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Research Article

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Ibuprofen Prevents Oxidant Lung Injury and In Vitro Lipid Peroxidation by Chelating Iron

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Abstract

Because ibuprofen protects from septic lung injury, we studied the effect of ibuprofen in oxidant lung injury from phosgene. Lungs from rabbits exposed to 2,000 ppm-min phosgene were perfused with Krebs-Henseleit buffer at 50 ml/min for 60 min. Phosgene caused no increase in lung generation of cyclooxygenase metabolites and no elevation in pulmonary arterial pressure, but markedly increased transvascular fluid flux (ΔW = 31±5 phosgene vs. 8±1 g unexposed, $P < 0.001$), permeability to albumin (^{125}I-HSA) lung leak index 0.274±0.035 phosgene vs. 0.019±0.001 unexposed, $P < 0.01$; ^{125}I-HSA lavage leak index 0.352±0.073 phosgene vs. 0.008±0.001 unexposed, $P < 0.01$), and lung malondialdehyde (50±7 phosgene vs. 24±0.7 μmol/g dry lung unexposed, $P < 0.01$). Ibuprofen protected lungs from phosgene (ΔW = 10±2 g; lung leak index 0.095±0.013; lavage leak index 0.052±0.013; and malondialdehyde 16±3 μmol/g dry lung, $P < 0.01$). Because iron-treated ibuprofen failed to protect, we studied the effect of ibuprofen in several iron-mediated reactions in vitro. Ibuprofen attenuated generation of · OH by a Fenton reaction and peroxidation of arachidonic acid by FeCl$_3$ and ascorbate. Ibuprofen also formed iron chelates that lack the free coordination site required for iron to be reactive. Thus, ibuprofen may prevent iron-mediated generation of oxidants or iron-mediated lipid peroxidation after phosgene exposure. This suggests a new mechanism for ibuprofen's action. (*J. Clin. Invest.* 1990. 86:1565–1573.) Key words: oxidants • iron-mediated reactions • toxic gases

Introduction

Ibuprofen is one of the most useful nonsteroidal anti-inflammatory agents available to humans. Originally intended as a therapy for arthritis, it is now available as a nonprescription antipyretic and analgesic. Recently, ibuprofen has been shown to ameliorate acute lung injury in animals induced by sepsis (1, 2), the pesticide paraquat (3), burn injury (4), smoke inhalation (5), and thrombin (6). Ibuprofen also attenuates experimental endotoxin shock (7, 8) and is undergoing trial as a treatment for septic shock in humans. Because many of these

entities are in part oxidant-mediated illnesses, we evaluated ibuprofen as a therapy for toxic oxidant gas inhalation. We chose the oxidant phosgene as a model of inhalational injury because it is extremely reactive and commercially important and characteristically produces acute noncardiogenic pulmonary edema as its most serious health effect in human beings (9). We report that ibuprofen markedly attenuates lung injury from phosgene. In this model ibuprofen appears to act as an iron chelator, possibly preventing iron-mediated oxidant generation and lipid peroxidation caused by phosgene.

Methods

Lung perfusion experiments

In initial studies we determined that exposure to 200 ppm phosgene for 10 min (2,000 ppm-min) consistently caused profound lung injury, characterized by increased transvascular fluid flux and wet/dry lung weight ratios (10). This dose of phosgene was used for all experiments. We used transvascular fluid flux during lung perfusion and lung wet/dry weight ratios to assess phosgene lung injury (10–13). Microvascular permeability to macromolecules was studied by measuring the flux of ^{125}I-albumin from the vascular space into lung water and lavage fluid. To assess injury biochemically, we measured perfusate and bronchoalveolar lavage levels of cyclooxygenase mediators to determine whether phosgene activated this pathway of arachidonate metabolism, and lung content of malondialdehyde to determine if phosgene caused peroxidation of lung lipids.

PHOSGENE EXPOSURES

We exposed rabbits to phosgene using a specially constructed facility. Phosgene was prepared by Matheson Gas Products, Inc., New York, and stored in 3-qt. cylinders at a concentration of 200 ppm in ultrapure air. Stability of the mixture was verified over 3 wk and periodically thereafter using the method of Noweir and Pfitzer (14). Cylinder pressure (1,800 psi) was reduced to a delivery pressure of 10 psi with a model B15 regulator coupled to a model 206 rotameter (both from Matheson Gas Products, Inc.) for adjustment of flows from 0 to 25 liter/min. Both regulator and rotameter were fitted with corrosion-resistant valves. The cylinder and valve assembly were enclosed under a single high-velocity (1,200 liters/min) laboratory exhaust hood to contain any accidental leakage of phosgene. Animal exposures were performed in a sealed plexiglass box placed inside a second smaller laboratory exhaust hood. The plexiglass box was connected to the valve and rotameter assembly by seamless ⅓ in. stainless steel tubing. The effluent of the box and exposure hood was exhausted into a scrubber (Mystaire HS-7, Heat Systems-Ultrasonics, Inc., Farmingdale, NY). This scrubber detoxifies phosgene by continuous exposure of effluent to a 0.2% aqueous solution of potassium hydroxide (pH = 9). This detoxification is based on the principle that phosgene reacts rapidly with water to form HCl and CO$_2$. HCl is subsequently neutralized by the caustic in the mist. Investigators wore positive pressure self-contained breathing devices while in the exposure facility (ISI Ranger SCBA Apparatus, Direct Safety Co., Phoenix, AZ) and carried Monotox personal safety alarms (MDA Scientific, Inc., Lincolnshire, IL) which provide audio alert of atmospheric phosgene concentrations greater than the threshold limit value of 0.1 ppm.

Male New Zealand White rabbits (Myrtle Farms, Nashville, TN) weighing 2.5–3.0 kg were maintained on laboratory diet (Carnation Rabbit Formula 18) and water *ad libitum*. On the day of the experiment, a protective water-based ointment was instilled into the rabbits' eyes to prevent conjunctival and corneal irritation. Rabbits were sealed into the plexiglass exposure box and the phosgene mixture was delivered into the box at 5 liter/min. The exposure dose of phosgene was adjusted by increasing the duration of exposure to the fixed (200 ppm) concentration of gas. Exposure dose was expressed as the concentration × time product (ppm-min) (15). At the end of timed exposures, phosgene was flushed from the box for 10 min by compressed air at a flow of 25 liter/min before the animal was removed. Anesthesia was not required, as animals appeared to experience little distress during exposures. Use of rabbits in these experiments was approved by the Animal Welfare and Use Committee.

