Pulmonary Delivery of Low Molecular Weight Heparins

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Received March 29, 2004; accepted July 26, 2004

Purpose. To investigate if pulmonary delivery of low molecular weight heparin (LMWH) formulated with tetradecyl-β-maltoside (TDM) or dimethyl-β-cyclodextrin (DMβCD) could be a feasible alternative to subcutaneous injections for the treatment of pulmonary embolism.

Methods. The pulmonary absorption of two LMWHs and unfractionated heparin formulated with TDM or DMβCD was studied in cell culture and rodent model. The *in vitro* study was performed by measuring the transport of radiolabeled enoxaparin and mannitol across human bronchial epithelial cells (Calu-3) in the presence or absence of varying concentrations of TDM or DMβCD. The changes in transepithelial electrical resistance (TEER) and enoxaparin metabolic stability were also investigated using Calu-3 cells. *In vivo* absorption studies were performed by measuring plasma anti-factor Xa activity after pulmonary administration of enoxaparin, dalteparin, or unfractionated heparin to anesthetized rats.

Results. *In vitro* experiments conducted in Calu-3 cells suggest that the addition of TDM or DMβCD to the apical chamber results in a significant increase in ^{3}H-enoxaparin and ^{14}C-mannitol permeability and a decrease in TEER across the Calu-3 cell monolayer. Enoxaparin incubated in Calu-3 cell extracts was stable for 8 h. *In vivo* studies indicate that both TDM and DMβCD enhance pulmonary absorption of LMWH. However, TDM was found to be more potent than DMβCD in both *in vitro* transport and *in vivo* absorption studies.

Conclusions. TDM and DMβCD enhance pulmonary absorption of LMWH both *in vitro* and *in vivo*, with TDM being more efficacious than DMβCD. Both agents increase drug transport by acting mainly on the membrane rather than interacting with the drug.

KEY WORDS: dimethyl-β-cyclodextrin; enoxaparin; low molecular weight heparins; permeability; pulmonary absorption; tetradecyl-β-maltoside; transepithelial electrical resistance.

INTRODUCTION

Deep vein thrombosis affects 2 million Americans annually, and an estimated 600,000 of these develop pulmonary embolism, a fatal complication that results in 200,000 deaths a year (1–3). Deep vein thrombosis and pulmonary embolism can occur in both healthy ambulatory individuals and bedridden hospitalized patients. A recent study has shown that one in 100 airplane travelers, with no previous history of clotting disorder, may develop deep vein thrombosis as a result of their lengthy flights (4). Of the thromboembolic disorders, pulmonary embolism is particularly important because in this acute thromboembolic episode, a thrombus originating from the circulation lodges in the pulmonary artery or one of its branches. As a result, death can occur within a few hours of onset due to partial or complete obstruction of pulmonary blood flow. Thus, rapid diagnosis and prompt treatment are critically important for the outcome of patients with pulmonary embolism (5).

In recent years, because of improved pharmacokinetic profiles and reduced cost of therapy, low molecular weight heparins (LMWHs) have enjoyed success as an alternative to unfractionated heparin (UFH) in the treatment of deep vein thrombosis and pulmonary embolism (6,7). However, these new generation drugs are still delivered via the traditional routes of administration such as subcutaneous and intravenous routes. Administration of an anticoagulant drug directly to the pulmonary circulation would be ideal for the treatment of pulmonary embolism. A pulmonary formulation of LMWH will allow direct administration of the drug into the lungs, and consequently this formulation is likely to reduce the mortality from an attack of pulmonary embolism. Further, a portable noninvasive formulation would also be beneficial for both patients with thromboembolic disorders and healthy individuals who are susceptible to deep vein thrombosis.

However, LMWHs do not get absorbed from the respiratory tract because of their excessive hydrophilicity and large surface charges. We have previously identified tetradecylmaltoside as a nonionic surfactant that enhances nasal and pulmonary absorption of insulin (8,9). Similarly, dimethyl-β-cyclodextrin (DMβCD), a derivative of cyclodextrin with seven glucose units, has been studied extensively for its safety and efficacy as an absorption promoter for nasal and pulmonary delivery of peptide drugs (9–11). Recently, we have shown that both tetradecyl-β-maltoside (TDM) and DMβCD enhance nasal absorption of enoxaparin, a low molecular weight heparin available in the U.S. market (12,13). Based on the efficacy of TDM and DMβCD in enhancing nasal absorption of LMWHs and peptide drugs, it is reasonable to assume that both of these agents can also increase pulmonary absorption of LMWH. However, there is no experimental data in support of the assumption that TDM and DMβCD enhance pulmonary absorption of LMWH, a polysaccharide with negative surface charges. On the other hand, because the lung provides a larger surface area and has a thin epithelial membrane, the bioavailability of a drug administered via the pulmonary route is expected to be much higher than that obtained via the nasal route. More importantly, clinical benefit offered by pulmonary LMWH in the treatment of pulmonary embolism could be of paramount significance because this delivery approach will permit to administer the drug directly at the site affected by the disease.

This study tests the hypothesis that TDM and DMβCD enhance pulmonary absorption of LMWH *in vitro* and *in vivo*. Enoxaparin and dalteparin, LMWHs that received widespread acceptation among the medical practitioners in the United States, have been used to evaluate the efficacy of TDM and DMβCD in enhancing pulmonary absorption of LMWH. The mechanistic aspects of these absorption enhancers have also been studied using human bronchial epithelial cells (Calu-3).

