Pharmaceutical Research, Vol. 22 No. 11, November 2005 (© 2005)
DOI: 10.1007/s11095-005-7676-z

Research Paper

Insulin Containing Nanocomplexes Formed by Self-Assembly from Biodegradable Amine-Modified Poly(Vinyl Alcohol)-Graft-Poly(L-Lactide): Bioavailability and Nasal Tolerability in Rats

Michael Simon,[1] Matthias Wittmar,[1] Thomas Kissel,[1] and Thomas Linn[2,3]

Received April 1, 2005; accepted July 28, 2005

Purpose. The bioavailability and local tolerability of insulin containing nanocomplexes from amine-modified poly(vinyl alcohol)-graft-poly(L-lactide) were studied in rats. Histology of the nasal epithelium was studied to document integrity of the mucosa.
Methods. Nanocomplexes (NC) were prepared by spontaneous self-assembly of insulin and the water-soluble amphiphilic polymer. Changes in blood glucose and insulin blood concentration were monitored in anesthetized rats using a glucose meter and enzyme-linked immunosorbent assay, respectively. Histological sections of the nasal cavity were examined after H&E staining by light microscopy.
Results. NC reduced blood glucose level in fasted healthy rats by 20% after 50–80 min and in streptozotocin induced diabetic rats by 30% within 75–95 min compared to basal levels. In both animal models significant concentrations of human insulin were detected, with relative bioavailabilities F_{rel} of 2.8 up to 8.3%. The more hydrophobic, lactic acid grafted polyester were more effective at a threefold higher polymer concentration, increasing the relative bioavailability F_{rel} of a 5 IU/kg dose from 2.8 to 5.7%. Histological examination of the nasal mucosa after 4 h showed no signs of toxicity at the site of nasal administration.
Conclusions. These results demonstrate that the NC significantly enhanced insulin absorption, suggesting that amphiphilic biodegradable comb-polymers offer a promising approach for nasal peptide delivery.

KEY WORDS: bioavailability in rats; insulin; nanocomplexes; nasal administration; nasal carrier; nasal histology.

INTRODUCTION

Recent progress in biotechnology led to an enormous increase in the number of peptide and protein drugs that usually require parenteral administration to become therapeutically useful (1,2). Nonparenteral administration across mucosal surfaces (e.g., rectal, buccal, vaginal, nasal, and pulmonary epithelia) have been considered as alternative routes of application to replace parenteral injections (3,4). Among them, nasal delivery of peptides attracted strong interest because of its favorable biopharmaceutical properties. The structure of the nasal epithelium, its high blood perfusion, and lower detrimental enzyme concentrations may result in higher bioavailabilities as compared to oral administration (5). Nasal drug administration facilitates self-medication, thereby improving patient compliance. However, protein and peptide drugs show frequently low and variable bioavailabilities after nasal administration, e.g., for insulin <1% (6). Therefore, nasal insulin therapy necessitates coadministration of absorption enhancers such as surfactants, bile salts, fusidic acid derivatives, medium-chain fatty acids, chelators, or enzyme inhibitors to increase absorption and hence bioavailability (7,8). Although these absorption enhancers significantly increase transmucosal peptide transport, local tolerability and safety concerns, related to membrane damage, ciliary toxicity, mucus discharge, and epithelial disruption, need to be overcome (9).

Consequently, the design of appropriate peptide carrier systems avoiding harmful additives has become a topic of intensive research. Nanoscale colloidal carriers from hydrophilic and biocompatible polymers, such as chitosan (10), were found to be useful for nasal vaccine (11) and insulin delivery (12) without employing additional penetration enhancers. Not only size and surface properties, but also the polymer composition and architecture affected the functional properties of nanocarriers, such as transport of macromolecules across biological surfaces. In general, nanoscale dimensions favor transport of particles across mucosal epithelia (13,14).

Moreover, nanocarriers with bioadhesive properties, e.g., NP coated with Carbopol, prevented rapid nasal clear-

[1] Department of Pharmaceutics and Biopharmacy, Philipps University, Ketzerbach 63, D-35032 Marburg, Germany.
[2] Medical Clinic and Policlinic 3, Justus-Liebig University Giessen, Rodthohl 6, D-35392 Giessen, Germany.
[3] To whom correspondence should be addressed. (e-mail: thomas.linn@innere.med.uni-giessen.de)

ance (15). Increasing residence time in the nasal cavity was thought to improve drug absorption (16,17). Through the design of polymer properties, nanoparticle characteristics such as hydrophilicity and surface charge can be manipulated. In particular, polycations such as poly(l-lysine), protamine, poly(ethylene imine), chitosan, and dextran derivatives seem to increase mucosal permeability (18–20). The combination of enhancing properties, mediated through cationic surfaces charges, with the formation of nanoparticulate carriers was achieved by encapsulating insulin into chitosan NP by ionotropic gelation, which yielded a significant blood glucose reduction in rabbits after nasal administration (21).

In an attempt to combine different enhancer principles—amphiphilicity, cationic charges, and bioadhesion—into one macromolecule, we studied water-soluble amine-modified polyesters, namely, poly(vinyl 3-(diethyl)propylcarbamate-*co*-vinyl acetate-*co*-vinyl alcohol)-as cationic backbone and the corresponding graft-polyesters with short (L-lactic acid) side chains, for their potential to form insulin nanocomplexes by spontaneous self-assembly (22). The objective of the present work was to evaluate two formulations of NC, composed of polymers P(26) and P(26)-2_{LL}, as a novel nasal delivery system for insulin in a rat model and to explore the influence of the different polymeric structures on absorption enhancement.