LUNG PERFUSION TECHNIQUES
Rabbits were anesthetized with pentobarbital sodium (25 mg/kg) and given 3,000 U of heparin by ear vein. The chest was opened, and the animal was killed by rapid exsanguination from the left ventricle. Right and left parasternal incisions were made along the costal cartilages to remove the sternum and open the chest widely. Stainless steel cannulas were secured in the left atrium and pulmonary artery with umbilical tape. The ligature around the pulmonary artery was also passed around the aorta, preventing loss of perfusate into the systemic circulation. The pulmonary circulation was washed free of blood with ~ 500 ml of perfusate before recirculating flow was established at 50 ml/min. The perfusate medium was protein-free Krebs-Henseleit buffer (16) maintained at a temperature of 37–38°C and pH of 7.35–7.40.

The lungs were ventilated with 5% CO_2 in air through a tracheostomy using an animal respirator (Harvard Apparatus Co., Inc., S. Natick, MA) delivering a tidal volume of 7 ml/kg at 18 breaths/min with a 2 cm H_2O positive end-expiratory pressure. Lungs were perfused through a 250-ml circuit which included a perfusate reservoir, roller perfusion pump (Sarns, Ann Arbor, MI), filter (Swank transfusion filter, Extracorporeal Medical Specialities, King of Prussia, PA), and a heat exchanger, connected by Tygon tubing. The reservoir collecting perfusate from the left atrium was suspended from a force displacement transducer (model FT10D, Grass Instrument Co., Quincy, MA) so that loss of reservoir weight from transvascular fluid flux could be continuously measured. The perfusate reservoir was placed below the lower most portion of the lung to keep left atrial pressure at zero.

Pulmonary arterial and left atrial pressures were monitored using pressure transducers (model P23ID, Gould-Statham Instruments, Inc., Hato Rey, Puerto Rico) connected to the inflow circulation, outflow circulation, and tracheal cannula. Pressure and force transducer measurements were recorded on a four-channel recorder (model 2400S, Gould, Inc., Recording Systems Div., Cleveland, OH). At the end of perfusion, lungs were dissected free from the thorax, and one lung was snap-frozen immediately in liquid N_2 and stored at $-70°C$ until later biochemical analysis. The wet/dry weight ratio of the remaining lung was determined by recording wet weight and drying the lung in a vacuum oven at 85°C for 72 h, or until dry lung weight was stable.

EXPERIMENTAL DESIGN
Lungs perfused immediately after exposure. To determine if pretreatment with ibuprofen could attenuate lung injury from phosgene, rabbits were pretreated with ibuprofen, exposed to phosgene, and studied immediately after exposure. Injury was assessed by transvascular fluid flux during lung perfusion. Lung perfusion was generally started 60 min after the beginning of phosgene exposure. Lungs were perfused for 60 min, during which time samples of effluent perfusate were collected at 0, 20, 40, and 60 min and frozen for later measurement of thromboxane B_2 (TXB$_2$), the metabolite of the vasoconstrictor thromboxane A_2, and 6-keto-PGF$_{1\alpha}$, the metabolite of prostacyclin. At the end of lung perfusion, wet/dry weight ratios were determined on one lung. The remaining lung was snap frozen for measurement of malondial-

dehyde. Three groups of rabbits were studied. The first group was sham exposed to air rather than phosgene. The second group was exposed to phosgene, but not pretreated. The third group was pretreated with ibuprofen 25 mg/kg given intravenously 30 min before phosgene exposure. After lung perfusion was begun, treatment was continued by adding 50 μg/ml of ibuprofen to the perfusate. This dose of ibuprofen is similar to that previously reported to attenuate thrombin-induced lung injury (6). In separate experiments, we measured the concentration of TXB$_2$ and 6-keto-PGF$_{1\alpha}$ in the bronchoalveolar fluid from sham-exposed animals ($n = 3$) and phosgene exposed animals ($n = 3$). The lungs of these animals were perfused for 60 min and then bronchoalveolar fluid was obtained.

Lung injury studied 4 h after exposure. To determine if ibuprofen could attenuate lung injury when given after exposure, rabbits were exposed to phosgene, treated immediately afterward with ibuprofen, and then studied 4 h later. In addition, other rabbits were pretreated with the food additive antioxidant butylated hydroxyanisole (BHA)[1] to determine if oxidant injury from phosgene could be ameliorated by antioxidant therapy (17, 18). Injury was assessed by transvascular fluid flux and lung wet/dry weight ratios. This protocol was used to mimic the usual situation occurring after an industrial accident, when phosgene characteristically produces symptomatic lung edema 4–8 h after exposure (9).

Four groups of rabbits were studied: unexposed controls, untreated phosgene controls, rabbits pretreated with BHA before phosgene exposure, and rabbits exposed to phosgene and treated afterward with ibuprofen. Rabbits treated with BHA were injected with 100 mg/kg intraperitoneally for 3 d before phosgene exposure. This dose of BHA is similar to that previously shown to prevent oxidant injury in rabbit lungs (17, 18). Rabbits treated with ibuprofen were injected with 12.5 mg/kg intravenously after phosgene exposure and 12.5 mg/kg intraperitoneally 2 and 4 h later. Ibuprofen was first injected 10 min after rabbits were removed from the exposure chamber to approximate emergency treatment which might begin at the site of a workplace accident. The ibuprofen treatment protocol was similar to that previously used to prevent septic (1, 2) and thermal (4) lung injury. After initial treatment, rabbits were returned to their cages and treatment was continued. Lungs were perfused 4 h later and wet/dry lung weight ratios were determined after perfusion.

MEASUREMENT OF TRANSVASCULAR FLUX OF ^{125}I-ALBUMIN
To assess the effect of phosgene and ibuprofen on albumin flux, we measured transvascular movement of ^{125}I-albumin in the isolated lung. Rabbits were exposed to 2,000 ppm-min phosgene, and their lungs were perfused immediately. After 30 min of perfusion at a left atrial pressure of zero, 1.5 μCi of ^{125}I-human serum albumin (HSA) (8.3 mCi/g albumin, Syncor Corp., Memphis, TN) was added to the reservoir, and left atrial pressure was increased to 10 mm Hg. 10 min later, 1.0 ml of pulmonary venous effluent was obtained, weighed, and counted for 1 min in a γ-counter to determine the number of counts in 1.0 g of circulating perfusate. The reservoir supplying perfusate to the lung was then changed to contain buffer without radioactivity. After lungs were perfused in a nonrecirculating manner for 5 min, 1.0 ml of pulmonary venous effluent was obtained and assessed for radioactivity to confirm that minimal ^{125}I-HSA counts remained in the vascular space. Perfusion was stopped and lungs were lavaged by repeatedly instilling and withdrawing 30 ml of normal saline into the tracheal cannula three times. The lavage fluid was thoroughly mixed and 1.0 ml of lavage fluid was weighed and counted to assess ^{125}I-HSA accumulation in the alveolar space. Lungs were then dissected free from the thorax and a portion of lung from the dorsal surface was weighed and counted to measure ^{125}I-HSA accumulation in lung tissue. Leak in-

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1. *Abbreviations used in this paper:* BHA, butylated hydroxyanisole; HSA, human serum albumin; Ppa, pulmonary arterial pressure; W/D ratio, wet/dry lung weight ratio; ΔW, cumulative lung weight gain from transvascular fluid flux.

dexes were then calculated using the following formulas: lavage leak index = ^{125}I-HSA counts per g of lavage fluid/^{125}I-HSA counts per g of perfusate; lung leak index = ^{125}I-HSA counts per g of lung/^{125}I-HSA counts per g of perfusate.