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MATERIALS AND METHODS

Materials

^{3}H-enoxaparin (specific activity of 250 μCi/mg) was purchased from American Radiolabeled Chemicals Inc., (St. Louis, MO, USA). D-[1-^{14}C]-Mannitol (specific activity of 53 mCi/mmol) and dimethyl-β-cyclodextrin (DMβCD) were obtained from Sigma Chemicals Company (St. Louis, MO, USA). Calu-3 cells, Modified Eagle's Medium (MEM), L-glutamine, fetal bovine serum (FBS), penicillin and streptomycin solution, and trypsin EDTA solution were purchased from ATCC (Rockville, MD, USA). Vitrogen 100 solution was obtained from Cohesion, Inc (Palo Alto, CA, USA). Transwell cell culture assembly with polycarbonate inserts (0.4-μm pore size, 1 cm^2 area) were obtained from Corning Costar Corporation (Cambridge, MA, USA). Enoxaparin (Lovenox, 3000 U of anti-factor Xa activity per 0.3 ml), and Dalteparin (Fragmin, 2500 U of anti-factor Xa activity per 0.2 ml) injections were obtained as sterile solutions from Aventis Pharmaceutical Products Inc. (Bridgewater, NJ,USA) and Pharmacia & Upjohn Company (Kalamazoo, MI, USA), respectively. Heparin sodium injection (10,000 U/ml) was purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ, USA). Tetradecyl-β-D-maltoside (TDM) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA).

Enoxaparin Degradation in Calu-3 Cell Extracts

Calu-3 cells were grown in MEM, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified 37°C incubator with 5% CO_2. Calu-3 cell homogenates were prepared as described previously (14). Briefly, Calu-3 cells were grown in culture flask (75 cm^2) until 80% confluence was achieved. Subsequently, the cells were washed with ice-cold normal saline, scraped, homogenized in 5 ml ice-cold media (Ultra-Turrax 725 Basic, IKA-WERKE, Staufen, Germany), and centrifuged at 12,000 rpm for 10 min at 4°C. The resultant supernatant was used as Calu-3 cell extracts. Protein content (0.52 mg/ml) of the extracts was determined as described by Bradford using a Coomassie blue dye assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Increasing concentrations of enoxaparin (0.5 to 500 U/ml) were incubated at 37°C for 8 h in saline or cell extracts. The resulting solutions were analyzed for anti-factor Xa activity by a colorimetric assay using Coatest Heparin Kit (Diapharma Group Inc., West Chester, OH, USA).

Transport and Transepithelial Electrical Resistance (TEER) Studies Across Calu-3 Cell Monolayers

Calu-3 cells were plated in 75 cm^2 culture flask (coated with fibronectin/collagen solution) and subcultured after achievement of 85–90% confluence. Media was changed every 2 days, and the passages used for the experimentation were between 8 and 15. The inserts of the Transwells (0.4-μm pore size, 1 cm^2 area) were coated with Vitrogen 100 solution. The coating procedures were performed by adding a freshly prepared chilled solution (8 ml Vitrogen-100, 1ml 10 × PBS, 1 ml 0.1 M sodium hydroxide, adjusted to pH 7.4) to the Transwell insert surface then incubating at 37°C for at least 1 h to induce the *in situ* gel formation. Transwells were then dried overnight under laminar airflow.

For transport studies, the Calu-3 cells were seeded at a density of 50,000 cells/well onto collagen-coated polycarbonate Transwells (0.4 μm pore size, 1 cm^2 area). After the cells had attached to the Transwells, the media was removed from the apical compartment to allow the monolayer to grow at the air-interface. The integrity of the confluent cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) using the EVOM epithelial voltohmeter (World Precision Instruments, Sarasota, FL, USA). The monolayers with TEER values above 400 Ω· cm^2 were used in the experiments (about 10–14 days). ^{14}C-mannitol flux was determined for each set of cells used in the transport experiments.

On the day of experiment, ^{3}H-enoxaparin and ^{14}C-mannitol were dissolved in culture media and mixed with TDM or DMβCD stock solution in saline. The final concentrations of TDM or DMβCD used in the experiments were 0.0625%, 0.125%, and 0.25% (w/v). The pH of the solutions was approximately 7.4. Prior to the initiation of transport experiments, 1.5 ml fresh media was added to the basolateral side and 0.5 ml of pre-warmed solutions containing ^{3}H-enoxaparin and ^{14}C-mannitol were added to the apical side in the presence or absence of different concentrations of TDM or DMβCD. Monolayers for transport experiments were kept in a 37°C/5% CO_2 incubator. Samples (100 μl) were withdrawn from the basolateral chamber at various time intervals (0, 15, 30, 45, 60, 90, 120 min). The basolateral chamber was replenished with fresh media (100 μl) after each sampling. TEER was recorded during the experiments.

The amount of ^{14}C-mannitol and ^{3}H-enoxaparin transported across cell monolayers was determined by a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Inc, Fullerton, CA, USA). The apparent permeability coefficient (P_{app}) was calculated from the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \cdot Co}$$

where dQ/dt is the flux of ^{14}C-mannitol or ^{3}H-enoxaparin across the cell monolayer (cpm/s). A is the surface area available for transport (1 cm^2 for 12 well Transwell system). C_0 is the initial concentration (cpm/ml) of mannitol or enoxaparin in the donor compartment.

