Immediate and Short-Term Cellular and Biochemical Responses to Pulmonary Single-Dose Studies of Insulin and H-MAP

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Purpose. It was previously reported that co-administration of H-MAP to the airways of the lungs significantly influenced the absorption, disposition, and effect of insulin in a dose-dependent fashion. Doses of H-MAP (16 mg/kg) and insulin (1.3 U/kg) required to achieve maximum pharmacokinetic and pharmacodynamic responses were determined. The purpose of the present study was to evaluate the effects of insulin and H-MAP spray-instilled (SI) to rats on the physiology of the lung. A short-term, single-dose study of insulin alone and combined with H-MAP was performed.

Methods. Solutions of either insulin (INS), H-MAP, or insulin plus H-MAP (INMA) were SI to the lungs of rats. Lipopolysaccharide solution (LPS) and sodium dodecyl sulfate solution (SDS) were used as positive controls, and normal saline (SAL) was used as negative control. Animals were sacrificed at various time points and bronchoalveolar lavage (BAL) was conducted. BAL fluid was analyzed for local markers of lung injury, such as total cell numbers, differential cell count, total protein content and enzyme activities of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and N-acetyl glucosaminidase (NAG).

Results. SI of any solution, including normal saline, seems to have a minor but detectable effect on the normal physiology of the lung. SI of positive control solutions resulted in most markers of immunity and lung injury being significantly elevated, notably enzyme activity and white cell infiltrate. In contrast, SI of INS produced a response similar to that of SAL. SI of INMA resulted in a small transient response characterized by a slight increase in the proportion of neutrophils at 24 h, which decreased with time and was comparable to that of SAL at 72 h. SI of H-MAP resulted in a response similar to that from INMA; however an additional transient increase in LDH activity was noted which may be related to the mechanism of action of H-MAP.

Conclusion. SI of INS, H-MAP, and INMA caused no apparent toxicity at the doses studied. A small, transient effect of H-MAP was noted which did not elicit the full complement of inflammatory markers and which was substantially smaller than the effect of either LPS or SDS.

KEY WORDS: insulin; H-MAP; pulmonary delivery; bronchoalveolar lavage (BAL); markers of lung injury.

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INTRODUCTION

More than 50 million people around the world suffer from Diabetes Mellitus and require daily insulin injections to control their blood glucose levels, avoiding related complications and thereby leading a normal life (World Health Organization, 1991 May–June report). Most patients need to self-administer at least two subcutaneous (SC) injections per day while three or four injections provide better control (1). Non-invasive routes of administration would be more desirable for insulin therapy, which would avoid the pain, inconvenience, and side effects at the injection site associated with the chronic SC administration of insulin (2). The large absorptive surface area of the lung, thin alveolar epithelial barrier, and extensive vasculature makes the pulmonary route the most promising non-invasive option. Several studies in animals and humans have shown that insulin is well absorbed by the lungs (3–6). Inhaled insulin in humans is more rapidly absorbed than it is from the SC injection site and shows less variability in intra-subject glucose response. However, the bioavailability of inhaled insulin is relatively low (10–57%) compared to that of SC injection (6–9). Co-administration of permeation enhancers (10–12) and/or proteolytic enzyme inhibitors (12–14) has effectively increased the bioavailability of insulin; however, associated side effects present limitations to this approach.

As an alternative option to improve the bioavailability of pulmonary insulin, we have recently reported the use of a novel compound, hydroxymethyl amino propionic acid (H-MAP) as facilitator of insulin transport in the rat lung (15). The mechanism of action of H-MAP cannot readily be explained as general permeation enhancement or protease inhibition. Reversible destabilization of the protein in the native state favoring a partially unfolded conformation, facilitating absorption through membranes into the bloodstream, is a possible explanation (16). However, formal studies to demonstrate the mechanism of action of H-MAP have yet to be conducted. Five increasing doses of insulin alone or combined with four increasing doses of H-MAP were administered intratracheally into fasted anesthetized rats using a micro spray-instillator. These studies demonstrated that the concomitant administration of H-MAP with insulin significantly influenced the pharmacokinetic and pharmacodynamic parameters of insulin in a dose-dependent fashion. Specifically, it was determined that the combination of a dose of H-MAP (16 mg/kg) and insulin (1.3 U/kg) produced the highest pharmacokinetic and pharmacodynamic responses (15).

After establishing the appropriate doses of H-MAP and insulin delivered to the lungs, it is important to determine the effects that insulin and H-MAP would have on the normal physiology of the lungs. Therefore, the goal of this study was to evaluate the effects that insulin alone and in the presence of H-MAP, administered at the doses determined in our previous study, may have on the lungs. Short-term, single-dose studies were conducted as a follow-up to the pharmacokinetic studies. These studies are intended as preliminary experiments to evaluate improving the bioavailability of insulin delivered to the lung. This will potentially result in lower doses to the lungs to achieve the required therapeutic effect.

Bronchoalveolar lavage (BAL) was used as a tool to accomplish the goal of this study. BAL is the sampling of the

lining fluid and cells of the lower respiratory tracts by instillation of sterile saline and subsequent aspiration of fluid, recovering cells and other soluble substances along with diluted lining fluid from the epithelial surface of the lung (17). BAL methods have been used to characterize inflammatory and immune responses of the lower respiratory tract in different forms of acute pulmonary diseases (18). The evaluation of soluble and cellular components in BAL will give preliminary insights into the effects of pulmonary administration of H-MAP and insulin solutions on the physiology of the lung when compared to appropriate controls. It should be noted that the present studies are intended to evaluate only the short-term effects following pulmonary administration of insulin and H-MAP solutions. Formal acute and chronic inhalation toxicity studies following drug administration by the pulmonary route will ultimately be necessary.