Five groups were studied: unexposed controls; untreated phosgene controls, phosgene-exposed rabbits pretreated with ibuprofen, phosgene-exposed animals pretreated with ibuprofen that had been iron saturated, and phosgene-exposed animals pretreated with the same amount of iron used to saturate ibuprofen. Ibuprofen was given intravenously (25 mg/kg) 30 min before exposure and 50 μg/ml was added to the perfusate at the start of lung perfusion. Ibuprofen was iron-treated by mixing it in saline with sufficient FeCl$_3$ to provide a 6:1 molar ratio of ibuprofen to iron (25 mg/kg ibuprofen mixed with 5.5 mg/kg of FeCl$_3$). This ratio was chosen because we hypothesized that the propionic acid group of ibuprofen could bind one of iron's six coordination sites (19). Rabbits treated with iron alone were given 5.5 mg/kg of FeCl$_3$ in 2 ml normal saline (pH adjusted to 7.0) by slow intravenous injection before phosgene exposure, and 2.25 mg/kg was added to the recirculating buffer during lung perfusion.

MEASUREMENT OF CYCLOOXYGENASE METABOLITES

TXB$_2$ and 6-keto-PGF$_{1\alpha}$ were measured by radioimmunoassay as described previously (20). The radioimmunoassays used are sensitive to 20 pg/ml and employ dextran-coated charcoal as the separation technique. Cross-reactivity of all rabbit antiserums was evaluated with the following heterologous ligands: PGF$_{2\alpha}$, PGE$_1$, PGE$_2$, 6-keto-PGF$_{1\alpha}$, and thromboxane B$_2$. Antiserum cross-reactivity was < 1% with all heterologous ligands except with 6-keto-PGF$_{1\alpha}$, which has a 4.5% cross-reactivity with PGF$_{2\alpha}$, 2.9% with PGE$_1$, and 2.3% with PGE$_2$ (20).

MEASUREMENT OF LUNG MALONDIALDEHYDE

Malondialdehyde was determined by the method of Buege and Aust (21). Weighed 250-mg portions of defrosted lungs were homogenized on ice with 2 ml of 1.15% KCl and mixed with 4 ml of the reagent (0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl, to which 0.01% butylated hydroxytoluene was added just before use). After incubation at 100°C for 20 min, the absorbance of the supernatant was measured at 532 nm. An extinction coefficient of 1.56 × 10^5 M^{-1} cm^{-1} and lung wet/dry weight ratios were used to determine micromoles of malondialdehyde per gram of dry lung.

In vitro experiments

IRON-MEDIATED · OH GENERATION

The ability of ibuprofen to suppress iron-mediated hydroxyl radical (· OH) generation from hydrogen peroxide (H$_2$O$_2$) was studied using an ascorbate-driven Fenton reaction (22). Sealed glass tubes containing 1 mM FeCl$_3$, 1.5 mM H$_2$O$_2$, 13 mM dimethylsulfoxide (DMSO), and 1 mM ascorbate in 1 ml of Hanks' balanced salt solution (HBSS, without Ca^{2+} or Mg^{2+}, pH 7.0) were incubated for 30 min at 37°C in a shaking water bath. Production of · OH was assessed by generation of methane (CH$_4$) from DMSO (23). The concentration of CH$_4$ in head space gas was measured with a gas chromatograph (Varian Associates, Inc., Palo Alto, CA; 6 ft × 2 mm stainless steel column packed with 60/80 Supelco Carbosieve B; flame ionization detector), using a CH$_4$ standard.

In parallel experiments the ability of ibuprofen to attenuate H$_2$O$_2$ consumption by the Fenton reaction was studied in a system identical to that above, but without DMSO. After incubation for 30 min at 37°C, reaction mixtures were alkalinized to pH 11 with 1 N NaOH and centrifuged at 3,000 g for 30 min to precipitate iron from solution. The concentration of H$_2$O$_2$ in supernatants was measured by the method of Thurman et al. (24) and compared to that of 1.5 mM H$_2$O$_2$ in HBSS also incubated at 37°C for 30 min.

IRON-MEDIATED LIPID PEROXIDATION

The ability of ibuprofen to suppress iron-mediated lipid peroxidation was studied using two methods. First, an iron-ascorbate system was used to oxidize arachidonic acid (25). Sodium arachidonate (160 μM)

was incubated with 150 μM FeCl$_3$ and 1 mM ascorbate in 3 ml of HBSS (pH 7.0) for 30 min at 37°C in a shaking water bath. Peroxidation of arachidonate was followed using the thiobarbituric acid assay of Buege and Aust (21), as described above.

Next, the ability of ibuprofen to inhibit spontaneous lipid peroxidation of brain homogenates was studied using a modification of the methods of Pacht and Davis (26). Lipids of brain homogenates are known to undergo spontaneous autooxidation when exposed to air at 37°C (27). This peroxidation can be inhibited by iron chelators such as deferoxamine or transferrin but not by scavengers of superoxide anion, H$_2$O$_2$, or · OH (26). Guinea pig brain homogenates were prepared as previously described (26, 27) and frozen under N$_2$ at −70°C until use. On the morning of the experiment, stock guinea pig brain homogenate was thawed, 0.5-ml aliquots were placed in borosilicate tubes and aliquots were diluted with 1.5 ml of phosphate buffered saline (PBS, pH 7.4) to a total volume of 2 ml. After vortexing, each tube was split into paired 1-ml samples. One sample was stored under N$_2$ at 4°C. The remaining sample was incubated at 37°C for 90 min. Both were then assayed for thiobarbituric acid reactive products as described above. The difference in absorbance at 532 nm between paired samples incubated at 37°C or stored at 4°C under N$_2$ was used to calculate malondialdehyde formed during the 90-min incubation.