TEER Reversibility Study

For TEER reversibility study, Calu-3 cells were grown on polycarbonate membrane filters as described above. On the day of the experiment, solutions of 0.125% TDM or 0.125% DMβCD in media were added to the apical chamber. After 60 min of exposure to TDM or DMβCD, solutions were removed and the cells were rinsed with saline. The cells were then incubated for 48 h (37°C, 5% CO_2) after adding pre-warmed media to the basolateral (1.5 ml) chamber. During the incubation, the media was removed from the apical chamber to allow the monolayer to recover at the air-interface. TEER values were measured in triplicate at 0.5, 1, 2, 4, 8, 12, 24, 30, 36, and 48 h.

Preparation of Formulations for *in Vivo* Pulmonary Absorption Studies

Stock solutions of TDM (1%) and DMβCD (1%) were prepared by dissolving the reagents in normal saline and stored at 4°C for 30 days or less. Stock solutions older than 30 days were not used in the experiments. Further, it was previously determined (data not shown) that there were no differences in the drug absorption when formulations containing reagents stored for 30 days were used, compared to formulations that contained freshly prepared solutions. On the day of the experiment, formulations for *in vivo* pulmonary absorption studies were prepared by mixing enoxaparin with saline or appropriate concentrations of TDM or DMβCD. The concentrations of TDM and DMβCD used in the formulations were 0.0625%, 0.125%, and 0.25% (w/v). The strength of the final formulation was such that each 100 μl of the solution contained an amount of enoxaparin sodium equivalent to 15 U of anti-factor Xa activity. Similarly, for subcutaneous absorption studies, formulations were made by diluting enoxaparin with appropriate amounts of saline and each 100 μl of solution contained 15 U of anti-factor Xa activity. The pulmonary formulations of dalteparin and unfractionated heparin were prepared as described above, with each 100 μl of final formulation containing 15 U of anti-factor Xa activity.

Pulmonary Absorption Studies in Rats

Male Sprague-Dawley rats (Charles River Laboratories, Charlotte, NC, USA) weighing between 250 and 350 g were used for *in vivo* absorption studies. Prior to the experiment, the animals were anesthetized by intramuscular injection of a mixture containing xylazine (20 mg/ml) and ketamine (100 mg/ml). Anesthesia was maintained with additional intramuscular injections of anesthetic solution as needed throughout the experiments. Formulations containing enoxaparin, dalteparin or unfractionated heparin (50 U/kg) plus saline, TDM or DMβCD were administered intratracheally as described earlier (9). The amount of formulation administered was 80–120 μl depending on animal body weight. For bioavailability studies, formulations were administered subcutaneously (50 U/kg) as a single injection under the back skin. After pulmonary and subcutaneous administration, blood samples (300 μl) were collected from the tip of the rat-tail at 0, 15, 30, 60, 120, 240, 360, and 480 min in citrated microcentrifuge tubes and placed on ice. Subsequently, the plasma was separated by centrifugation (1600 × *g* for 5 min) and the plasma samples thus obtained were stored at −20°C until further analysis. Enoxaparin absorption was determined by measuring plasma anti-factor Xa levels using a colorimetric assay kit (Chromogenix Coatest Heparin Kit, Diapharma Group Inc., West Chester, OH, USA).

All animal studies were approved by the Texas Tech University Health Sciences Center (TTUHSC) Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Pharmacokinetic Analysis

Standard pharmacokinetic analysis (Kinetica, Version 4.0, Innaphase Corp. Philadelphia, PA, USA) was performed for enoxaparin absorption-time profiles. Area under the plasma concentration vs. time curve ($AUC_{0\rightarrow480}$) was calculated by the trapezoidal method. The area under the first moment curve ($AUMC_{0\rightarrow\infty}$) for plasma anti-factor Xa activity-time profile was estimated from a plot of the product of plasma anti-factor Xa concentration and time (c×t) vs. time. The mean residence time (MRT) was calculated by dividing $AUMC_{0\rightarrow\infty}$ with $AUC_{0\rightarrow\infty}$. Relative bioavailabilities (F_r) were estimated by comparing $AUC_{0\rightarrow480}$ for pulmonarily administered enoxaparin with that of subcutaneously administered enoxaparin.

Statistical Analysis

Permeation coefficients and pharmacokinetic parameters of different formulations were compared by paired *t* test or one-way ANOVA. When the differences in the means were significant, post-hoc pair wise comparisons were conducted using Newman-Keuls multiple comparison (GraphPad Prism, version 3.03, GraphPad Software, San Diego, CA, USA). Differences in p values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Transport of Enoxaparin Across Calu-3 Cell Monolayers

Calu-3 cell monolayer has been used as an *in vitro* model for studying drug transport across bronchial epithelial cells. Because these cells form polarized monolayers and express functional tight junction, it has been proposed that Calu-3 cells can be an attractive model for *in vitro* pulmonary drug delivery studies (15,16). The transport of ^{3}H-enoxaparin across Calu-3 cells was studied in the presence or absence of increasing concentrations of two chemically different absorption promoters, TDM or DMβCD. The permeation of ^{14}C-mannitol across Calu-3 cells was also studied concomitantly in order to determine if TDM and DMβCD enhance transport of enoxaparin via paracellular route by loosening cell-cell tight junctions.