MATERIALS AND METHODS

Materials

Polymers were synthesized and characterized as previously described (23). As abbreviation for the polymers, we use P(X)-Y_{LL}. P(26) carried 26 amino groups per PVA molecule (DP = 300). P(26)-2_{LL} had an additional 48 short lactide chains per backbone with an average chain length of about three lactic acid units. Human recombinant insulin (26.2 IU/mg) was a gift from Aventis Pharma AG (Frankfurt, Germany).

Nanocomplexes Preparation

Insulin stock solutions with concentrations of 4.3, 5.7, and 11.4 mg/mL for 4.0, 5.0, and 10 IU/kg doses, respectively, were prepared in two steps: (1) insulin powder was dissolved in 87% (v/v) 1.15×10^{-2} N HCl in 10-mL Pyrex® tubes; (2) 13% (v/v) of 0.1 *N*-Tris(hydroxymethyl)amino methane solution was added, resulting in a clear Tris buffer solution with low ionic strength ($I = 0.01$) and pH 7.4. Polymer stock solutions in various concentrations, 2.15 mg/mL for P(26) and 7.30, 9.70, and 29.70 mg/mL for P(26)-2_{LL}, were prepared in Tris buffer. An equal volume of the filtered stock solutions of insulin and polymer were mixed resulting in defined ratios (Table I) of insulin/polymer in the final NC solution as previously described (22). The colloidal polymer–insulin complexes were formed after mixing of aqueous buffer solutions containing insulin and polymer via spontaneous self-assembly.

Animal Preparation and Dosing

Male Wistar rats (Charles River Wiga, Sulzfeld, Germany) weighing about 250 g were fasted overnight with free access to water. Diabetic rats were obtained after streptozotocin treatment (55 mg/kg) and included into the experiment when their blood glucose levels exceeded 250 mg/dL. Rats were anesthetized by ether inhalation for ~2 min in a sealed glass container, followed by intraperitoneal injection of avertin. To prepare avertin, stock solution was prepared with 25.0 g 2,2,2-tribromoethanol in 15.5 mL *tert*-amyl-alcohol (Sigma-Aldrich, Deisenhofen, Germany) mixed for 12 h in a dark bottle at room temperature. Working solution was made fresh before use of 0.5 mL Avertin stock added with 39.5 ml 0.9% NaCl (Braun-Melsungen, Melsungen, Germany) and filtered through a 0.2-μm filter. The animals were dosed with 10 μL/g body weight by intraperitoneal injection and remained anesthetized for 60–90 min (healthy) and 30–40 min (diabetic) in a supine position. NC suspensions (~20 μl) were carefully delivered to the right nostril only, by using a Hamilton syringe with an attached polyethylene tube, which was inserted about 0.5 cm into the nostril. The animal study was conducted according to the principles outlined in the *Guide for the Care and Use of Laboratory Animal Resources* (National Academy Press, Washington, DC) and had received approval from the local Animal Review Committee.

Glucose and Insulin Measurements

Blood glucose levels were directly measured in blood collected from the tip of the rat tail using a glucose meter (Glucometer Elite; Bayer Corp., Leverkusen, Germany).

Table I. Characterization of Administered Solutions/Nanocomplexes

Formulation	Insulin single dose (IU)[a]	Insulin conc. (IU/kg)	Polymer conc. (mg/kg)	Particle size (mean ± SD) (nm)	Zeta potential (mV)	Mass ratio (insulin/polymer)
Group of healthy rats						
INS Sol, s.c.	0.125	0.5	–	–	–	–
INS Sol, i.n.	1.0	4.0	–	–	–	–
INS/P(26) NC	1.0	4.0	0.076	394 ± 61	17.7 ± 2.7	2.0:1.0
INS/P(26)-2_{LL} NC	1.0	4.0	0.260	250 ± 39	18.8 ± 3.3	1.0:1.7
Group of diabetic rats						
INS Sol, s.c.	0.1875	0.75	–	–	–	
INS/P(26)-2_{LL} NC	1.25	5.0	0.324	275 ± 52	18.4 ± 3.4	1.0:1.7
INS/P(26)-2_{LL} NC	1.25	5.0	0.973	448 ± 25	5.0 ± 1.1	1.0:5.1
INS/P(26)-2_{LL} NC	2.50	10	0.649	290 ± 62	22.9 ± 2.5	1.0:1.7

[a] Based on an average of 250 g body weight per animal.

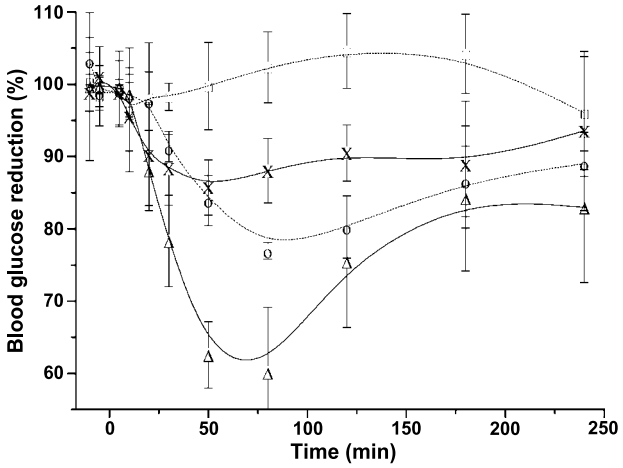

Fig. 1. Blood glucose reduction in healthy rats: —△— insulin (s.c.) with 0.5 IU/kg; —o— NC composed of P(26)-2_{LL} with 4.0 IU/kg insulin; —X— NC composed of P(26) with 4.0 IU/kg insulin; —□— control solution with 4.0 IU/kg insulin in buffer.

