However, no difference was

Fig. 1 A commercial form of propofol (*Prop*), Diprivan, attenuated the cytotoxicity of exogenous NO from 1 mM SNAP in cultured human bronchial epithelial (IB-3) cells. The effect of propofol was dose-independent within the range of doses tested (1–100 μM) Data are shown as means ± SEM derived from three independent cultures. The table of multiple comparisons between treatment groups shown uses the * symbol to indicate statistical significance ($p<0.05$) and *NA* to indicate that the comparison was not applicable



Cell Counts	No Additives	SNAP	SNAP + 1 μM Prop	SNAP + 10 μM Prop	SNAP + 100 μM Prop	100 μM Prop
No Additives	NA	*	*	*	*	ns
SNAP	*	NA	*	*	*	*
SNAP + 1 μM Prop	*	*	NA	ns	ns	*
SNAP + 10 μM Prop	*	*	ns	NA	ns	*
SNAP + 100 μM Prop	*	*	ns	ns	NA	*
100 μM Prop	ns	*	*	*	*	NA

Fig. 2 An immunoblot for nitrotyrosine-containing proteins performed on samples harvested on day 3 is shown. An illustrative example of the immunoblots is shown, whereas the plot shows a summary of data derived from all immunoblots. Treatment with NO resulted in the appearance of bands at 215 and 66 kDa. All doses of the propofol (*Prop*) decreased the tyrosine nitration. The table of multiple comparisons shown uses the * symbol to indicate statistical significance ($p<0.05$) and *NA* to indicate that the comparison was not applicable



66/215 kDa	No Additives	SNAP	SNAP + 1 μM Prop	SNAP + 10 μM Prop	SNAP + 100 μM Prop	100 μM Prop
No Additives	NA	*/*	*/*	*/*	*/ns	ns/ns
SNAP	*/*	NA	*/*	*/*	*/*	*/*
SNAP + 1 μM Prop	*/*	*/*	NA	*/*	*/*	*/*
SNAP + 10 μM Prop	*/*	*/*	*/*	NA	ns/*	*/*
SNAP + 100 μM Prop	*/ns	*/*	*/*	ns/*	NA	*/ns
100 μM Prop	ns/ns	*/*	*/*	*/*	*/ns	NA

noted among the groups exposed to NO. In these groups, cell death outpaced cell growth by day 3 and this trend became more evident on day 4. Live cell numbers on day 3 in all groups exposed to NO were significantly lower than those in the negative control group and Diprivan control group ($p<0.001$). Though no difference was noted between the three groups exposed to NO and Diprivan, a significant difference was noted between the group exposed to NO only and those groups exposed to NO and Diprivan ($p=0.025$). Similarly, on day 4 live cell numbers in the negative control group were significantly higher than those of the four groups exposed to NO ($p<0.001$). The day 4 live cell numbers in the three groups exposed to both NO and Diprivan were significantly higher than those of the group exposed to NO only ($p<0.001$). However, there was no difference in the live cell numbers in the three groups exposed to NO and Diprivan throughout the experiment. That is, the apparent cytoprotective effect of Diprivan was dose-independent within the range that we tested.

Immunoblotting analysis

To our surprise, we found two bands that closely matched the size of our 215 and 66 kDa nitrotyrosine-containing protein controls (Fig. 2). Although we cannot be certain about the identity of the protein bands in our cultures, it seems possible that they are similar to the controls (albumin and myosin) and probable that they reflect the overall nitration of proteins. Homogenates from cells exposed to NO alone had significantly higher intracellular nitrotyrosine-containing protein concentrations than those in the negative control group ($p<0.001$). We also found that the concentrations of intracellular nitrotyrosine-containing protein in homogenates from those cells exposed to both NO and Diprivan were significantly lower than those from cells exposed to NO alone ($p<0.001$). Concentrations of 10 μM or 100 μM Diprivan resulted in significantly lower nitrotyrosine-containing protein concentrations than did a concentration of 1 μM ($p<0.001$). However, there was no significant difference between the effects at 10 μM and 100 μM.