MATERIALS AND METHODS

Materials

Porcine Zinc Insulin and H-MAP were obtained from Emisphere Technologies, Inc. (Tarrytown, NY). Lipopolysaccharide (from *E. coli* serotype 055:B5) and sodium dodecyl sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). The solution for BAL, 0.9% sodium chloride injection USP was purchased from Baxter Healthcare Corp. (Deerfield, IL).

Animals

Female Sprague-Dawley rats (Hilltop Laboratory Animals Inc., Scottdale, PA) weighing 225–250 g were housed in a 12 h light/12 h dark cycle and constant temperature environment of 22 °C. A standard diet (Prolab RMH 3000, PMI Nutrition International, Inc., Brentwood, MO) and water were supplied *ad libitum,* but animals were fasted 16 h before treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, an AALAC approved facility.

Treatments

Animals were anesthetized with an i.p. injection of ketamine:xylazine (50:3.4 mg/kg). The endotracheal tube of a small animal spray-instillator (Penn Century Inc., Philadelphia, PA) was placed at the level of the tracheal bifurcation to the main bronchii using a fiber optic laryngoscope (Dolan-Jenner Industries Inc., Lawrence, MA). Solutions (100 μL) of either insulin alone (INS, 1.3 U/kg), H-MAP alone (HMAP, 16 mg/kg), or insulin (1.3 U/kg) plus H-MAP (16 mg/kg) (INMA) were spray-instilled (SI) into the lower airways of the rats using a Hamilton syringe attached to the endotracheal tube of the spray-instillator. After administration, the tube was removed and respiration of animals was monitored visually until they recovered from anesthesia. In addition to the test solutions, positive and negative control solutions were also spray-instilled to the rats. An immunostimulant, lipopolysaccharide solution (LPS, 0.1 μg/ml), and an irritant, sodium dodecyl sulphate solution (SDS 0.1%), were used as positive controls. Normal saline solution was used as negative control. All solutions were prepared just prior to administration into animals. Animals were sacrificed at various time points (24, 48, and 72 h) after SI of test solutions and bronchoalveolar lavage (BAL) was conducted. An additional control for the basal values of all parameters was obtained by lavaging the lungs of rats without any treatment. The experimental layout is shown in Table I.

Broncho Alveolar Lavage (BAL)

Rats were anesthetized with a mixture of ketamine/xylazine (50:3.4 mg/kg) and then exsanguinated by severing the abdominal aorta. Lungs were resected and weighed. Wet lung weight was obtained by correcting the weight of the lung to the body weight of each animal and expressed as wet lung weight per 100 g of body weight.

BAL was performed as follows. Lungs were first lavaged with two separate 3-ml aliquots of normal saline solution, left in the lungs for 30 sec, withdrawn, re-instilled for an additional 30 sec, and finally withdrawn. After centrifugation (500 *g* for 10 min) the resultant cell-free supernatant was analyzed for enzyme activity. The lungs were then lavaged 5 more times with 4-ml aliquots each of saline solution. These samples were also centrifuged (GR-15S centrifuge, Beckman, Palo Alto, CA) for 10 min at 500 *g* and the supernatant separated. The cell pellets from all 7 lavages for each rat were combined, and resuspended in 1 ml of saline.

Sample Analysis

Total cell numbers were determined by analyzing the cell pellet collected from all 7 lavages using a Neubauer hemacytometer. The differential count of white blood cells was performed as follows. Slides were prepared using a Cytospin Shandon Cytocentrifuge 2 (Shandon, Scientific Unlimited, Cheshire, UK) Cells, at least 100 per rat, were evaluated after automated staining of the slides (Hematology Slide Master, Intelligent Medical Imaging, Inc., Palm Beach Gardens, FL) with Wright-Giemsa Stain (Fisher Scientific, Pittsburgh, PA).

Table I. Summary of Experimental Conditions

Treatment	Dose	Time points (hours)	Number of rats per time point	Number of rats per treatment
Insulin	1.3 U/kg	24, 48, 72	5, 5, 5	15
H-MAP	16 mg/kg	24, 48, 72	7, 5, 5	17
Insulin + H-MAP	1.3 U/kg + 16 mg/kg	24, 48, 72	6, 5, 5	16
LPS	0.1 μg/ml	24, 48, 72	5, 5, 5	15
SDS	0.1%	24, 48, 72	7, 5, 5	17
Saline	—	24, 48, 72	5, 6, 5	16
Untreated controls	—	—	5	5

Enzyme activities for lactate dehydrogenase (LDH), alkaline phosphatase (ALP), N-acetyl glucosaminidase (NAG) and total protein content were determined in the cell-free supernatant collected from the two first lavages with assays modified for use on the Cobas Fara II Autoanalyzer (Hoffman-LaRoche, Branchburg, NJ). Protein concentration in the BAL fluid was determined using Pierce-Coomasie Plus Protein Assay Reagent (Pierce, Rockford, IL). Sample protein concentration was determined from a standard curve using BSA as a standard (Sigma Chemical Co., St. Louis MO). Activities of LDH and ALP were assayed using commercially available kits and normal controls purchased from Sigma Chemical Co. (St. Louis MO); NAG activity was assayed using a commercially prepared kit purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

Histology

After BAL, lungs of animals were fixed in 10% neutral buffered formalin. Preserved lungs were then embedded in paraffin, sectioned at 5 μm and stained with hematoxylin-eosin for microscopic evaluation.