Blood samples (~200 μl) for insulin determination were collected in Microvettes® CB300 (Sarstedt, Nümbrecht, Germany) at the same time points of −10 and −5 min before formulation application and at various time intervals up to 4 h postadministration. Glucose content was calculated as a percentage of the mean value of the first three measurements for each animal. The values of the blood glucose baselines were in the range of 75–105 mg/dL for healthy rats, and 350–500 mg/dL for diabetic rats. The quantitative determination of human insulin in serum was carried out using an enzyme immunoassay enzyme-linked immunosorbent assay (ELISA) (Cat. EIA-2935; DRG Instruments GmbH, Marburg, Germany), which exhibited no cross-reactivity to rat insulin.

Analysis of Data

The areas under the curve (AUC) for insulin concentration against time in relation to glucose percentage against time were calculated for the various groups using the trapezoidal method. Statistical analysis was performed by one-way analysis of variances (ANOVA) using the Origin 6.0 software (OriginLab, Northampton, MA, USA). Throughout the experiment, the level of significance was chosen as $p < 0.05$.

Histological Studies

From healthy rats, three animals per group were randomly selected. The animals were decapitated, then the mandible, brain, and excess soft tissue were removed, and the residuals were fixated in formalin solution and decalcified in 10% Titriplex® III solution adjusted to pH 7.2 (Merck KG, Darmstadt, Germany) for 3 weeks. All regions were processed through to Paraplast® embedding (Carl Roth GmbH, Karlsruhe, Germany) using routine histological methods. Sections were cut serially at 7-μm thickness with a Reichert-Jung Biocut 2030, mounted, and stained with hematoxylin and eosin. Cross-sections of the nasal cavity were examined with the light microscope (Olympus BHZ), according to the method of Chandler *et al.* (24).

RESULTS AND DISCUSSION

Nondiabetic Rats

Previously, we described nanocomplex (NC) formation between insulin and amphiphilic cationic charge-containing biodegradable copolyesters composed of a hydrophilic PVA backbone grafted with short hydrophobic lactic acid groups (22). From a range of compounds we selected two polymers, the charge modified backbone P(26) and a corresponding graft-polyester P(26)-2_{LL}, to evaluate the influence of poly lactic acid (PLA) grafting. Insulin NC revealed interesting properties for a nasal peptide delivery system: positive zeta potential ~+20 mV, small particle sizes in the range of 200–450 nm, high insulin loading efficiency, and bioadhesive attributes as demonstrated for NP of DEAPA-PVA-g-PLGA (25). The dose for insulin was selected based on the expected pharmacological effect and the respective route of administration. Polymer concentrations ranged from 0.076 to 0.973 mg/kg body weight and reflected optimal polymer/insulin ratios from systematic investigations of the complexation process (Table I).

First, we studied the absorption enhancing properties of both formulations after nasal application in a nondiabetic, fasted rat model. Figure 1 shows the blood glucose time profiles, Fig. 2 the insulin time curves, and Table II the corresponding pharmacokinetic and pharmacodynamic parameters: $C_{max/min}$, $T_{max/min}$, AUC, and $F_{rel/dyn}$.

The pharmacodynamic effect of insulin NC *vs.* a subcutaneous reference is shown in Fig. 1. In the control group intranasally receiving a buffered solution with 4.0 IU/kg insulin, only a slight decrease in blood glucose concentration was observed, in accordance with data found in the literature (9). The stable glucose concentration (95–105%) in this group over the experimental period of 240 min indicated that the animals were not stressed by anesthesia and blood sampling procedures. The subcutaneous reference of 0.5 IU/kg

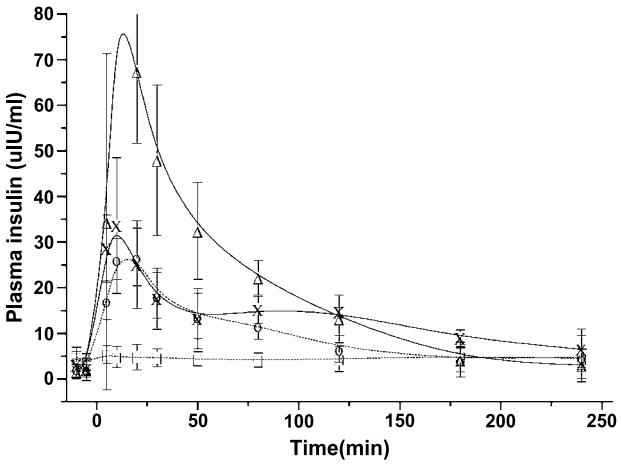

Fig. 2. Plasma insulin from healthy rats: —△— insulin (s.c.) with 0.5 IU/kg; —o— NC composed of P(26)-2_{LL} with 4.0 IU/kg insulin; —X— NC composed of P(26) with 4.0 IU/kg insulin; —□— control solution with 4.0 IU/kg insulin in buffer.

Table II. Pharmacokinetics and Pharmacodynamics of Insulin in a Healthy Rat Model

Analysis of pharmacodynamics

Formulation	n	T_{min} (min)	C_{min} (% basal glucose)	$AUC_{0–240\ min}$ (% min)	F_{dyn} (%)[f]
INS Sol s.c.[a]	4	72.5 ± 15.0	56.3 ± 4.2	4488 ± 795	100.0 ± 17.7
INS Sol i.n.[b]	4	107.5 ± 56.2	96.6 ± 2.0	348 ± 72	0.96 ± 0.2
INS-DEAPA26[c]	4	52.5 ± 20.6*/–	83.5 ± 2.1*/–	2292 ± 470	6.38 ± 1.3*/–
INS-DEAPA26/2[d]	4	82.5 ± 28.7*/–	78.2 ± 4.1*/–	2770 ± 324	7.72 ± 0.9*/–
One-way ANOVA[e]			$P > 0.05$		$P > 0.05$