IRON CHELATION STUDIES

Activity of ibuprofen as an iron chelator was studied by determining its ability to present iron in a chelated form that facilitates binding of iron to apotransferrin, thereby increasing the absorbance of transferrin at 465 nm (28). FeCl$_3$ 5 mM was added to ibuprofen 30 mM and incubated for 15 min at 37°C in a shaking water bath. An aliquot was then combined with human apotransferrin in PBS (pH 7.4) with 100 mM NaHCO$_3$. The final mixture containing 0.5 mM FeCl$_3$ was gently agitated for 1 h at 37°C and dialyzed successively against double glass distilled H$_2$O to eliminate NaHCO$_3$ and ibuprofen, and against the buffer to eliminate free iron. Absorbance at 465 nm was measured to determine if iron binding to transferrin had occurred (28). We measured absorbance at 465 nm under the following conditions: FeCl$_3$ 0.5 mM + ibuprofen 3 mM; FeCl$_3$ 0.5 mM + apotransferrin 0.05 mM; apotransferrin 0.05 mM alone, as a negative control; FeCl$_3$ 0.5 mM + the iron chelator trisodium nitrilotriacetate 0.05 mM + apotransferrin 0.05 mM, as a positive control; and FeCl$_3$ 0.5 mM + ibuprofen 3 mM + apotransferrin 0.05 mM.

DETERMINATION OF REACTIVE FREE COORDINATION SITES IN IRON CHELATES

We also determined whether iron chelated with ibuprofen has a free coordination site. A free coordination site is necessary for iron to mediate electron exchanges in free radical reactions. We tested for the presence of a free coordination site by studying the ability of azide to react with such sites and thereby cause a shift in the visible absorption spectrum of the chelate (29). We studied iron-ibuprofen chelates and, as a positive control, chelates of iron with ethylenediamine tetracetic acid (EDTA). FeCl$_3$ 300 μM and EDTA 5 mM or FeCl$_3$ 300 μM and ibuprofen (0.3–3.6 mM) in HBSS (pH 7.0) were scanned at 345–500 nm and 25°C in the presence of 50–1,500 mM NaN$_3$ using a spectrophotometer (model DU-80, Beckman Instruments, Inc., Palo Alto, CA) programmed for a scanning rate of 120 nm/min. Demonstration of an azide-dependent shift in absorption was used to determine whether a chelate should be catalytically reactive.

Reagents and pharmaceuticals

A water-soluble formulation of ibuprofen was provided by Upjohn Pharmaceuticals, Kalamazoo, MI. Dimethylthiourea was purchased from Fike Chemicals, Nitro, WV. Except where indicated, all other reagents were from Sigma Chemical Co., St. Louis, MO.

Statistical analysis

Pulmonary arterial pressure (Ppa), cumulative lung weight gain from transvascular fluid flux (ΔW), and perfusate levels of cyclooxygenase

mediators were analyzed by two-way analysis of variance with randomized split block design (30). Values for ΔW, wet/dry lung weight ratio, lavage and lung leak indexes for ^{125}I-HSA, and lung malondialdehyde were analyzed by one-way analysis of variance (30). Comparisons among groups were performed with Duncan's multiple range test (31). Results are presented as mean±standard error of the mean (mean±SEM). Significance was assumed when $P < 0.05$.

Results

Lung perfusion experiments

Lungs perfused immediately after exposure. Exposure to 2,000 ppm-min phosgene caused profound lung injury which was apparent even in lungs studied immediately after exposure. Compared to unexposed lungs, in which Ppa increased gradually during 60 min of perfusion, Ppa was significantly lower in untreated phosgene exposed lungs (Fig. 1 *A*). Despite lower
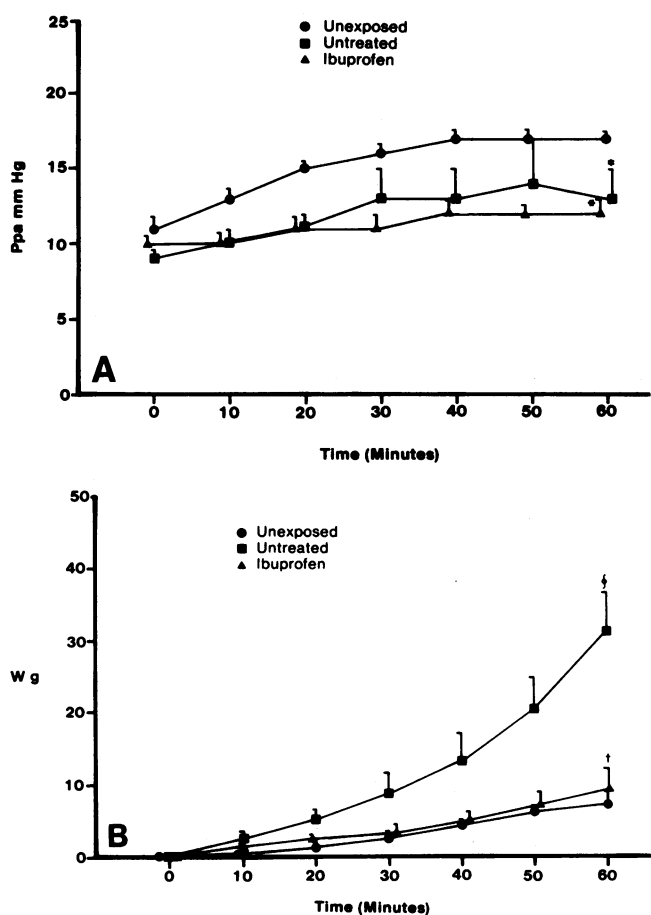
Figure 1. (*A*) Pulmonary arterial pressure in lungs perfused immediately after phosgene exposure. Compared to unexposed control lungs ($n = 5$), untreated lungs exposed to 2,000 ppm-min phosgene ($n = 5$) caused a decrease in Ppa during lung perfusion. Pretreatment with ibuprofen ($n = 5$) had no effect on Ppa compared to untreated phosgene lungs. *$P < 0.01$ compared to unexposed lungs over the course of perfusion. (*B*) ΔW in lungs perfused immediately after exposure. Exposure to 2,000 ppm-min phosgene ($n = 5$) caused a profound increase in ΔW compared to unexposed lungs ($n = 5$). Pretreatment with ibuprofen ($n = 5$) attenuated the increase in transvascular fluid flux from phosgene. †$P < 0.01$ compared to untreated phosgene lungs over the course of perfusion; §$P < 0.001$ compared to unexposed lungs over the course of perfusion.