Lung Function Test

Animals were anesthetized with 0.875 mL/kg of a 0.75: 0.05:0.075 Ketamine (100 mg/mL): Xylazine (100 mg/mL): Acepromazine (10 mg/mL) mixture administered IM. Depth of anesthesia was assessed by loss of corneal reflex and lack of response to interdigital pinch. Animals were placed on a heated pad, the body temperature was measured by a rectal probe, and maintained at 37°C (Homeothermic blanket control unit, Harvard Apparatus Ltd., Edenbridge, KY). The rats were tracheostomized, intubated and pressure ventilated as described below.

The experimental layout followed the commonly used Konzett Roessler arrangement (19). Rats were anesthetized, tracheostomized and intubated to allow positive pressure ventilation of 1 ml air/breath at 65 breaths/min (Harvard Rodent Ventilator, Model 683, Harvard Apparatus, Southnatick, MA). A bronchospasm transducer (model 7020, Ugo Basile, Biological Respiratory Apparatus, Varesse, Italy) (20) was connected to a side arm, and employed to measure pulmonary inflation pressure as an indicator of airway resistance related to the delivery of the test solutions. All previously described solutions, INS, H-MAP, INMA, LPS, and SDS, were administered by intratracheal SI after a stable baseline pulmonary pressure was observed (20–30 min). Before dose administration, the ventilation tube was temporarily detached and the delivery tube of the SI was inserted into the intubation tube. The delivery tube was removed and the ventilation tube reconnected following SI. The entire dose process took less than 30 sec. Data collection and analog to digital conversion was performed by a data acquisition module (DI-170, 4 channel serial i/o data acquisition module, Dataq Instruments, Akron, OH). Changes in pulmonary inflation pressure measured by the bronchospasm transducer were monitored by a data acquisition software package (Windaq data acquisition for Windows, Dataq Instruments, Akron OH). The animals were positively pressure ventilated and maintained under anesthesia for the entire duration of the study (approximately 2 h) to evaluate the effect of anesthesia and experimental conditions on pulmonary inflation pressure over time. This reduced the duration of the study and limited the discomfort to the animal. No previous treatment was applied to the animals in such way that each animal served as its own control.

Statistical Analysis

Data was analyzed with ANOVA and General Linear Model Procedure (The SAS system, SAS Institute, Cary NC). Differences between treatments and times were determined by Duncan's Multiple Range comparisons test. A probability level of < 0.05 was considered to be statistically significant. Duncan's test was chosen among other multiple comparison tests because it is statistically more powerful and it makes "pairwise" comparisons among sample means of all treatments, rather than group comparisons (21).

RESULTS

Wet Lung Weight

Figure 1 shows the corrected wet lung weights at 24, 48, and 72 h after intratracheal spray-instillation of the test solutions. The effects of time on wet lung weight were not significantly different between all the groups, but the effects of the treatments showed statistically significant differences ($p < 0.05$). Spray-instillation (SI) with LPS produced the highest wet lung weight, which was significantly higher (almost 2-fold) than that after treatment with any other solution. There was no significant difference between the wet lung weights after spray-instillation of any other solution ($p > 0.05$).

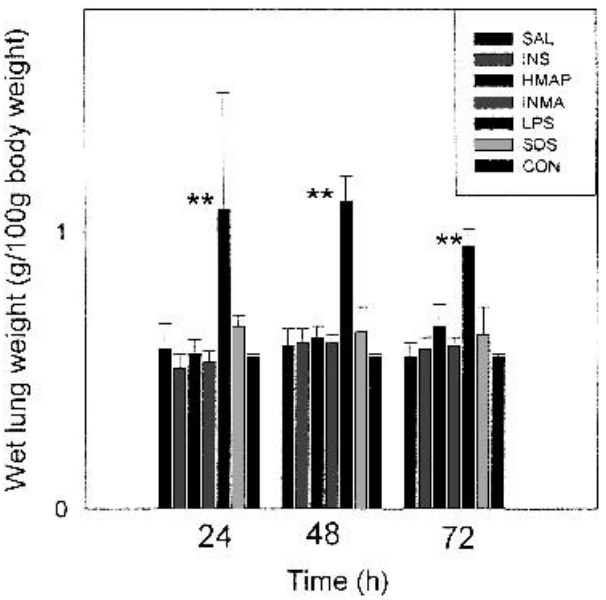

Fig. 1. Corrected wet lung weights 24, 48, and 72 h after a single intratracheal spray-instillation of the different test solutions. Normal saline (SAL), insulin (INS), HMAP, insulin plus HMAP (INMA), lipopolysaccharide (LPS), sodium dodecyl sulphate (SDS) and control (CON).

Cellular Constituents in BAL

The number of white cells found in the bronchoalveolar lavage fluids from the lungs of rats at the different times after SI of test solutions is shown in figure 2. The number of cells was not significantly influenced by time. SI of SAL caused a significant increase in the number of white cells in BAL fluid compared to untreated controls. SI of LPS resulted in the highest white cell infiltrate at all times among all the groups. SI of SDS also resulted in a white cell infiltrate significantly higher than SAL and untreated controls. SI of HMAP resulted in a slightly elevated white cell infiltrate compared to untreated controls, however, it was not statistically different than that of SAL and considerably smaller than that of LPS.

Figure 3A shows the number of neutrophils as a percentage of the total number of cells in the BAL fluids from the lung after SI of different test solutions. The effects of treatment and time on neutrophils in BAL were significantly different ($p < 0.05$) among groups. The percentage of neutrophils in the BAL after SI of test solutions was highest at 24 h and decreased with time for all treatments. SI of LPS resulted in the highest neutrophil infiltrate to the lungs of rats. SI of INMA, SDS or HMAP solutions resulted in a significantly greater proportion of neutrophils compared to SAL or INS solutions or untreated controls. However, the percentage of neutrophils in BAL after SI of INMA was four tenths of that resulting from SI of LPS and SI of H-MAP led to one-third of that resulting from SI of LPS.