Analysis of pharmacokinetics

Formulation	n	T_{max} (min)	C_{max} (μIU/mL)	$AUC_{0–240\ min}$ (μIU/mL min)	F_{rel} (%)[f]
INS Sol s.c.[a]	4	17.5 ± 9.6	78.7 ± 37.1	4861 ± 669	100.0 ± 13.7
INS Sol i.n.[b]	4	37.5 ± 55.5	5.9 ± 2.4	1098 ± 393	2.82 ± 1.0
INS-DEAPA26[c]	4	8.8 ± 2.5*/–	35.7 ± 13.8*/–	3237 ± 464	8.32 ± 1.2*/**
INS-DEAPA26/2[d]	4	15.0 ± 5.8*/–	29.1 ± 5.1*/–	2270 ± 360	5.84 ± 0.9*/**
One-way ANOVA[e]			$P > 0.05$		$P > 0.05$

[a] Insulin solution, administered by subcutaneous injection.
[b] Insulin solution administered intranasally.
[c] Insulin/P(26)-polymer nanocomplexes administered intranasally.
[d] Insulin/P(26)-2_{LL}-polymer nanocomplexes administered intranasally.
[e] Significance level of comparisons made between treated and control groups(*/), respective between nasally treated groups (/**), denoted when significant different otherwise (–).
[f] Calculation according to formula: $F = \frac{AUC_{Test} Dose_{s.c.}}{AUC_{s.c.} Dose_{Test}} \times 100$.

insulin yielded a C_{min} = 56% of the basal glucose concentration after 72 min. Corresponding insulin data in Fig. 2 showed values of C_{max} and T_{max} of about 79 μIU/mL and 18 min, respectively. AUC values resulting from s.c. application were used as benchmark to calculate the relative bioavailability $F_{dyn/rel}$.

Both the NC of insulin/P(26)-2_{LL} and -P(26) increased the insulin plasma level up to 29.1 ± 5.1 and 35.7 ± 13.8 μIU/mL, respectively, about 10–15 min after nasal instillation. The administration of insulin solution without polymer (control group) resulted in a low plasma level of 5.9 ± 2.4 μIU/mL. The partially elevated insulin absorption in the control group could be attributable to an effect of the anesthetic agent as suggested by Mayor and Illum (26).

It was not possible to dose fully conscious rats as control for the investigation of the effects of avertin, because nonanesthetized rats, as obligatory nose breathers, sneeze vigorously, and nasally administered solutions are discharged from the nasal cavity. Therefore, relative bioavailabilities between the different treatment groups were assessed, with all animals anesthetized in the same way. Those treated with insulin NC exhibited elevated insulin levels with F_{rel} of 5.84 ± 0.9% for insulin/P(26)-2_{LL} and 8.32 ± 1.2% for insulin/P(26), in comparison to the insulin control group with F_{rel} of 2.82 ± 1.0%. These findings suggest that the changes in blood glucose concentrations are a direct consequence of elevated insulin blood levels and not a decrease, e.g., due to other hormonal counterregulatory effects. The AUC values for both types of NC were significantly different compared to the control group ($p < 0.05$). The observation that the insulin control group exhibited no glucose change despite slightly elevated human insulin levels could be attributable to an autoregulation effect observed in healthy rats: Small amounts of absorbed exogenous insulin depresses the endogenous secretion of insulin by β cells, and consequently the amount of exogenous insulin does not reach a sufficient level to affect glycaemia (27). Otherwise, the exogenous insulin concentration in NC treated groups was too high for compensation by regulatory mechanism.

A correlation between applied insulin dose and insulin plasma level requires additional experiments and is beyond the scope of this investigation.

Improved insulin absorption after nasal administration of NC formulations was supported by the corresponding glucose data: The decrease in plasma glucose levels in both groups treated with nasally applied NC was significantly different ($p < 0.05$) from that induced by the insulin control solution. Insulin NC with P(26) lowered the basal glucose concentration by about 17% within 52 min and the NC with insulin/P(26)-2_{LL} about 22% after 82 min, respectively. The decrease lasted up to 180 min as values close to basal levels were recorded at the last measuring time point at 240 min.

The relative bioavailability F_{dyn} for insulin/P(26)-2_{LL} was in the range of 7.7% and for insulin/P(26) at about 6.38%, in contrast to 0.96% for the control group. This reflects a significant and distinctive metabolic response as consequence of insulin NC application. Although the pharmacological difference between both NC groups is not significant ($p < 0.05$), the glucose–time curve exhibited a differential course: The glucose minimum for P(26)-2_{LL} NC is more clearly pronounced and lasted for a longer time than the NC composed of insulin/P(26), indicating improved properties for the more hydrophobic polymers.

DIABETIC RATS

In diabetic rats insulin deficiency causes severe changes in metabolism among others, a reduction in liver protein synthesis, decreased activity of the sympathetic nervous system, dehydration, glycosuria, and osmotic diuresis (28,29).

Thus, streptozotocin-diabetic rats showed a different response profile to environmental stress factors (30). For instance, duration of anesthesia is significantly reduced and variability of blood glucose levels is increased. As this was observed for different control groups especially after 120 min, the pharmacological data were evaluated only for a timeframe of up to 120 min.

After establishing the penetration enhancement of P(26)-2_{LL}-insulin NC, we investigated in more detail formulation aspects, by varying polymer and insulin concentrations in the diabetic rat model. Figure 3 shows the glucose time courses, Fig. 4 the insulin time curves, and Table III the corresponding pharmacokinetic and pharmacodynamic parameters: $C_{max/min}$, $T_{max/min}$, AUC, and $F_{rel/dyn}$.