Ppa, ΔW was markedly elevated by phosgene (Fig. 1 *B*). We did not detect TXB$_2$ in the recirculating lung perfusate in either control or untreated phosgene lungs, and levels of 6-keto-PGF$_{1\alpha}$ were similar in both groups (478±40 pg/ml with phosgene vs. 544±50 in controls at 60 min). Phosgene exposure also did not increase, and may have decreased, the levels of TXB$_2$ and 6-keto-PGF$_{1\alpha}$ in bronchoalveolar fluid (TXB$_2$ 68±10 pg/ml with phosgene [$n = 3$] vs. 151±15 in controls [$n = 3$] and 6-keto-PGF$_{1\alpha}$ 494±18 pg/ml with phosgene vs. 2,816±1,059 in controls). Phosgene also caused substantial peroxidation of lung lipids, as demonstrated by a doubling of lung malondialdehyde (Fig. 2), indicating that phosgene produces oxidant lung injury.

Pretreatment with ibuprofen markedly protected against lung injury after exposure to phosgene. Pulmonary arterial pressure in lungs treated with ibuprofen was not significantly different from the pressure in untreated phosgene-exposed lungs (Fig. 1 *A*). Ibuprofen, however, substantially decreased the elevated transvascular fluid flux seen after phosgene (Fig. 1 *B*). Ibuprofen also significantly blocked formation of malondialdehyde in phosgene-exposed lungs (Fig. 2). This effect could not be explained by in vitro interference with the thiobarbituric acid reaction, since ibuprofen (50 μg/ml) did not change a standard curve run with dilutions of malondialdehyde hemiacetal.

Lungs perfused 4 h after exposure. When rabbits exposed to phosgene were studied 4 h later, injury was so profound that untreated lungs could be perfused for only 10 min before perfusate filled the tracheal cannula. Both ΔW and wet/dry (W/D) lung weight ratio were markedly increased in untreated phosgene-exposed lungs compared to unexposed controls (Fig. 3, *A* and *B*), but Ppa was not different (14±1 mm Hg in controls vs. 15±1 mm Hg with phosgene). To additionally test whether phosgene causes oxidant lung injury, we treated rabbits with the antioxidant BHA. BHA pretreatment prevented the increase in ΔW (0.29±0.12 for BHA vs. 1.20±0.39 g/min for untreated phosgene, $P < 0.05$) and W/D ratio (10.6±1.0 for BHA vs. 14.2±1.4 for untreated phosgene, $P < 0.05$).
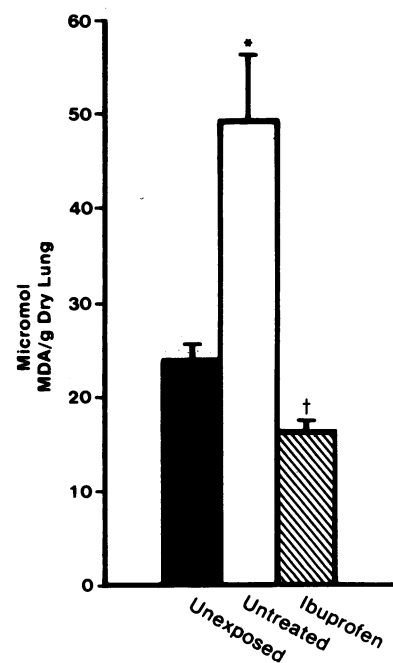
Figure 2. Lung malondialdehyde in lungs perfused immediately after exposure to phosgene. Compared to unexposed lungs ($n = 5$), exposure to 2,000 ppm-min phosgene ($n = 5$) caused a marked increase in lung malondialdehyde. Pretreatment with ibuprofen ($n = 5$) significantly decreased lung malondialdehyde compared to untreated phosgene exposed lungs. *$P < 0.01$ compared to unexposed lungs; †$P < 0.001$ compared to untreated phosgene lungs.

Ibuprofen markedly reduced phosgene lung injury even when given after exposure. Posttreatment with ibuprofen decreased both ΔW and W/D ratio compared to untreated phosgene lungs (Fig. 3, A and B), but Ppa was similar in the two groups (14±1 mm Hg).

Effect of phosgene on transvascular flux of ^{125}I-albumin. There was negligible transvascular flux of ^{125}I albumin in unexposed control lungs, as measured by lavage fluid or lung leak indexes (Fig. 4, A and B). Phosgene exposure markedly increased both lavage and lung leak indexes. Pretreatment with ibuprofen significantly decreased movement of ^{125}I-albumin into lavage fluid and lung water after phosgene (Fig. 4). In contrast, iron-treated ibuprofen failed to prevent the increase in albumin permeability caused by phosgene (Fig. 4). Iron-treated ibuprofen did not cause lung injury in a normal rabbit unexposed to phosgene (lavage leak index 0.017; lung leak index 0.040); nor did iron alone exacerbate phosgene lung injury (Fig. 4). This suggests that the ability to bind iron is important to the mechanism by which ibuprofen protects against the lung injury caused by phosgene.

In vitro experiments

Iron-mediated · OH generation. When combined with an appropriate reducing substance, iron is a potent catalyst for the generation of · OH from H_2O_2 (22). As shown in Fig. 5, 1 mM $FeCl_3$ in the presence of ascorbate catalyzed production of 1,012±12 ppm CH_4 from 1.5 mM H_2O_2. As previously reported (29), increasing the solubility of iron with EDTA enhanced CH_4 production. Addition of 1 mM ibuprofen decreased CH_4 generation by two-thirds, and 6 mM ibuprofen completely blocked CH_4 generation. The reaction was also inhibited by the iron chelator deferoxamine 2 mM (1.4±0.3 ppm CH_4, $n = 5$) and dimethylthiourea 15 mM (45±2 ppm CH_4, $n = 5$).