Figure 3B shows the number of lymphocytes as a percentage of total cell number in the BALs after SI of different test solutions. Time following administration did not influence the percentage of lymphocytes among the treatments. SI of LPS and SDS resulted in the highest lymphocyte infiltrate. No significant difference was observed after SI of any other solution and the untreated control group. Figure 3C shows the number of monocytes as a proportion of total number of cells present in the BAL after SI of the different test solutions. No significant difference was observed in time or treatment effects on the percentage of monocytes after SI of any of the test solutions, although there was a trend to a high value at 72 h after administration of SDS.

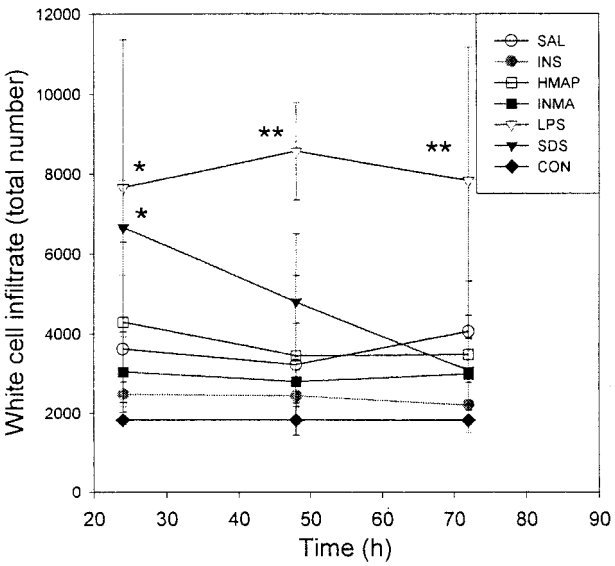

Fig. 2. Total nucleated cells present in the BALs from the lungs of rats 24, 48, and 72 h after a single intratracheal spray-instillation of the different test solutions. Normal saline (SAL), insulin (INS), HMAP, insulin plus HMAP (INMA), lipopolysaccharide (LPS), sodium dodecyl sulphate (SDS) and control (CON).

Differential cell counts are reported as a percentage of the total number of cells present. Consequently, fluctuations in the number of a particular cell type will impact on the proportion of all others. The proportion of macrophages present in the BAL 24, 48, and 72 h after SI of different test solutions is depicted in figure 3D. The effects of treatment and time on the percentage of macrophages in the BAL were significantly different. Untreated animals exhibited a large proportion of macrophages and small proportion of neutrophils. Animals treated with LPS, INMA, and INS exhibited an elevated proportion of neutrophils at 24 h; consequently, the proportion of macrophages was smaller than that of untreated controls. The proportion of macrophages for the treatment group increased at 48 and 72 h following treatment. SI of LPS produced the greatest inflammatory cell proliferation, indicated by the large proportion of neutrophils, which is reflected in a smaller proportion of macrophages and was significantly different from those obtained with other treatments. SI of SDS, HMAP, and INMA resulted in a significantly smaller number of macrophages as compared to SAL, INS, and untreated controls. However, this proportion was 2.7 times higher for HMAP and INMA than that after SI of LPS.

In addition, there was a difference in the macrophage population among treatments and times. After 24 h from SI of SDS solution, BALs in that group showed several macrophages with inclusions, vacuolation and in some cases degenerating cells. None of these were observed either at 48 or 72 h. Vacuolation was also observed within the macrophage population in most BALs after 24 h SI of LPS. These vacuoles were observed only in the macrophages from the lungs of few animals after 72 h. In addition, some macrophages with inclusions were observed in some BALs after 24 h SI of HMAP solution, and they were not observed after 48 or 72 h. Some macrophages with inclusions were also observed in some BALs after 48 h SI of INMA; however, they were observed only in a few BALs after 72 h and they were not observed 24 h after SI.

Enzyme and Protein Constituents in BAL

Figure 4A shows total protein concentration in the BAL after SI of different test solutions. The effects of treatment and time after treatment were significantly different on total protein concentration in BAL. Overall, SDS resulted in the highest total protein concentration in BAL, standing alone from the rest of the treatments with the highest effect observed 24 h post-SI. Although there was a trend to elevated total protein concentration after 24 h of SI of HMAP compared to untreated controls, this was not significantly different from those after SI of INMA, SAL, and INS at any time. A significantly elevated total protein concentration was also observed after 72 h LPS solution.

The LDH activity in the BAL fluid of rats after SI of different test solutions is shown in figure 4B. The effects of treatment ($p < 0.05$) and time ($p = 0.05$) on LDH activity were significantly different. As previously described for other parameters, LPS resulted in the most significantly elevated LDH activity among all treatments. LDH activity after SI of