When transport of ^{3}H-enoxaparin was conducted without any absorption enhancer in the apical chamber, little drug permeation to the basolateral chamber was noted during the course of 2-h experiment (Fig. 1). Such a little increase in the drug permeation suggests that Calu-3 cells form a tight monolayer that restricts the movement of the drug from the apical to basolateral side. However, in the presence of TDM or DMβCD (0.0625–0.25%) in the apical fluid, a dose-

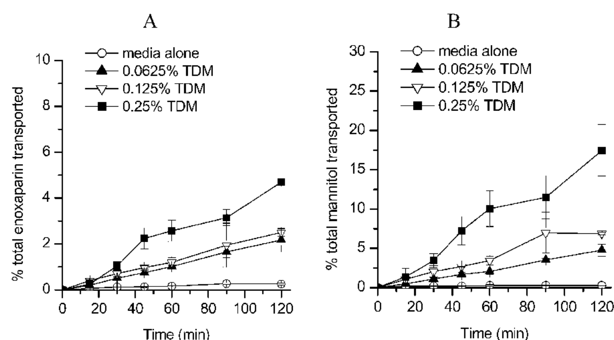

Fig. 1. Percentage of ^{3}H-enoxaparin (A) or ^{14}C-mannitol (B) transported across Calu-3 cell monolayers in the presence of different concentrations of TDM. Data represent mean ± standard deviation, n = 3.

dependent increase in ^{3}H-enoxaparin transport was observed (Figs. 1A and 2A). TDM increases the movement of enoxaparin from the apical to basolateral side even when the concentration of TDM was 0.0625%. There was a 4-fold increase in the P_{app} when the concentration of TDM was increased from 0.0625% to 0.25% (Table I). The difference between the permeability coefficients obtained from transport studies in presence of either agents and that obtained in the absence of the agents are statistically significant ($p < 0.05$). However, increase in the permeation produced by TDM was significantly higher than that produced by DMβCD ($p < 0.05$). Similar results were obtained when the permeability of ^{14}C-mannitol was studied. Like that of enoxaparin transport, there was a dose dependent increase in the permeability of ^{14}C-mannitol in the presence of TDM or DMβCD (Figs. 1B and 2B). Likewise, increase in the permeability of mannitol produced by TDM was higher than that produced by DMβCD. Increase in overall mannitol permeability in the presence of TDM or DMβCD also suggests that both agents perhaps cause loosening of tight junctions and enhance transport of enoxaparin via the paracellular route. This result agrees with the data obtained from *in vivo* nasal absorption of enoxaparin formulated with TDM or DMβCD (12,13).

Enoxaparin Degradation in Calu-3 Cell Extracts

Previously, it has been argued that metabolic changes in drug under investigation may show false increase in enoxaparin permeability during an *in vitro* study (17). In fact, any change or degradation of polysaccharide chain of LMWH can result in the loss of anti-factor Xa activity of enoxaparin. In order to rule out such nonspecific degradation or disappearance of enoxaparin, stability of enoxaparin in Calu-3 cell extract was studied. In this regard, increasing concentrations of enoxaparin (0.5, 5, 50, or 500 U/ml) were incubated for 8 h in either Calu-3 cell extracts or saline. Subsequently, the samples were assayed for anti-factor Xa activity. Interestingly, no differences were noted between the anti-factor Xa activity of the different concentrations of enoxaparin incubated in Calu-3 cell extracts or enoxaparin incubated in saline (0.5 ± 0.01 vs. 0.5 ± 0.01, 4.9 ± 0.1 vs. 5.3 ± 0.01, 52 ± 2.2 vs. 53 ± 1.2, 516 ± 23 vs. 506 ± 38 U/ml). The data suggest that no depolymerization or degradation of enoxaparin molecule occurred during the course of study and anti-factor Xa activity

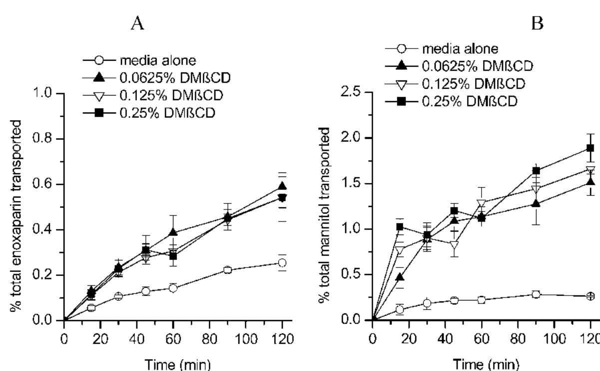

Fig. 2. Percentage of ^{3}H-enoxaparin (A) or ^{14}C-mannitol (B) transported across Calu-3 cell monolayers in the presence of different concentrations of DMβCD. Data represent mean ± standard deviation, n = 3.

Table I. Effects of TDM or DMβCD on ^{3}H-Enoxaparin and ^{14}C-Mannitol Transport Across the Calu-3 Cell Monolayer[a]

Formulations	$P_{app} \times 10^{-7}$ (cm/s)	
	Enoxaparin	Mannitol
No enhancer	0.35 ± 0.02	1.4 ± 0.3
0.0625% TDM	4.63 ± 0.66[b]	39.9 ± 6.5[b]
0.125% TDM	5.20 ± 0.12[b]	57.8 ± 1.4[b]
0.25% TDM	16.69 ± 0.62[b]	127.2 ± 26.7[b]
0.0625% DMβCD	0.78 ± 0.02[b]	7.6 ± 0.4[b]
0.125% DMβCD	0.86 ± 0.06[b]	8.4 ± 1.2[b]
0.25% DMβCD	1.01 ± 0.04[b]	14.5 ± 1.5[b]

[a] Data represent mean ± standard deviation, n = 3.
[b] Significantly different from those obtained without enhancer, $p < 0.05$.

of the drug was most likely maintained throughout the study. In agreement with this data, no degradation of enoxaparin was observed when the drug was incubated with 16HBE14o^{-} cells, a different bronchial epithelial cell line (13). However, this result is in contrast to that obtained when transport of radiolabeled insulin was studied in human bronchial epithelial cells. Insulin was degraded significantly during the course of transport study (17). Therefore, it can be argued that enzymatic degradation may not pose a problem for successful pulmonary delivery of LMWH. However, it is important to recognize that large amounts of various enzymes present in the respiratory fluid may adversely affect the stability of LMWH administered via the pulmonary route. In order to rule out the possibility of degradation of enoxaparin in the respiratory fluid, future studies will be directed to investigate the stability of enoxaparin in the bronchoalveolar lavage fluid obtained from rats or human volunteers.