We administered an insulin dose of 5.0 IU/kg, maintaining the polymer concentration constant at a mass ratio of 1:1.7 insulin/polymer (option 1). At a constant insulin dose, we increased the polymer concentration threefold to a 1:5.1 mass ratio, to probe if an increase in insulin bioavailability could be related to the amount of instilled polymer (option 2). Finally, we increased concentration of NC in the suspension reaching a dose of 10.0 IU/kg at a constant 1:1.7 mass ratio (option 3).

After the subcutaneous administration of insulin solution (0.75 IU/kg), an immediate and distinctive decrease in glycemia was observed (Fig. 3). The $AUC_{0–120\ min}$ values for glucose/time and the $AUC_{0–240\ min}$ for insulin/time resulting from s.c. application were used as reference to calculate the bioavailabilities $F_{dyn/rel}$.

The NC of P(26)-2_{LL} insulin according to option 1 caused a clear blood glucose reduction as expected. The minimum was achieved after 93 min with 20% decrease in the blood glucose basal levels and a relative bioavailability F_{dyn} of 8.5%. Figure 4 depicts the corresponding rapid increase in human insulin levels for option 1 up to 18 μIU/mL within 14 min, corresponding to a bioavailability F_{rel} of about 2.8%.

The largest decrease in blood glucose was achieved with option 2, containing a threefold higher polymer concentration. The formulation according to option 2 exhibited a fast

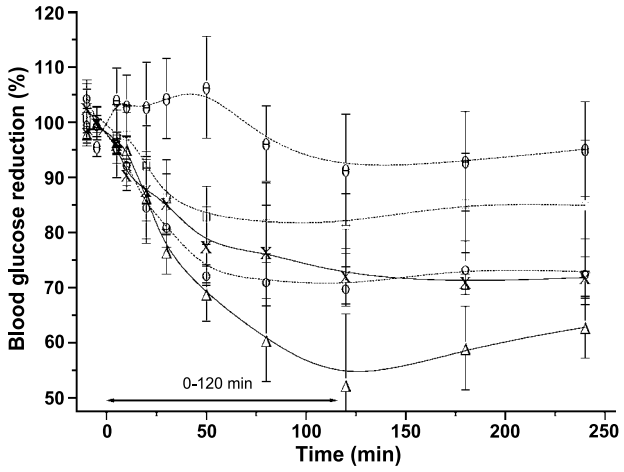

Fig. 3. Blood glucose reduction in diabetic rats: —△— insulin (s.c.) with 0.75 IU/kg; option 1: —□— NC composed of P(26)-2_{LL} (1.7:1.0 m/m polymer/insulin) with 5.0 IU/kg insulin; option 2: —o— NC composed of P(26)-2_{LL} (5.1:1.0 m/m) with 5.0 IU/kg insulin; option 3: —X— NC composed of P(26)-2_{LL} (1.7:1.0 m/m) with 10.0 IU/kg insulin; —θ— control solution with 5.0 IU/kg insulin in buffer.

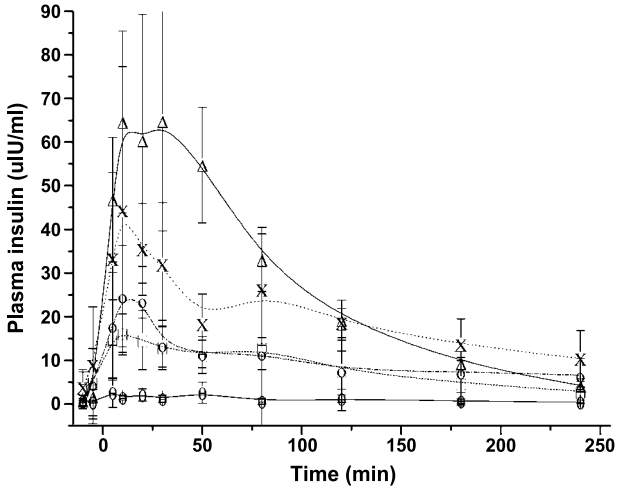

Fig. 4. Plasma insulin from diabetic rats: —△— insulin (s.c.) with 0.75 IU/kg; ··· X ··· NC composed of P(26)-2LL with 10.0 IU/kg insulin (1.7:1.0); — o — NC composed of P(26)-2LL with 5.0 IU/kg insulin (5.1:1.0); — □ — NC composed of P(26)-2LL with 5.0 IU/kg insulin (1.7:1.0); — θ — control solution with 10.0 IU/kg insulin in buffer.

onset of insulin action in the first 50 min, which is comparable to the s.c. application (Fig. 3). The blood glucose decrease reached a maximum of 30% of the basal values after 75 min. That correlates with the fast increasing blood insulin concentration to 30 μIU/mL after 14 min (Fig. 4). The resulting bioavailabilities with F_{dyn} 11.8% and F_{rel} 5.67% were significantly higher compared to option 1 ($p < 0.05$). Then we evaluated the effect of increasing the dose to 10.0 IU/kg at a higher concentration, but not increasing the volume. Following nasal administration, blood insulin concentrations rose to 47 μIU/mL after 17 min. The bioavailability F_{rel} with 5.3% was significantly increased compared to option 1. This increase could be attributed to a higher local NC concentration, leading to a higher concentration gradient at the site of absorption. Furthermore, the absolute polymer concentration was twofold higher compared to option 1.