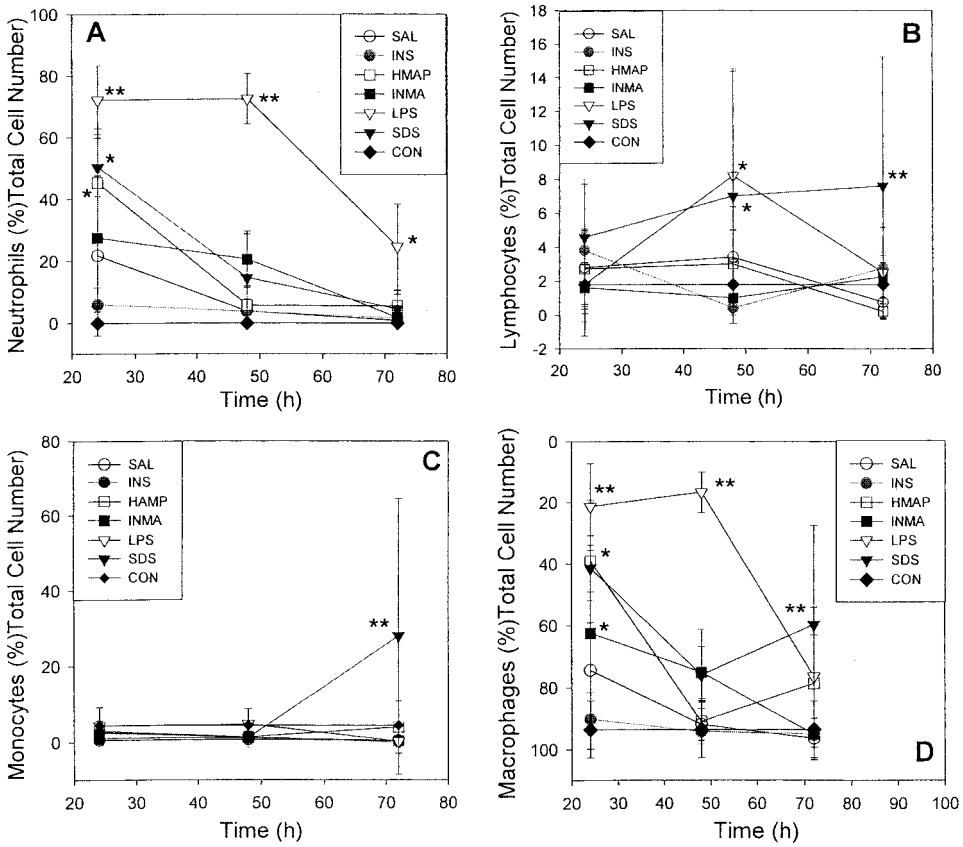

Fig. 3. Percentage of neutrophils (A), lymphocytes (B), monocytes (C) and macrophages (D) present in the BALs from the lungs of rats 24, 48, and 72 h after a single intratracheal spray-instillation of the different test solutions. Normal saline (SAL), insulin (INS), HMAP, insulin plus HMAP (INMA), lipopolysaccharide (LPS), sodium dodecyl sulphate (SDS), and control (CON).

SDS and HMAP solutions was significantly elevated compared to that after INMA, SAL, or INS solutions or untreated controls. However, the activity elicited after SI of HMAP was half of that by LPS. It should be noted that LDH activity resulting from SI of HMAP was elevated at 24 h, but decreased gradually with time.

The ALP activity in the BAL after SI of different test solutions is shown in figure 4C. The effects of time on ALP activity were not significantly different among treatments. LPS resulted in the most significantly elevated ALP activity among all treatments. SI of SDS also resulted in a higher ALP activity as compared to that resulting from any other solution. There were no significant differences in ALP activity after SI of any of test solutions and they were comparable to that of SAL and untreated control groups. Figure 4D shows the NAG activity in BALs after SI of different test solutions. Time had no significant effect on the NAG activity among treatments. The highest NAG activity compared to untreated controls was observed after SI of LPS, HMAP, and SAL solutions and no significant difference was observed among those treatments. NAG activity was also similar for SDS, INS, INMA, and control groups. Although the NAG activity elicited by HMAP was higher at some time points, it was not significantly different from that of SAL.

Table II summarizes the findings of statistical analyses of the data for each measure of effect found in BAL fluids compared with negative control groups. In addition, to further elucidate the nature of the enzymatic response to pulmonary SI of different test solutions, enzyme activities of LDH, ALP, and NAG were measured in the BAL and plasma of the same animals. Comparable ratios of activity in the plasma vs. BAL fluid were obtained for all enzymes for treated and negative control rats. This indicates that SI of HMAP or LPS elicited no systemic response and changes in enzyme activities observed in BAL fluid are due only to a local response of the lung.

Histology

The lungs of untreated control animals were considered normal histologically. The lungs of animals that received SI with SAL had minimal histologic changes. At 24 h, slight perivascular edema containing scant eosinophils and a few mast cells was observed. Fewer cells were observed at 48 h and the tissue was considered to be within normal limits (figure 5A). Similarly, the lungs of animals 24 h after SI of INMA consisted mainly of normal parenchyma; however, perivascular edema involving a few small vessels and foci of alveolar septal thickening were observed on histologic examination. These alterations persisted through 48 h with a little associated inflammation (Fig. 5B); normal histology was observed after 72 h. At 24 h post-treatment, the lungs of HMAP-treated animals had a multiple alveolar foci containing increased numbers of alveolar macrophages admixed with a few neutrophils. Some alveolar septal thickening was also observed after 48 h (Fig. 5C), diminishing after 72 h. These