The concentration of H_2O_2 in reaction mixtures containing 1 mM $FeCl_3$ and ascorbate decreased by over 80% to 0.26±0.04 mM ($n = 4$), compared to 1.3±0.01 mM when H_2O_2 was incubated in buffer alone ($n = 4$). [6 mM ibuprofen attenuated H_2O_2 consumption (0.42±0.02 mM H_2O_2 left, $n = 4$) and 12 mM ibuprofen inhibited it (0.96±0.04 mM, $n = 4$).] These results suggest that at lower concentrations relative to that of iron, ibuprofen acts as a · OH scavenger, but at higher concentrations it inhibits the Fenton reaction and prevents H_2O_2 consumption by rendering iron nonreactive.
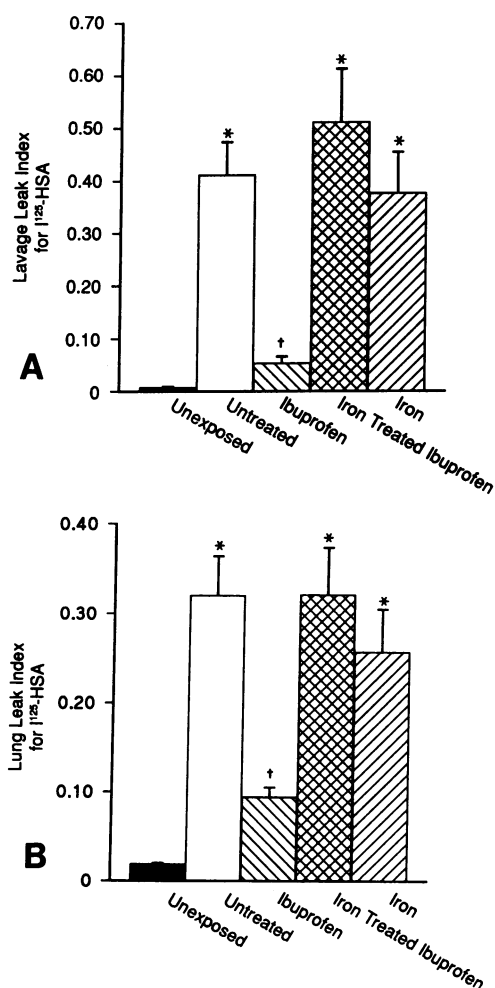
Figure 4. Effect of ibuprofen pretreatment on (A) lavage leak index and (B) lung leak index for ^{125}I-HSA. Lung perfusion was begun immediately after exposure to air or phosgene (2,000 ppm-min). After 30 min of lung perfusion, ^{125}I-HSA was added to the vascular space and lungs were perfused at a left atrial pressure of 10 mm Hg for 10 min. Movement of albumin from the vascular space into lung tissue and the alveolar space was then assessed by counting radioactivity in samples of lung and alveolar lavage fluid ($n = 6$ in untreated phosgene-exposed lungs; $n = 3$ in all other groups). Phosgene markedly increased both lavage and lung leak indices compared to control lungs ($P < 0.01$). Pretreatment with ibuprofen prevented the increase in lavage and lung leak for ^{125}I-albumin caused by phosgene. Iron treatment of ibuprofen eliminated its protective effect. The lavage fluid leak index was expressed as counts per minute of ^{125}I-albumin per gram of lavage fluid, normalized to counts per minute per gram of perfusate. Lung leak index was calculated as counts per minute of ^{125}I-albumin per gram of lung, normalized to counts per minute per gram of perfusate. $*P < 0.01$ compared to unexposed lungs; $\dagger P < 0.01$ compared to untreated phosgene lungs or lungs that received iron-treated ibuprofen.
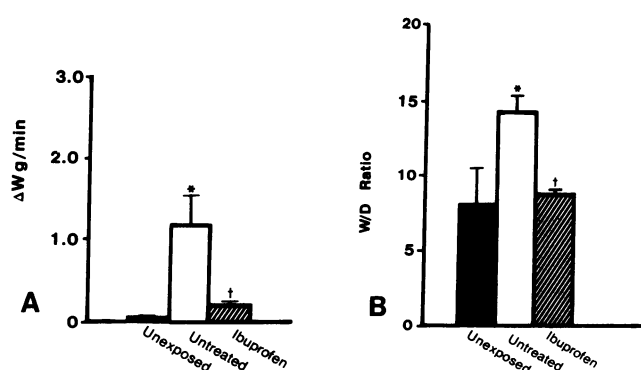

Figure 3. (A) Effect of ibuprofen on lung injury 4 h after exposure to 2,000 ppm-min phosgene. Ibuprofen markedly attenuated the phosgene-induced increase ΔW in lungs treated after exposure and perfused 4 h later. (B) Effect of ibuprofen on lung injury from 2,000 ppm-min phosgene. Ibuprofen markedly attenuated the phosgene-induced increase in W/D ratio of lungs treated after exposure and studied 4 h later. $n = 17$ in unexposed lungs; $n = 10$ in untreated phosgene-exposed lungs; $n = 5$ in ibuprofen treated phosgene-exposed lungs. $*P < 0.05$ compared to unexposed lungs; $\dagger P < 0.05$ compared to untreated phosgene lungs.
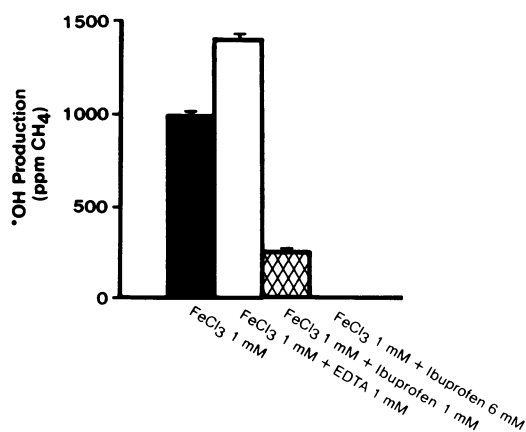
Figure 5. Ibuprofen inhibits ·OH generation by an iron-ascorbate Fenton reaction. Reaction mixtures contained 13 mM DMSO, 1 mM $FeCl_3$, and 1.5 mM H_2O_2 in 1 ml HBSS (pH 7.0). The reaction was initiated by adding 1 mM ascorbate and ·OH was monitored by evolution of CH_4 from DMSO. Generation of ·OH was increased by 1 mM EDTA and markedly inhibited by ibuprofen. Ibuprofen 6 mM completely prevented ·OH generation. Each bar represents a mean of at least five experiments.

Iron-mediated lipid peroxidation. It is well recognized that iron in the presence of a reducing substance will facilitate lipid peroxidation (32). $FeCl_3$ + ascorbate caused substantial peroxidation of arachidonic acid which was inhibited in a concentration-dependent manner by ibuprofen (Fig. 6). Peroxidation in this system was completely prevented by 300 μM deferoxamine (0.07 ± 0.02 μM malondialdehyde, $n = 5$). Incubation of rat brain homogenate at 37°C for 90 min also resulted in lipid peroxidation (61 ± 8 nmol malondialdehyde/

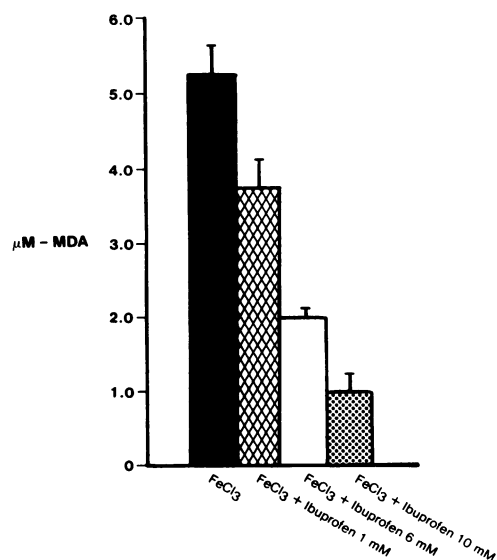*Figure 6.* Ibuprofen attenuates iron-mediated lipid peroxidation. $FeCl_3$ 150 μM in the presence of 1 mM ascorbate caused marked peroxidation of arachidonic acid. Ibuprofen caused a dose-dependent inhibition of lipid peroxidation, measured as concentration of malondialdehyde in the reaction mixture. Each bar represents a mean of at least five experiments.