Systematic investigations of the complex assembly demonstrated that a defined ratio of both binding partners is necessary for optimal NC formation (22). For insulin/P(26)-2_{LL} NC, the optimal insulin/polymer ratio is 1.0:1.7 (m/m) (see Table I). A threefold increase in the ratio for P(26)-2_{LL} NC from 1.7 to 5.1 increases the average complex size from 275 to 448 nm and reduces the zeta potential from about +18.4 to +5.0 mV (Table I). Increasing the ratio obviously reduces the specific advantages of nanocarriers; however, the bioavailability of 5.0 IU insulin as a single dose increases. We assume the spontaneous occurrence of complex rearrangement processes and an overall increase in free polymer concentration especially for higher polymer ratios (22). Increased bioavailability could therefore be likely related to a higher polymer concentration, resulting in a more direct interaction between polymer and epithelium, rather than the nanoparticle character of the carrier. Similar observations were published by Dyer *et al.* (31). They reported insulin–chitosan solution to be significantly more effective than the NP formulation, and they also observed an influence of chitosan concentration (20). Other researchers demonstrate the superiority of

Table III. Pharmacokinetics and Pharmacodynamics of Insulin in a Diabetic Rat Model

Analysis of pharmacodynamics

Formulation	n	T_{min} (min)	C_{min} (% basal glucose)	$AUC_{0–120\ min}$ (% min)	F_{dyn} (%)[f]
INS Sol, s.c.[a]	4	140.0 ± 34.6	50.7 ± 10.2	3576 ± 581	100.0 ± 16.3
INS Sol, i.n.	4	110.0 ± 663	87.7 ± 5.8	487 ± 93	2.04 ± 0.4
5.0 IU with 1.7[b]	4	93.3 ± 23.1$^{-/-}$	80.3 ± 6.1$^{*/-}$	2038 ± 261	8.54 ± 1.1$^{*/**}$
5.0 IU with 5.1[c]	4	75.0 ± 33.2$^{-/-}$	70.2 ± 4.2$^{*/-}$	2803 ± 213	11.76 ± 0.9$^{*/**}$
10.0 IU with 1.7[d]	4	160.0 ± 34.6$^{-/**}$	68.8 ± 3.1$^{*/-}$	2703 ± 401	5.67 ± 0.1$^{*/**}$
One-way ANOVA[e]			$P > 0.05$		$P > 0.05$

Analysis of pharmacokinetics

Formulation	n	T_{max} (min)	C_{max} (μIU/mL)	$AUC_{0–240\ min}$ (μIU/mL min)	F_{rel} (%)[f]
INS Sol, s.c.[a]	3	16.7 ± 11.5	67.5 ± 23.9	6456 ± 1063	100.0 ± 16.5
INS Sol, i.n.	4	21.3 ± 20.1	3.8 ± 2.9	261 ± 178	0.30 ± 0.2
5.0 IU with 1.7[b]	4	13.8 ± 7.5$^{*/-}$	17.9 ± 6.9$^{*/-}$	1194 ± 521	2.77 ± 1.2$^{*/**}$
5.0 IU with 5.1[c]	4	13.8 ± 7.5$^{*/-}$	30.0 ± 10.6$^{*/-}$	2438 ± 476	5.67 ± 1.1$^{*/-}$
10.0 IU with 1.7[d]	4	16.7 ± 5.8$^{*/**}$	47.0 ± 29.9$^{*/**}$	4533 ± 2201	5.27 ± 2.6$^{*/-}$
One-way ANOVA[e]			$P > 0.05$		$P > 0.05$

[a] Insulin solution administered by subcutaneous injection.
[b–d] Insulin/P(26)-2_{LL}-polymer nanocomplexes administered intranasally (option 1^b, option 2^c, option 3^d).
[e] Significance level of comparisons made between treated and control groups(*/), respective between nasally treated groups (/**), denoted when significant different otherwise (–).
[f] Calculation according to formula: $F = \frac{AUC_{Test} \times Dose_{s.c.}}{AUC_{s.c.} \times Dose_{Test}} \times 100$.