Evaluation of TEER and Monolayer Integrity

Estimation of TEER is a useful tool in studying paracellular transport of solutes because any changes in TEER across cell monolayers is the result of the changes in paracellular ion flux and epithelial tight junctional stability (18). Similarly, TEER reversibility studies are used as an indicator for cell viability after exposure to an absorption enhancer (19). Thus, TEER values across Calu-3 cells were measured after the treatment with TDM or DMβCD and were expressed as a fraction of initial TEER, plotted as a function of time (Fig. 3). TEER values were reduced to 51.9% of the initial in the presence of 0.25% TDM after 2 h, whereas for 0.25% DMβCD, TEER values decreased to 70.7% of the initial value (Figs. 3A and B). The reduced TEER and *in vitro* permeability data presented thus far suggest that TDM and DMβCD increase mannitol permeability and both agents enhance drug transport across Calu-3 cells monolayer via the paracellular route.

Recovery of monolayer integrity was evaluated by TEER reversibility study. TEER was measured following a 60-min exposure of Calu-3 cell monolayer to either 0.125% TDM or DMβCD. In the absence of any enhancer, TEER was maintained for the course of the entire measurement period. In contrast, addition of formulations containing either TDM or DMβCD resulted in different degree of reductions in TEER (Fig. 3C). Compared to DMβCD, TDM produced

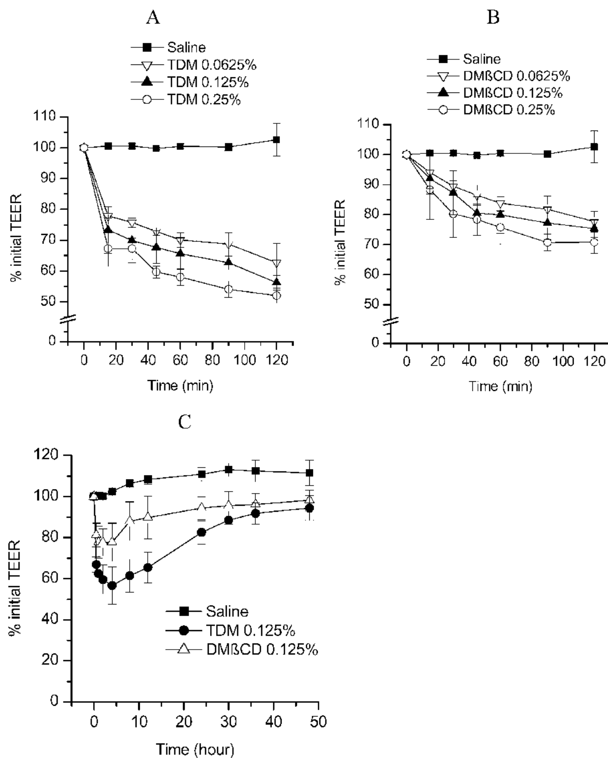

Fig. 3. Changes in TEER of Calu-3 cell monolayers in the presence of different concentrations of (A) TDM or (B) DMβCD. (C) Changes in TEER of Calu-3 cell monolayers measured over a 48-h period of recovery following a 60-min exposure to solutions containing TDM or DMβCD. Data represent mean ± standard deviation, n = 3.

more pronounced decrease in the TEER values, and the reversibility of TEER for TDM-treated monolayers was slower than that for DMβCD treated monolayer.

Diminished TEER values was recovered almost completely to baseline (>95% of initial values) 30 h after the treatment with DMβCD and 48 h after the treatment with TDM. This data suggest that disruption or loosening of tight junction caused by TDM or DMβCD can revert to original physiologic state and reform the disrupted tight junctions. Therefore, we can argue that neither of the agents causes any irreversible damage to the cell monolayer and the monolayer integrity can be recovered after acute exposure to TDM or DMβCD. The *in vitro* reversibility data agree with *in vivo* reversibility data reported previously (9). In a recent study, we have shown that the effects of 0.25% TDM or DMβCD on rat respiratory epithelium are reversible and the epithelium reestablishes its normal physiologic barrier 120 min after exposure to these agents (9). Overall, the effects of TDM and DMβCD on the respiratory membrane are reversible and acute exposure of TDM or DMβCD is less likely to cause extensive damage or cellular toxicity in respiratory epithelial cells.