Discussion

This study confirms our hypothesis that clinically relevant concentrations of Diprivan attenuate the cytotoxicity of NO added to human bronchial epithelial cell cultures. The clinical implications of our study are twofold. First, our data raise concerns regarding whether human bronchial epithelial cells may be damaged by NO inhalation therapy or by overproduction of endogenous NO in situations such as septic shock. Second, our data support the idea that propofol, or other peroxynitrite scavengers, might benefit patients exposed to oxidants such as NO.

We chose to use bronchial epithelial cells in this study because they represent a lung cell type that is likely to be injured by either endogenous or exogenous NO overexposure. Endogenous NO overproduction is common in patients suffering from the inflammatory consequences of sepsis and/or ischemia [20, 21, 22, 23]. Some studies have shown that intrapulmonary NO formation correlates with lung injury due to endotoxemia [24] and ischemia [25]. Exposure to supra-physiologic levels of NO is becoming more common with the increasing popularity of inhaled NO therapy. Numerous reports have raised concerns regarding whether this therapy could result in intrapulmonary oxidative injury [8, 26, 27, 28]. In support of this concern is the finding that inhibition of endogenous overproduction of NO improves outcomes in models of endotoxemia and ischemia [29, 30]. Whether the cytoprotective effect of Diprivan against NO-mediated injury demonstrated here in cell cultures accurately translates to a therapeutic effect of Diprivan in patients remains uncertain. This study represents the first to recognize a possible cytoprotective effect of Diprivan against NO mediated injury.

That Diprivan attenuated the cytotoxicity of NO at concentrations as low as 1 μM suggests that this protective effect of Diprivan may be clinically relevant even at routinely used concentrations. Previous studies have shown that peak plasma concentrations of propofol with the administration of Diprivan reach 40–60 μM with the induction of anesthesia [19]. Using single bolus injections (2.5 mg/kg), Cockshott et al. found that blood concentrations of propofol generally decrease from 30 μM to 7 μM over the first 15 min and then decrease from 7 μM to 0.5 μM over 2–3 h [31]. During a constant infusion of Diprivan, as seen during the maintenance of anesthesia or sedation, a concentration of 10–25 μM can be expected [19]. Gepts et al. found that the half-life of propofol is longer after stopping a continuous infusion than it is after administering a bolus [32]. Thus, any cytoprotective effects of Diprivan may be expected to last for several hours after cessation of its administration.

One limitation of this study relates to the method of NO supplementation. As mentioned earlier, 2 mM SNAP is expected to produce a concentration of NO that peaks around 2 h and then gradually decreases to baseline over the next 15 h [8]. Therefore, the cells in our study were exposed to dynamic pulses of NO rather than constant concentrations. In clinical situations, patient exposure to NO is generally more constant whether the source is exogenous (e.g. in NO inhalation therapy) or endogenous (e.g. in sepsis).

Our findings support those of others, who have shown that excessive NO exposure increases nitration of tyrosine residues [7, 33]. Tyrosine nitration correlates with impaired cardiac [34] and pulmonary [35] functions. Al-

though the exact mechanism by which Diprivan protects against NO-induced toxicity is not well studied, some investigators have shown that NO and NO derivatives may be inactivated by directly nitrating propofol [36, 37]. Whether tyrosine nitration is directly responsible for cell death or merely an indicator of cell injury remains uncertain. Others have also found a strong association between nitration of tyrosine and cell death [8]. Decreases in tyrosine nitration might therefore be useful indicators of propofol's ability to protect against NO-mediated cell injury.

We, therefore, conclude that Diprivan effectively attenuates the cytotoxicity of NO overexposure in cultured human bronchial epithelial (IB-3) cells. Ordinary anesthetic/sedative use of Diprivan probably affords cytoprotection against NO-induced oxidation. The cytoprotective effect of Diprivan may involve inhibition of tyrosine nitration.

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