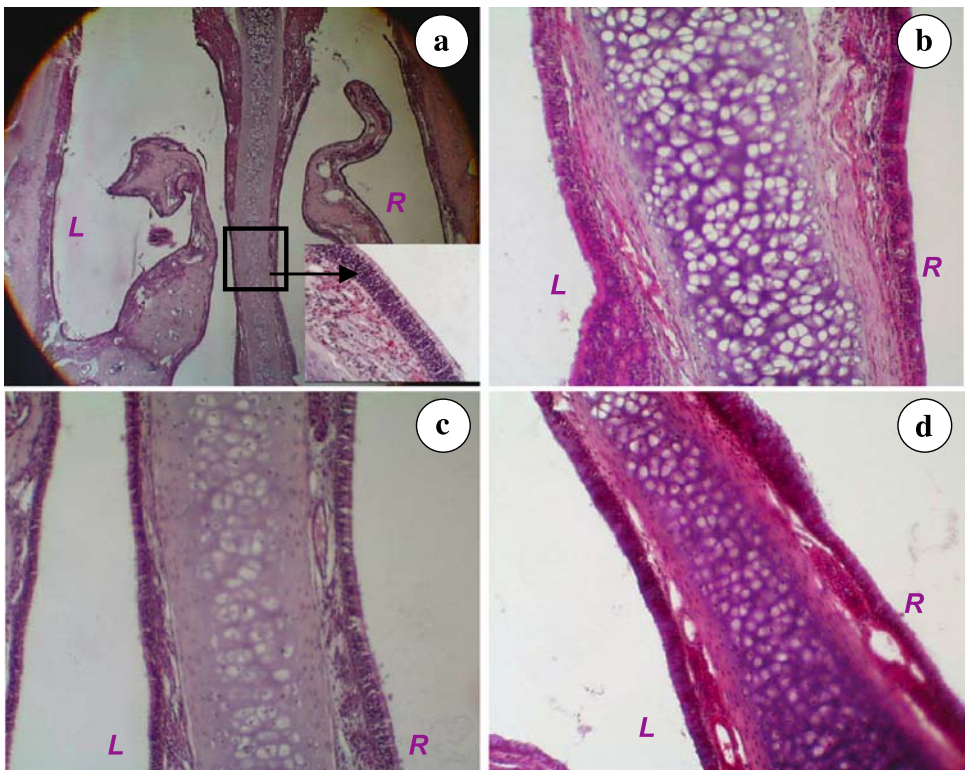

Fig. 5. Photomicrographs of vertical sections through the anterior rat nasal cavity showing two sides of the nasal septum 4 h after application of 20–25 μm insulin solution into the right side (R). Normal respiratory epithelium populated with goblet cells covers the undosed left side (L) in each case. Treated tissue on the right side can be compared with untreated tissue on the left of the septum; epithelium thickness on the right side was 134 ± 14 μm and on the left side 137 ± 15 μm (n.s.). (a) Section overview from control group (insulin, s.c.), magnification ×20. (b–d) Septum region from (a) indicated by the black rectangle, magnification ×100. (b) Nasal insulin application without polymer (nonpolymeric control). (c) NC composed of insulin and P(26)-2_{LL}. (d) NC composed of insulin and P(26). Panels (b)–(d) show no clear difference of the epithelial lining between dosed side and untreated side.

chitosan–insulin NP against solutions (12), but also discuss whether the effect of the nanoparticulate system is more likely due to an interaction of chitosan to epithelial membranes, in terms of bioadhesion and transient opening of the tight junctions (32).

It is generally accepted that two major pathways are available for transport of drugs across the epithelial membrane, namely, the paracellular and transcellular route. The tight junctions can be influenced by interaction of the epithelia with polymeric materials; in fact, we are currently investigating with the Caco 2 cell line to check if the cationic poly(vinyl 3-(diethyl amino) propylcarbamate-*co*-vinyl acetate-*co*-vinyl alcohol)-graft-poly(L-lactide) can interact with the junctional complex, temporarily opening the paracellular transport pathway as reported for chitosan (32).

HISTOLOGICAL STUDIES

Increased absorption of peptides after nasal application has been reported in a number of studies after coadministration with absorption enhancers such as surfactants, including bile salts and their derivatives, chelators, and enzyme inhibitors. Many absorption enhancers are irritants to mucous membranes, and the histological effects of some agents on nasal mucosa have been reported. Even after a single application of formulations containing enhancer such as laureth-9 (1% w/v) cellular erosion, cell–cell separation, dense mucous coating, and subsequent reduction of epithelial height from rat nasal epithelium were observed within 5 min after nasal application (9). Therefore, it is desirable to evaluate the histopathological effects. The anatomical division of the rat nasal cavity by the midline septum enables the assessment of histopathological effects by direct comparison of the treated and untreated side. Errors due to interanimal variability and tissue sampling are thus minimized. The major structural features in each region of the rat nasal cavity, including the septum and the turbinates, could be identified in the overview micrographs (Fig. 5a). In particular, the area of the nasal septum, which consists of the respiratory epithelium, is of interest, because it is the projected deposition site for the administered dose. The magnification in Fig. 5a shows the typical anatomy of the septum: cartilage, covered by ciliated pseudostratified columnar epithelium, populated with goblet cells. A basic requirement for comparability and reproducibility of the histological and pharmacological results is that the administered dose is deposited on a restricted area. For small volumes of 20–30 μl per dose, no leakage via the septal window in more posterior regions was reported. Volumes in this range are known to be retained exclusively at the site of application (9).

Histological tissue preparation was performed at the end of blood measurements (after 4 h), so that toxicological effects of longer-lasting adhesion of polymer residues were taken into account. In each animal treated with NC or insulin control solution, all regions of the dosed side of the cavity were covered by an intact, undamaged epithelium layer, comparable to that observed in the sections of the nondosed side. On the dosed side, no epithelial disruption occurred on septal and nasoturbinate surfaces either for insulin/P(26)-2_{LL} NC (Fig. 5c) or for insulin/P(26) NC (Fig. 5d). Moreover, the treated and untreated side of the nasal cavity in each cross-section matched each other with respect to the height of the respiratory epithelia. Repeated dosing and recovery studies are necessary to establish the full toxicological implications of a chronic administration. However, it should be noted that no acute adverse effects were registered in our single-dose experiment for both NC preparations.

CONCLUSION

As an alternative application route for insulin, the intranasal administration along with pulmonary delivery represents one of the most promising options for treating diabetic patients. The commercial exploitation of nasal insulin delivery depends on the development of new and safe enhancers, increasing the absorption and hence bioavailability. In this work, we report on the feasibility of two types of insulin NC from a novel class of amphiphilic comb-like polyesters demonstrating enhanced insulin absorption across rat nasal mucosa. These data indicated that amphiphilic NC with grafted lactic acid side chains were superior to more hydrophilic backbones. The relative effect availabilities (glucose) and bioavailability (insulin) suggest that significant enhancement factors can be attained using NC. Both nanocomplex preparations were shown to be nontoxic after a single nasal administration. The effect of lowering blood glucose levels observed in healthy rats could be reproduced in diabetic rats. Therefore, NC, on the basis of insulin/P(26)-2_{LL} NC, may present a promising delivery system for peptides enhancing mucosal absorption. Further work is warranted to elucidate the enhancer mechanism.