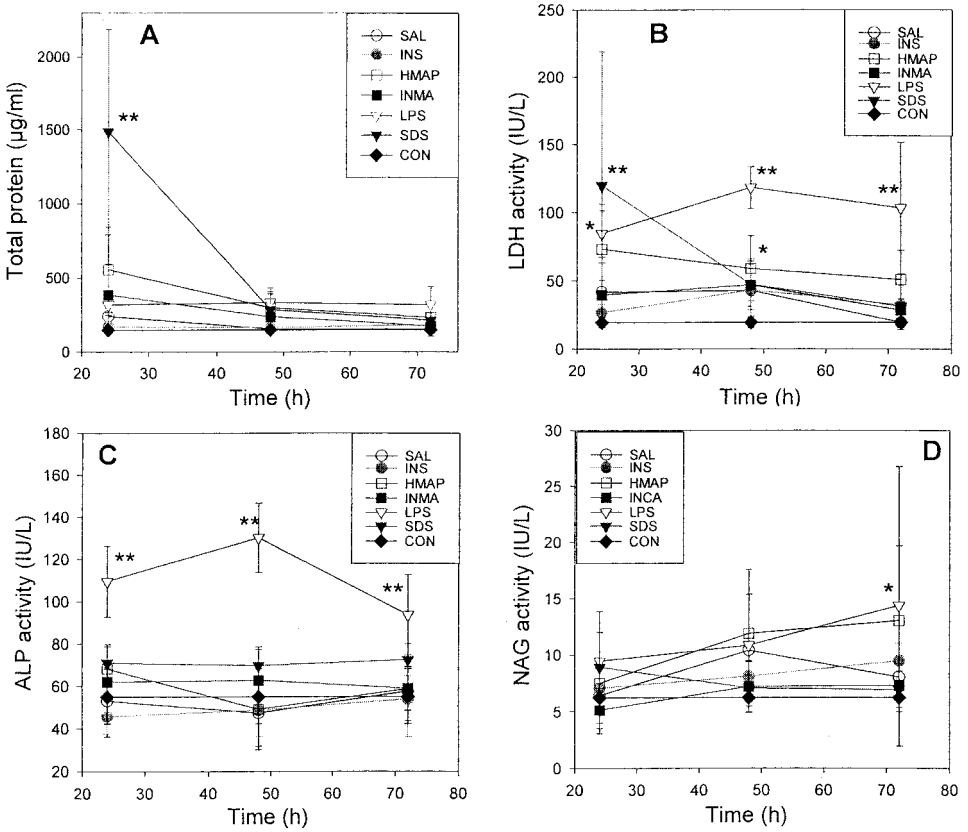

Fig. 4. Total protein concentration (A), LDH (B), ALP (C), and NAG activities (D) of the cell-free BALs from the lungs of rats 24, 48, and 72 h after a single intratracheal spray-instillation of the different test solutions. Normal saline (SAL), insulin (INS), HMAP, insulin plus HMAP (INMA), lipopolysaccharide (LPS), sodium dodecyl sulphate (SDS), and control (CON).

animals also had a prominent eosinophilic perivasculitis at 48 h post-treatment. These vascular lesions were less severe in the lung sections from the animals sampled at 72 h post-treatment.

As with other parameters, treatment of rats with LPS produced the most severe pulmonary histopathology compared to that resulting from treatment with other solutions. An increase in the alveolar exudate consisting of alveolar macrophages, neutrophils, and eosinophils was observed after 24 h, representing early acute alveolar damage. Also, acute perivascular edema and perivasculitis involving multiple small vessels were observed. In some vessels, endothelial cell nuclei were particularly prominent suggesting endothelial cell injury. The lung damage increased with time. At 48 h (Fig. 5D), a mild, diffuse alveolar exudate was more prominent and alveolar lining cells (type II pneumocytes) were hypertrophied producing a thickened alveolar wall and proliferative alveolitis characteristic of diffuse alveolar damage. Lymphocytic/monocytic perivascular cuffs were prominent, and endothelial swelling and hypertrophy was also evident. This general pattern of lung pathology persisted after 72 h of SI of LPS but was less severe than at 48 h, particularly in the vascular compartment.

Lung Function

There was no significant difference in pulmonary resistance (all time points range = 0.046–0.048) $cmH_20/mL/min$ within or between treatment groups ($P < 0.05$) and less than 1% RSD across time points (0.48–0.95%) in resistance for all groups.

Table II. Statistical Significance of All Parameters

Treatment	Total cells	Neutroph	Lymph	Mono	Macroph	Total proteins	LDH	ALP	NAG
LPS	$S^{s,u}$	$S^{s,u}$	$S^{s,u}$	$N^{s,u}$	$S^{s,u}$	$N^{s}S^{u}$	$S^{s,u}$	$S^{s,u}$	$N^{s}S^{u}$
SDS	$S^{s,u}$	$S^{s,u}$	S^{su}	$S^{s}N^{u}$	$S^{s,u}$	$S^{s,u}$	$S^{s,u}$	$S^{s,u}$	$N^{s,u}$
INS	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$
HMAP	$N^{s}S^{u}$	$S^{s,u}$[a]	$N^{s,u}$	$N^{s,u}$	$S^{s,u}$	$N^{s}S^{u}$	$S^{s,u}$,[a]	$N^{s,u}$	$N^{s}S^{u}$,[b]
INMA	$N^{s}S^{u}$	$S^{s,u}$,[a]	$N^{s,u}$	$N^{s,u}$	$S^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$	$N^{s,u}$

S = significantly different; N = not significantly different. Compared to ^{s} = saline and ^{u} = untreated controls.

[a] Elevated at 24 hours.

[b] Elevated at 72.