Pulmonary Absorption Studies in Rats

The pulmonary absorption studies were performed by monitoring the changes in plasma anti-factor Xa activity after intratracheal administration of enoxaparin, dalteparin or unfractionated heparin formulated with TDM or DMβCD. Initial studies were performed with enoxaparin to determine the optimal concentration at which absorption-enhancing agents produce maximum increase in LMWH absorption and produce therapeutic anti-factor Xa level in rodent model. Anti-factor Xa level greater than 0.2 U/ml was considered to be therapeutic as reported previously (20). When enoxaparin was administered into rat without absorption enhancer, there was a modest increase in anti-factor Xa level. In fact, the level of anti-factor Xa level produced by enoxaparin without absorption enhancers was below the therapeutic level for anti-thrombotic effect in male Sprague-Dawley rats. However, the anti-factor Xa levels produced by all three enoxaparin plus TDM formulations were higher than therapeutic anti-factor Xa level required for anti-thrombotic effect in rats. Further, administration of pulmonary formulations containing enoxaparin plus varying concentrations of TDM produced a dose dependent increase in anti-factor Xa levels (Fig. 4A). An increase in TDM concentration was accompanied by an increase in the plasma anti-factor Xa levels as confirmed by a plot of $AUC_{0\rightarrow480}$ values for plasma anti-factor Xa vs. time (r = 0.92). Pharmacokinetic analysis of the data also suggests that with the increase in TDM concentration, there was an increase in C_{max} (Table II) and decrease in T_{max} for enoxaparin formulations containing TDM. Nevertheless, there was no change in absorption rate constant K_a or MRT with the increase in TDM concentration. Altogether, the pulmonary absorption studies with enoxaparin plus TDM formulations suggest that TDM enhances pulmonary absorption of enoxa-

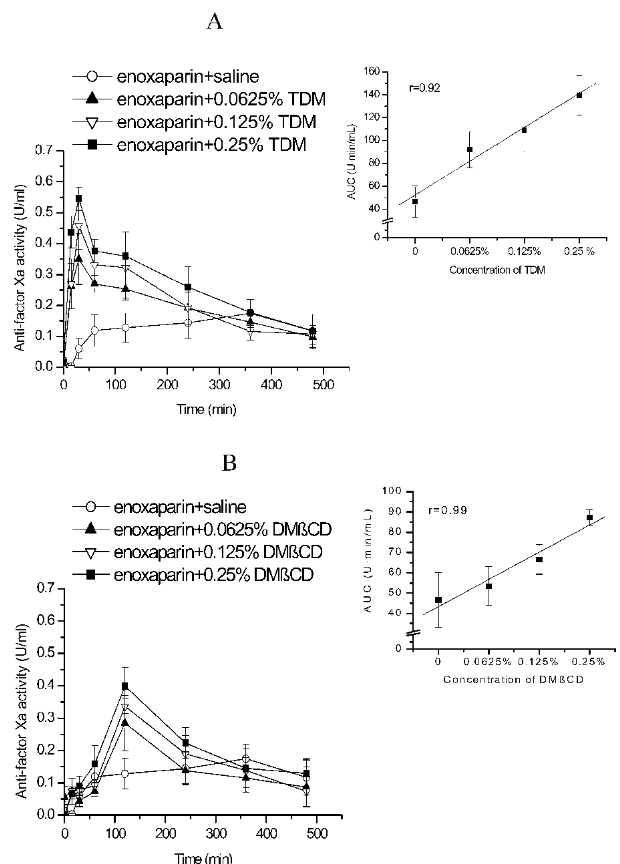

Fig. 4. Changes in plasma anti-factor Xa activity after pulmonary administration of enoxaparin (50 U/kg) in the absence or presence of different concentrations of TDM (A) or DMβCD (B). Data represent mean ± standard deviation, n = 5 to 6.

Table II. Pharmacokinetic Parameters for Subcutaneous and Pulmonary Formulations Containing Enoxaparin (50 U/kg) in Saline or Various Concentrations of TDM or DMβCD[a]

Formulations	C_{max} (U/ml)	T_{max} (min)	K_a (min^{-1})	MRT (min)	$AUC_{0\to480}$ (U · min/ml)	$F_{relative}$ (%)
No enhancer	0.18 ± 0.04	360 ± 48	0.003 ± 0.0005	419 ± 88	46.7 ± 13.5	33.8 ± 8.8
0.0625% TDM	0.35 ± 0.03[b]	45 ± 7[b]	0.003 ± 0.0008	293 ± 69	91.9 ± 15.8[b]	66.6 ± 12.5[b]
0.125% TDM	0.46 ± 0.07[b]	47 ± 8[b]	0.007 ± 0.003	325 ± 89	109.1 ± 19.2[b]	79.0 ± 15.2[b]
0.25% TDM	0.54 ± 0.04[b]	47 ± 15[b]	0.005 ± 0.001	297 ± 92	129.2 ± 18.5[b]	93.6 ± 14.7[b]
0.0625% DMβCD	0.28 ± 0.08	150 ± 24[b]	0.004 ± 0.001	319 ± 49	53.5 ± 9.4	38.7 ± 7.5
0.125% DMβCD	0.33 ± 0.05[b]	160 ± 25[b]	0.006 ± 0.002	251 ± 19	66.5 ± 7.4	48.2 ± 5.8
0.25% DMβCD	0.40 ± 0.02[b]	120 ± 20[b]	0.005 ± 0.001	339 ± 61	87.1 ± 4.0[b]	63.1 ± 3.2[b]
Subcutaneous	0.36 ± 0.03	120 ± 24	0.003 ± 0.0005	374 ± 61	138.1 ± 10.5	—

[a] Data represent mean ± standard deviation, n = 5 to 6.
[b] Significantly different from those obtained without enhancer, $p < 0.05$.

parin. This data agree with the studies on the effect of TDM on enhancing pulmonary and nasal absorption of insulin and calcitonin (8,9,21).

Similar dose-dependent increase in anti-factor Xa level was observed when enoxaparin formulated with increasing concentration of DMβCD was administered by intratracheal instillation to anesthetized rats (Fig. 4B). Like enoxaparin formulations with TDM, there was an increase in C_{max} and decrease in T_{max} for DMβCD based formulations (Table II). Relative and absolute bioavailabilities were calculated to compare the efficacy of enoxaparin plus TDM formulations with that of DMβCD-based pulmonary formulations. The bioavailability data presented in Table II suggest that TDM is more efficacious than DMβCD, a well characterized and extensively studied absorption promoter, in enhancing pulmonary absorption of LMWH.