Table I. Iron-chelating Activity of Ibuprofen*

Reaction mixture	Absorbance at 465 nm
Transferrin	0.010
$FeCl_3$ + transferrin	0.040
$FeCl_3$ + trisodium nitriloacetate + transferrin	0.230
$FeCl_3$ + ibuprofen	0.080
$FeCl_3$ + ibuprofen + transferrin	0.240

* Preparation of reaction mixtures is described in Methods.

ml, $n = 10$). The addition of 440 μM ibuprofen to brain homogenates inhibited malondialdehyde formation by over 80% (12 ± 7 nmol/ml, $n = 10$).

Iron chelation studies. When transferrin binds Fe^{3+}, its absorbance increases at 465 nm (A_{465}) (28). A chelator which enhances the solubility of iron greatly facilitates Fe^{3+} binding to transferrin (28). Table I shows that transferrin alone demonstrated little absorbance at 465 nm, and absorption remained relatively unchanged after the addition of $FeCl_3$. However, when iron was solubilized by the chelator NTA, there was an increase in A_{465}. This was also seen when ibuprofen was added with $FeCl_3$, indicating that ibuprofen chelates iron.

Determination of free coordination sites in iron chelates. For catalytic activity, iron requires at least one coordination site that is open or occupied by a readily dissociable ligand such as water (29). A chelate which contains a free coordination site and thus allows iron reactivity, such as Fe^{3+}-EDTA, readily combines with azide at a free coordination site, causing a spectral shift. In contrast, a chelate with an compound that decreases iron reactivity, such as deferoxamine, leaves no coordination site open and shows no spectral shift when azide is added (29). Sodium azide (NaN_3) caused a concentration-dependent shift in the visible spectrum of Fe^{3+}-EDTA, indicating the presence of a free coordination site at which transitional metal reactivity can occur (data not shown). In contrast, Fe^{3+}-ibuprofen demonstrated a spectral shift with 1 M azide only at low ratios of ibuprofen to iron (Table II). At higher ratios (12:1 ibuprofen to Fe^{3+}) sufficient ibuprofen was present to bind all coordination sites, render iron nonreactive with azide, and prevent an azide-induced increase in absorbance (Table II).

Discussion

Our objectives in this investigation were to establish a simple model of lung injury from inhaled toxic gas and determine if

Table II. Effect of Azide on the Spectral Absorbance of Fe^{3+}-Ibuprofen*

Molar ratio of ibuprofen to Fe^{3+}	Increase in absorbance at 355 nm
1:1	0.107
3:1	0.098
6:1	0.089
12:1	−0.050

* Spectra were obtained from 345 to 500 nm on a reaction mixture with 300 μM Fe^{3+} and 0.3–3.6 mM ibuprofen in HBSS (pH 7.0) before and after the addition of 1 M NaN_3. Maximal shift in absorbance occurred at 355 nm.

the course of injury could be favorably influenced using ibuprofen. In the wake of a civil disaster of the proportions of the recent catastrophe in Bhopal, the need for such research is obvious. If such an accident occurred in the U.S., the number of injured might easily exceed the capacity of nearby medical facilities for handling the critically ill. Any therapy that attenuates lung injury might greatly decrease mortality and diminish the number of injured requiring mechanical ventilation for survival.

To accomplish this goal, we studied phosgene inhalation. Phosgene ($COCl_2$) is a colorless, heavier-than-air, oxidant gas with a lethal exposure dose (LC_{50}) in humans of 500 ppm-min (50 ppm inhaled for 10 min) (9). Phosgene is 10 times more toxic than chlorine (9), and can react oxidatively at amine, hydroxyl, and thiol groups and with unsaturated carbon–carbon bonds to form a wide array of chemical modifications to biological molecules (33).

The phosgene model we have developed closely reproduces the pathological findings and time sequence for development of phosgene lung injury in humans (9). 4 h after exposure to 2,000 ppm-min, rabbit lungs were markedly congested and edematous. Lung malondialdehyde content was nearly doubled by phosgene exposure (Fig. 2), suggesting that oxidant injury from phosgene initiates substantial peroxidation of lung lipids. Unlike oxidants, such as *tert*-butyl hydroperoxide (11–13) and hydrogen peroxide (34), which stimulate lung production of thromboxane and cause pulmonary hypertension when administered into the vascular space, phosgene did not cause thromboxane generation and actually lowered Ppa in perfused lungs compared to unexposed controls. Nevertheless, phosgene caused a marked increase in lung ΔW (Figs. 1 *B* and 4 *A*) and W/D ratios (Fig. 3 *B*). Phosgene also substantially altered the ability of lung microvessels to retain ^{125}I albumin within the vascular compartment. Both lung and lavage fluid leak indexes were markedly increased by phosgene exposure (Fig. 4, *A* and *B*). Taken as a whole, these results indicate that phosgene increases alveolar-capillary permeability, providing an experimental explanation for the clinical occurrence of noncardiogenic pulmonary edema in humans (9).

Our study presents two major findings. First, ibuprofen markedly attenuates lung injury from phosgene. This is best illustrated in Fig. 1 *B*, in which ibuprofen significantly reduced the increase in transvascular fluid flux caused by phosgene, and in Fig. 4, *A* and *B*, in which ibuprofen prevented the phosgene-induced increase in lung and lavage fluid leak indexes for ^{125}I-albumin. As shown in Fig. 3, *A* and *B*, this protective effect was present even when ibuprofen was given after phosgene exposure. Because ibuprofen did not decrease Ppa compared to untreated lungs, the protective effect is unlikely to be due to changes in vascular pressure. Nor can the protective effect of ibuprofen be attributed to inhibition of thromboxane synthesis, since phosgene exposure did not stimulate lung production of cyclooxygenase metabolites.

Second, our results may shed new light on how ibuprofen acts as an anti-inflammatory agent. The mechanism by which ibuprofen prevents oxidant lung injury from phosgene is suggested in Fig. 2, which shows that ibuprofen markedly inhibits lipid peroxidation after phosgene. It is now well recognized that once initiated by membrane attack from a primary oxidant, continued lipid peroxidation requires the presence of iron (32). Without iron or another transitional metal to facili-

tate electron transfer reactions, lipid peroxidation stops spontaneously (32). Because iron chelators markedly inhibit lipid peroxidation (25, 26), we hypothesized that ibuprofen might attenuate phosgene lung injury in part by chelating iron, and reasoned that treatment of ibuprofen with iron might eliminate its protective effect. As shown in Fig. 4, iron-treated ibuprofen is ineffective.