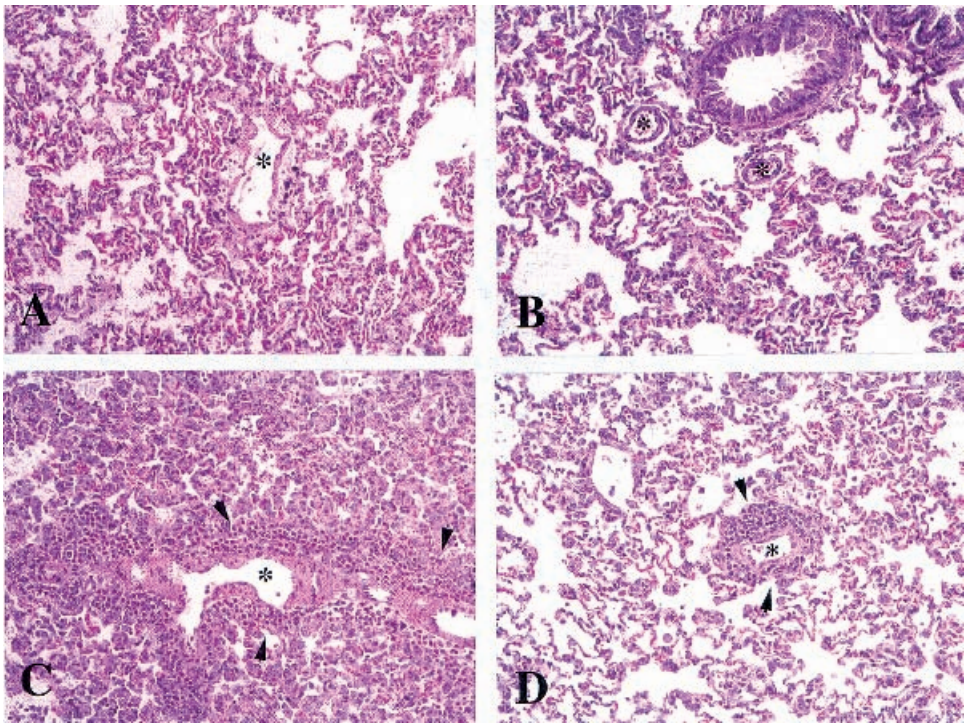

Fig. 5. All photomicrographs taken at 200× original magnification of sections removed from animals at 48 h post-treatment. (A) Lung of rat treated with saline by spray instillation. Asterisk marks the lumen of a small vein with perivascular fluid accumulation containing scant eosinophils and mast cells. (B) Lung of rat treated with INMA by spray instillation. Asterisks mark two vascular lumina adjacent to a bronchiole. The perivascular spaces are slightly distended. (C) Lung of rat treated with HMAP by spray instillation. Asterisk marks the lumen of an arteriole lumen. Arrows indicate an acute eosinophilic perivascular cuff several cell layers thick. Alveolar septal thickening is also evident in this section. (D) Lung of rat treated with LPS by spray instillation. Asterisk marks lumen of a small arteriole. The lymphocytic/monocytic perivascular cuff (arrows) demonstrated was characteristic of the vascular pathology seen throughout this section. Focal alveolar septal thickening and alveolar exudates are also apparent.

DISCUSSION

This report evaluates the short-term pulmonary response after intratracheal SI of insulin and H-MAP solutions compared to SI of negative controls (SAL and untreated animals) and positive controls (LPS, an immunostimulant and SDS, an irritant). The potential of LPS and SDS to elicit immune reaction or cause lung injury have been well documented (22–25), and they are suitable as positive controls for comparison of with H-MAP and insulin. LPS from *E. coli* is known to stimulate an immune response in the lungs and has been used as positive control in other studies dealing with toxicity (22–24). SDS has been used as permeation enhancer for drugs administered by several routes (25); however, depending on the dose used, it is known to irritate and disrupt the mucosa in the respiratory tract (26–27). Untreated and saline spray instillation treated animals are the most appropriate negative control studies for comparison with the experimental groups.

The evaluation of BAL fluids helps to define composition and concentrations of soluble and cellular components for diagnostic, prognostic, and therapeutic purposes (18,28). Total and differential cell counts in BAL are used to detect pulmonary injury. Changes in the number of white cells and in the cell profile indicate the type and degree of lung injury (18). The biochemical indicators of lung injury are analyzed in the cell-free BAL. Total protein concentration is measured as an indicator of increased permeability of the alveolar-capillary barrier and is considered as a corollary of the inflammatory response (28). Increases in enzymatic activities in BAL have been used as markers of pulmonary responses to inhaled substances (28). For this study lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and N-acetyl-glucosmiidase (NAG) were selected to evaluate the effects of H-MAP and insulin solutions on the lungs. Extracellular LDH, a cytoplasmic enzyme, was used as indicator of cytotoxicity because it is usually found in low concentrations extracellularly, except in the presence of damaged cells. ALP, a more specific enzyme, is considered a marker for type II cell proliferation following a type I cell injury (28). NAG, a lysosomal enzyme, is a good indicator of increased phagocytic activity in response to inhaled particles (28).

SI of a solution itself, as in the case of simple SAL, seems to have an effect on the normal physiology of the lung as evidenced by the histological findings and the significant increase in total number of white cells in BALF ($p < 0.05$). Other parameters such as wet lung weight, the proportions of neutrophils and lymphocytes, total protein concentration and the activities of LDH and NAG were consistently higher for SAL as compared to the untreated control group (CON), although the differences were not statistically significant.

In contrast to SAL, the irritant effect of SDS on the lungs of rats was indicated by an immediate increase in most of the BAL parameters. Increased permeability of the alveolar-capillary barrier was indicated by the elevated total protein content (4.7-fold increase), which in part may due to epithelial cell lysis. A 2-fold increase in LDH and a 1.3 fold increase in ALP also indicated cell damage and lysis; while inflammation was indicated by 1.4-fold increase in the total number of white cells, 3-fold increase in the proportion of neutrophils and elevated protein content. Additionally, a 2.6-fold increase in the proportion of lymphocytes, significant increase in monocytes and a significantly lower proportion of macrophages (from 88 to 63%) were observed when compared to SAL. The irritant effect of SDS was also observed in the population of macrophages showing inclusions and vacuolation, and degenerating cells.