The pharmacokinetic profiles of subcutaneous or pulmonary administration of LMWH are compared in Fig. 5. The results show that TDM based pulmonary formulation produces a quicker onset of anti-factor Xa activity compared to subcutaneous LMWH. The T_{max} for pulmonary formulation was 40–55 min, whereas T_{max} for subcutaneous formulation was 160–250 min. In this regard, it can be argued that in terms of onset of action, pulmonary LMWH is superior to subcutaneous preparation. The quicker onset of action of an anticoagulant formulation is clinically important for acute thromboembolic episodes. In particular, pulmonary LMWH with quicker onset of action would be ideal for pulmonary embolism where rapid anti-coagulant effect is highly desirable.

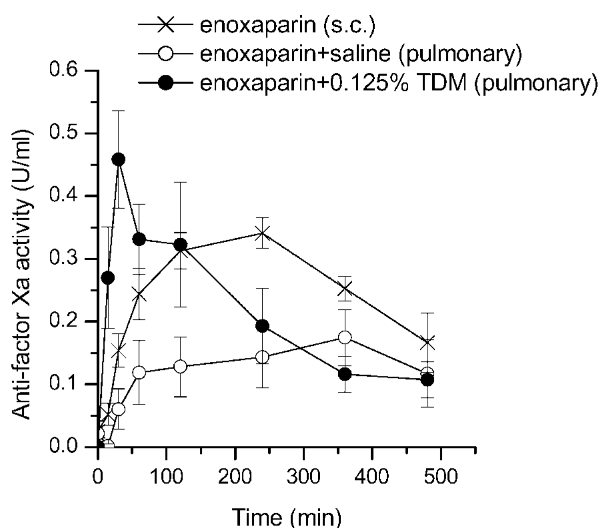

Fig. 5. Changes in plasma anti-factor Xa activity after subcutaneous and pulmonary administration of enoxaparin (50 U/kg) in the absence or presence of TDM. Data represent mean ± standard deviation, n = 5 to 6.

The *in vivo* pulmonary studies presented above suggest that both TDM and DMβCD can enhance pulmonary absorption of enoxaparin and pulmonary enoxaparin has a quicker onset of action compared to subcutaneous enoxaparin. However, it is not known if TDM and DMβCD will similarly enhance pulmonary absorption of other LMWHs and unfractionated heparin. To investigate the effect of TDM and DMβCD on other heparin preparations, the pulmonary absorptions of dalteparin, a LMWH with a molecular weight of 5000 Da, and unfractionated heparin (MW 15,000 to 20,000 Da) were studied in the absence or presence of TDM or DMβCD. When dalteparin was administered via the pulmonary route without any absorption promoter, there was no significant increase in anti-factor Xa levels. However, formulation of dalteparin in 0.125% TDM or DMβCD lead to a rapid and substantial increase in anti-factor Xa level (Fig. 6B). A comparison between the data presented in Figs. 6A and 6B and $AUC_{0\to480}$ values in Table III suggest that there are no differences between the anti-factor Xa levels produced by dalteparin and enoxaparin. Differences in the C_{max} and $AUC_{0\to480}$ values for these two formulations with 0.125% TDM or DMβCD are not statistically significant ($p > 0.05$). A comparable absorption profile of two LMWHs suggest that slight differences in the chemistry and molecular weight of two LMWHs perhaps do not play any role in their absorption. In fact, no differences in the pharmacokinetic profiles of enoxaparin or dalteparin were observed when the drugs were administered nasally after formulating with TDM or DMβCD (13,22). This also agrees with the fact that these two absorption promoters enhance drug absorption by increasing the permeability of the drug across bronchial epithelial cells as observed in *in vitro* studies.

Unlike LMWH formulations, TDM or DMβCD produced a modest increase in anti-factor Xa levels when formulated with unfractionated heparin (Fig. 6C). The C_{max} value for anti-factor Xa activity obtained following the administration of pulmonary formulations containing unfractionated heparin and TDM or DMβCD was below the level required

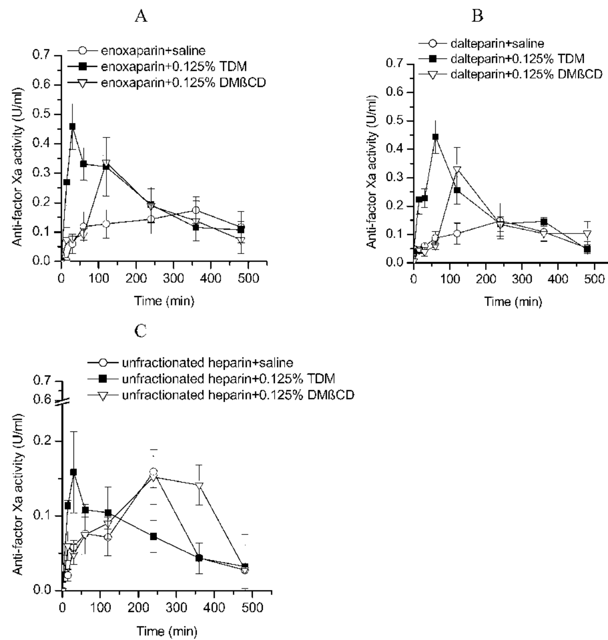

Fig. 6. Changes in plasma anti-factor Xa activity after pulmonary administration (50 U/kg) of enoxaparin (A), dalteparin (B), or unfractionated heparin (C) in the absence or presence of 0.125% TDM or DMβCD. Data represent mean ± standard deviation, n = 5 to 6.

for antithrombotic effect in rodent model (<0.2 U/ml). The reduced absorption of unfractionated heparin might be due to the fact that the unfractionated heparin fragments are much larger and bulkier than the enoxaparin and dalteparin. In fact, the average molecular weight of heparin is 3- to 6-fold higher than that of LMWH. The data on pulmonary absorption of three heparin preparations suggest that unfractionated heparin may not be suitable for pulmonary delivery, whereas LMWHs could be excellent candidates for pulmonary delivery.