Next, we sought to determine if ibuprofen could attenuate iron-mediated free radical reactions in vitro. Fig. 5 demonstrates that ibuprofen can prevent iron-mediated generation of $\cdot OH$ from H_2O_2. Our measurements of H_2O_2 consumption suggest that at lower concentrations relative to that of iron, ibuprofen probably acts as a $\cdot OH$ scavenger. At higher relative concentrations, ibuprofen may inhibit the Fenton reaction by acting as a chelator which renders iron nonreactive, so that H_2O_2 is not consumed. We also found that ibuprofen can attenuate iron-mediated peroxidation of arachidonic acid (Fig. 6) and spontaneous lipid peroxidation in brain homogenates. To more clearly define whether ibuprofen can chelate iron, we took advantage of the observation that the rate of Fe^{3+} binding to transferrin is greatly enhanced by compounds that chelate Fe^{3+} and thus increase its solubility, and that binding of Fe^{3+} to transferrin causes an increase in its absorbance at 465 nm (28). Table I shows that $FeCl_3$ alone causes only a fraction of the increase in A_{465} that occurs when incubations are performed with transferrin in the presence of the chelators NTA or ibuprofen.

Finally, we sought to determine whether iron chelated with ibuprofen still has free coordination sites which might mediate electron transfer reactions. To test this we determined the effect of azide on the spectra produced by Fe^{3+}-ibuprofen chelates. If a reactive coordination site exists in a chelate, azide will shift the spectra. If a reactive site does not exist, azide will not shift the spectra. Fe^{3+}-EDTA chelates have a free coordination site and azide causes a progressive change in the visible spectrum of the chelate (data not shown). Fe^{3+}-ibuprofen, however, undergoes a spectral shift with azide only at lower ratios of ibuprofen to iron (Table II). When enough ibuprofen is present to occupy all available coordination sites, iron is rendered nonreactive and Fe^{3+}-ibuprofen chelates demonstrate no increase in absorbance with azide (Table II).

To our knowledge, this report presents the first evidence that ibuprofen may protect against injury in an intact biological system by interfering with iron-mediated reactions. The ability of ibuprofen to inhibit iron-mediated oxidation of arachidonic acid was first reported in 1979 by Peterson et al. (35). These investigators suggested that ibuprofen inhibited in vitro lipid peroxidation by complexing with iron, and that formation of complexes with iron might underlie the activity of ibuprofen as a cyclooxygenase inhibitor. Later, Hiller et al. (36) reported that ibuprofen prevents $\cdot OH$-mediated inactivation of a bacteriophage. More recently, Aruoma and Halliwell (37) showed that ibuprofen can scavenge $\cdot OH$, and suggested that it can also chelate iron (37). We speculate that the propionic acid group of ibuprofen chelates iron, whereas the isobutylbenzene portion of the molecule scavenges $\cdot OH$. Chemical hydroxylation by $\cdot OH$ could account for formation of 2-(4-[β-hydroxyisobutyl]phenyl)-propionic acid as a major urinary metabolite of ibuprofen (38). Our studies of H_2O_2 consumption by the Fenton reaction and azide-induced spectral shift of Fe^{3+}-ibuprofen chelates suggest that at high con-

centrations relative to iron, ibuprofen may render iron nonreactive by binding all reactive sites. This would not only block generation of $\cdot$OH by a Fenton reaction, but would also inhibit lipid peroxidation, even if peroxidation of lipids was initiated by oxidants other than $\cdot$OH. At lower concentrations of ibuprofen to iron, reactive sites on iron may still exist, but iron-bound ibuprofen could function as a "site-directed" $\cdot$OH scavenger which would be present at the site of iron-mediated $\cdot$OH generation. This may allow ibuprofen to react with $\cdot$OH before it can attack nearby biological molecules. Thus, iron chelation may play a central role in ibuprofen's mechanism of action. Indeed, replacement of the carboxylic acid group on ibuprofen by an ester, alcohol, amide, or tetrazole substantially reduces the compound's anti-inflammatory activity (38).

Iron-chelating compounds have been previously demonstrated to have anti-inflammatory activity. Deferoxamine suppresses both acute and chronic experimental arthritis (39) and attenuates carrageenan-induced pleurisy (40). The iron chelator 2,3-dihydroxybenzoic acid (41) and iron-binding protein apolactoferrin (42) prevent complement- and neutrophil-mediated acute lung injury in rats, and deferoxamine protects cultured pulmonary endothelium from the cytotoxicity caused by phorbol-activated neutrophils (43). These studies suggest that iron chelating or site-directed $\cdot$OH scavenging activity may contribute to ibuprofen's anti-inflammatory activity.

Ibuprofen has other effects which could partially explain its activity. Ibuprofen blocks neutrophil adherence in vitro (6, 44) and neutrophil aggregation and degranulation both in vitro and in vivo (45). Ibuprofen has also been shown to attenuate neutrophil migration in vitro (6) and prevent migration of leukocytes into ischemic reperfused myocardium (46). Whether these effects can be explained by iron chelation or site-directed scavenging of $\cdot$OH is open to speculation. We did not see prominent influx of neutrophils into rabbit lungs immediately after phosgene. Currie et al. (47), however, have observed neutrophil influx into rat lungs 24 h after exposure to phosgene, and it is therefore conceivable that ibuprofen may also protect from phosgene in part by preventing leukocyte influx into lungs or by scavenging leukocyte-derived $\cdot$OH.

Our results have several important implications. First, should studies with other toxic gases confirm our findings in phosgene-exposed rabbits, ibuprofen might become a life-saving therapy for victims of toxic gas inhalation. Second, a number of studies demonstrate that the protective effects of ibuprofen on lung injury cannot be explained by cyclooxygenase inhibition (6, 48, 49). Our results suggest that in addition to inhibiting cyclooxygenase, ibuprofen may attenuate lung injury by preventing iron-mediated generation of oxidants and lipid peroxidation. Finally, our findings may shed light on the mechanism by which ibuprofen protects from septic lung injury. Brigham et al. (50) have recently shown that reactive oxygen species mediate the direct toxicity of endotoxin for cultured pulmonary artery endothelium. Also, a major mediator of the effects of endotoxin is tumor necrosis factor (51, 52), which has recently been shown to dramatically increase $\cdot$OH production within cells (53). Because ibuprofen prevents the hypotension and organ injury caused by tumor necrosis factor infusion (54), ibuprofen might protect against septic lung injury in part by scavenging or inhibiting the iron-dependent generation of reactive oxygen species produced in response to endotoxin or cytokines.

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