The immunological effect of LPS on the lungs of rats was indicated by the histological findings and the significant increase in most of the BAL parameters as compared to those of SAL. Lung edema was observed by histological analysis and also indicated by a 1.8-fold increase in wet lung weight. A 3-fold increase in LDH and a 2.1-fold increase in ALP indicated severe cytotoxicity and cell damage (which was also microscopically observed), while a significant increase in NAG indicated increased phagocytic activity of macrophages. Severe inflammation was microscopically observed in the lung tissue and also indicated by a 4.4-fold increase in the number of white cells and an 8-fold increase in the proportion of neutrophils. The significant increase in the proportion of neutrophils was reflected in a significantly lower proportion of macrophages in which some vacuolation was observed.

Contrary to the positive controls, all parameters for INS were comparable to those of SAL and untreated control indicating that SI of INS is comparable to SI of SAL. Therefore, no major detrimental effects could be attributed to INS.

No significant differences were observed between SAL and INMA in wet lung weight, total protein content, LDH, ALP or NAG activities, total number of white cells, or the proportions of lymphocytes and monocytes. However, an increase in the proportion of neutrophils was observed (3.3-fold), which was reflected in the proportion of macrophages. Indeed, the proportion of neutrophils was highest at 24 h but it decreased with time and was comparable to that of SAL and untreated control at 72 h. This indicated a small, transient inflammatory response, observed also during microscopic analysis. However, the significance of this response is questionable since no other markers of inflammation (e.g., ALP activity, and percentage of eosinophils and basophils) were elevated (18,28).

SI of H-MAP resulted in no significant differences in wet lung weight, total protein content, activities of ALP or NAG, total number of white cells, and in the proportions of lymphocytes or monocytes compared to SI of SAL. However, it resulted in modest increases in LDH activity (1.8-fold) and in the proportion of neutrophils (2.7-fold), reflected in the proportion of macrophages. As in the case of INMA, the proportion of neutrophils was elevated at 24 h but decreased dramatically by 48 h and, after 48 h it was comparable to that of SAL and untreated controls. Again, this indicated a mild transient inflammatory reaction, observed also during microscopic analysis. As in the case of INMA, no other markers of inflammation were elevated, which also makes this response of questionable significance (18,24). It should be noted that this was a local response as evidenced by the comparable ratios of all enzyme activities in BAL vs. plasma in the animals.

In other studies, Edwards *et al.* (29–30) reported that the inhalation of large porous insulin particles was also accompanied by a relatively low inflammatory response characterized by increase in the proportion of neutrophils; however, no other indicators of inflammation were measured in those studies. The particles used in these studies were prepared with poly(lactic-co-glycolic acid), a polymer that is rendered as "safe" and is commonly used as suture and implant material. Therefore, an increase in the proportion of neutrophils may be simply a reaction of the lungs to inhaled substances in general.

It is interesting to speculate that the increase in LDH activity after SI of H-MAP in our studies may in part be due to a phenomenon other than cell damage. In general, when cell damage occurs in the lung, an increase in LDH activity is accompanied by a significant increase in ALP activity and total protein content (18,28); however, neither of those parameters was elevated for H-MAP with respect to SAL control. In addition, it should be noted that the increase in LDH activity was very modest compared to that observed for LPS. It is possible that the increase in LDH activity may be related to the mechanism of action of H-MAP. Previous studies provide evidence that the mechanism of action of the family of compounds to which H-MAP belongs, is not explained through classical protease inhibition (31) or general permeation enhancement (32); rather they suggest that specific compound/protein interactions facilitates the transport through biological membranes. These compounds appear to reversibly destabilize the native state of the protein molecule favoring a partially unfolded conformation that satisfies the standard definition of the molten globule state (16). It also has been suggested that the transport process is passive and transcellular (16). Partially unfolded protein conformations have been reported to move through lipid bilayer of the membrane via passive diffusion by a novel translocation process (33–34). The unfolded protein is moved from the cis side of the membrane through a hydrophilic, hetero-oligomeric transmembrane channel composed of integral membrane proteins. This channel is gated across the membrane by signal-anchor sequences and the exit of the protein from the transport channel is followed by folding of the protein on the trans side of the membrane mediated by "chaperones" (33–34). Therefore, a plausible explanation for the mild increase in the LDH activity may have been the result of the reversible interaction of H-MAP with the lung epithelial and endothelial cell membranes. However, this explanation should be viewed with caution as no mechanistic studies have been conducted to challenge the hypothesis.

Concentrations of H-MAP employed in these studies may exceed the anticipated dose for humans. In addition, the available airway surfaces and cell numbers in the rat are small in comparison with the delivered dose. Low concentrations of LPS, known to elicit a mild response, were adopted and statistical analyses were performed at a high level of sensitivity (21,24). Consequently, extremely sensitive conditions have been adopted to challenge the effects of H-MAP *in vivo*. A small, transient effect was noted, which did not elicit the full complement of inflammatory markers and which was substantially smaller than either the effect of LPS or SDS.

The absence of effect of any agent on lung function justifies the approach taken in the present study of evaluating the effect of the delivery agent on lung biochemistry. Subtle effects which did not appear to influence lung function, such as significant changes in enzyme activity induced by LPS and SDS as positive controls, could not have been evaluated in any other way. Nevertheless, thorough toxicological evaluation based on therapeutically relevant multiple dosing regimens are required in which evaluating gross effects such as changes in pulmonary function may be valuable.

In summary, based on the results of this study, SI of H-MAP and INMA solutions cause a transient local response of the lung at the doses studied. This effect consisted of a small inflammatory reaction evidenced by the neutrophil infiltration in BAL fluid, which decreased with time. More extensive studies of the use of H-MAP are required to evaluate its mechanism of action and safety in the lungs.

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