The data presented in this manuscript is entirely consistent with our previous studies on nasal delivery of LMWHs (12,13). In the earlier studies, we have identified alkylmaltoside and cyclodextrin as absorption enhancers for nasal delivery of enoxaparin. The present study showed that LMWH formulated with dimethyl-β-cyclodextrin or tetradecylmaltoside, the most potent enhancers from two different families of excipients, could produce therapeutic anti-factor Xa level after administration via the pulmonary route. This study provides additional insights as to the superiority of pulmonary LMWH over nasally administered LMWH. In fact, a comparative analysis of the data obtained from nasal and pulmonary delivery of LMWH has shown that relative bioavailability of enoxaparin administered via the pulmonary route is 4- to 6-fold higher than the bioavailability of nasally administered enoxaparin (data not shown). Further, this study showed that the amounts of cyclodextrin and alkylmaltoside required to produce therapeutic anti-factor Xa activity from LMWH administered via the pulmonary route were 2–5 times less than those required for nasal administration of LMWH. More importantly, pulmonary delivery of LMWH, as opposed to nasal delivery described in earlier studies, will permit administration of the drug directly to the lung for the treatment of pulmonary embolism. Because almost one-third of deep vein thrombosis patients may develop pulmonary embolism and death can occur within hours of the onset of pulmonary embolism, administration of an anticoagulant drug directly to the lung could be extremely important to reduce the mortality from such an attack. Nasal delivery, although noninvasive compared to subcutaneous LMWH, may not be that much help in the event of an attack from pulmonary embolism.

In conclusion, two structurally distinct absorption enhancers, TDM and DMβCD, were tested for their effects on increasing the permeability and absorption of LMWH following administration via the pulmonary route. *In vitro* studies in Calu-3 cells suggest that addition of either TDM or DMβCD to the apical chamber results in a significant increase in ^{3}H-enoxaparin or ^{14}C-mannitol permeability and decrease in TEER across the Calu-3 cells. *In vivo* studies showed that both TDM and DMβCD enhance pulmonary LMWH absorption. While both agents are effective in enhancing *in vitro* and *in vivo* pulmonary absorption of LMWHs, TDM is found to be more efficacious than DMβCD. Data obtained from *in vitro* and *in vivo* studies are consistent with the fact that both agents increase drug transport by increasing the permeability of the respiratory membrane, rather than forming a complex with the drug. The *in vitro* TEER reversibility experiments coupled with previous *in vivo* reversibility studies suggest that the effects of TDM and DMβCD on the respiratory membrane are reversible and diminish with time. In summary, pulmonary LMWH with a quick onset of action could be used as an alternative to subcutaneous injection for the treatment

Table III. Pharmacokinetic Parameters for Pulmonary Formulations (50 U/kg) of Enoxaparin, Dalteparin, or Unfractionated Heparin (Heparin) With or Without TDM or DMβCD[a]

Heparins	Enhancers	C_{max} (U/ml)	T_{max} (min)	K_a (min^{-1})	MRT (min)	$AUC_{0\rightarrow480}$ (U · min/ml)
Enoxaparin	Saline	0.18 ± 0.04	360 ± 48	0.003 ± 0.0005	419 ± 88	46.7 ± 13.5
	0.125% DMβCD	0.33 ± 0.05[b]	160 ± 25[b]	0.006 ± 0.002	251 ± 19	66.5 ± 7.4
	0.125% TDM	0.46 ± 0.07[b]	47 ± 8[b]	0.007 ± 0.003	325 ± 89	109.1 ± 19.2[b]
Dalteparin	Saline	0.19 ± 0.04	320 ± 69	0.007 ± 0.001	253 ± 33	42.4 ± 3.8
	0.125% DMβCD	0.33 ± 0.07[b]	120[b]	0.004 ± 0.001	332 ± 54	62.9 ± 14.2
	0.125% TDM	0.44 ± 0.05[b]	40 ± 9[b]	0.005 ± 0.001	234 ± 21	104.5 ± 2.5[b]
Heparin	Saline	0.16 ± 0.02	240	0.004 ± 0.001	361 ± 97	34.1 ± 4.2
	0.125% DMβCD	0.17 ± 0.02	200 ± 40	0.003 ± 0.001	299 ± 66	43.6 ± 4.8
	0.125% TDM	0.19 ± 0.04	60 ± 30[b]	0.003 ± 0.001	491 ± 160	43.1 ± 6.2

[a] Data represent mean ± standard deviation, n = 5 to 6.

[b] Significantly different from those obtained without enhancer, $p < 0.05$.

of pulmonary embolism. Future studies will be directed to investigate the effects of the proposed formulations on rodent model of pulmonary embolism.

ACKNOWLEDGMENT

This work was supported in part by a New Investigator Award (0265182Y) from the American Heart Association, Texas Affiliate, Austin, TX.

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