REFERENCES

1. L. Andersson, L. Blomberg, M. Flegel, L. Lepsa, B. Nilsson, and M. Verlander. Large-scale synthesis of peptides. *Biopolymers* **55**:227–250 (2000).
2. A. E. Pontiroli. Peptide hormones: review of current and emerging uses by nasal delivery. *Adv. Drug Deliv. Rev.* **29**:81–87 (1998).
3. D. J. Chetty and Y. W. Chien. Novel methods of insulin delivery: an update. *Crit. Rev. Ther. Drug Carrier Syst.* **15**(6):629–670 (1998).
4. M. Simon and T. Kissel. Away with the needle. Noninvasive administration routes for insulin: improved quality of life for diabetics. *Pharm. Unserer Zeit* **30**(2):136–141 (2001).
5. S. Gizurarson and E. Bechgaard. Study of nasal enzyme activity towards insulin. *In vitro*. *Chem. Pharm. Bull.* **39**(8):2155–2157 (1991).
6. M. J. Deurloo, W. A. Hermens, S. G. Romeyn, J. C. Verhoef, and F. W. Merkus. Absorption enhancement of intranasally administered insulin by sodium taurodihydrofusidate (STDHF) in rabbits and rats. *Pharm. Res.* **6**(10):853–856 (1989).
7. M. Hinchcliffe and L. Illum. Intranasal insulin delivery and therapy. *Adv. Drug Deliv. Rev.* **35**:199–234 (1999).
8. A. Sanchez, P. Ygartua, and D. Fos. Role of surfactants in the bioavailability of intranasal insulin. *Eur. J. Drug Metab. Pharmacokinet.* **3**:120–124 (1991).
9. S. G. Chandler, L. Illum, and N. W. Thomas. Nasal absorption in the rats: II. Effect of enhancers on insulin absorption and nasal histology. *Int. J. Pharm.* **76**:61–70 (1991).
10. C. Prego, M. Garcia, D. Torres, and M. J. Alonso. Transmucosal macromolecular drug delivery. *J. Control. Release* **101**(1–3): 151–162 (2005).
11. M. Koping-Hoggard, A. Sanchez, and M. J. Alonso. Nanoparticles as carriers for nasal vaccine delivery. *Expert Rev. Vaccines* **4**(2):185–196 (2005).

12. R. Fernandez-Urrusuno, P. Calvo, C. Remunan-Lopez, J. L. Vila-Jato, and M. J. Alonso. Enhancement of nasal absorption of insulin using chitosan nanoparticles. *Pharm. Res.* **16**(10): 1576–1581 (1999).
13. J. Brooking, S. S. Davis, and L. Iluum. Transport of nanoparticles across the rat nasal mucosa. *J. Drug Target.* **9**(4):267–279 (2001).
14. A. Vila, A. Sanchez, C. Evora, I. Soriano, O. McCallion, and M. J. Alonso. PLA–PEG particles as nasal protein carriers: the influence of the particle size. *Int. J. Pharm.* **292**(1–2):43–52 (2005).
15. H. Takeuchi, H. Yamamoto, and Y. Kawashima. Mucoadhesive nanoparticulate systems for peptide drug delivery. *Adv. Drug Deliv. Rev.* **47**(1):39–54 (2001).
16. P. Dondeti, H. Zia, and T. Needham. Bioadhesive and formulation parameters affecting nasal absorption. *Int. J. Pharm.* **127**:115–133 (1996).
17. M. R. Jimenez-Castellanos, H. Zia, and C. T. Rhodes. Mucoadhesive drug delivery systems. *Drug Dev. Ind. Pharm.* **19**:143–194 (1993).
18. D. N. Granger, P. R. Kvielys, M. A. Perry, and A. E. Taylor. Charge selectivity of rat intestinal capillaries—influence of polycations. *Gastroenterology* **91**:1443–1446 (1986).
19. Y. Maitani, Y. Machida, and T. Nagai. Influence of molecular weight and charge on nasal absorption of dextran and DEAE-dextran in rabbits. *Int. J. Pharm.* **76**:43–49 (1992).
20. L. Illum, N. F. Farraj, and S. S. Davis. Chitosan as novel nasal delivery system for peptide drugs. *Pharm. Res.* **11**:1186–1189 (1994).
21. R. Fernandez-Urrusuno, P. Calvo, C. Remunan-Lopez, J. L. Vila-Jato, and M. J. Alonso. Enhancement of nasal absorption of insulin using chitosan nanoparticles. *Pharm. Res.* **16**(10): 1576–1581 (1999).
22. M. Simon, M. Wittmar, U. Bakowsky, and T. Kissel. Self-assembling nanocomplexes of insulin and water-soluble branched poly[(vinyl-3-(diethyl amino)propylcarbamate-*co*-(vinyl acetate)-*co*-(vinyl alcohol)]-graft-poly(D,L-lactid): a novel carrier for transmucosal delivery of peptides. *Bioconjug. Chem.* **15**(4):841–849 (2004).
23. M. Wittmar, Charge modified, comb-like graft-polyesters for drug delivery and DNA vaccination: synthesis and characterization of poly(vinyl dialkylaminoalkylcarbamte-co-vinyl acetate-co-vinyl alcohol)-graft-poly(D,L-lactide-co-glycolide)s, Dissertation, Philipps University Marburg, 2004. http://archiv.ub.uni-marburg.de/diss/z2004/0075/ (accessed 01/13/04).
24. S. G. Chandler, L. Illum, and N. W. Thomas. Nasal absorption in the rats: I. A method to demonstrate the histological effects of nasal formulations. *Int. J. Pharm.* **70**:19–27 (1991).
25. L. A. Dailey, E. Kleemann, M. Wittmar, T. Gessler, T. Schmehl, C. Roberts, W. Seeger, and T. Kissel. Surfactant-free, biodegradable nanoparticles for aerosol therapy based on the branched polyesters, DEAPA-PVAL-g-PLGA. *Pharm. Res.* **20**(12): 2011–2020 (2003).
26. S. H. Mayor and L. Illum. Investigation of the effect of anesthesia on nasal absorption of insulin in rats. *Int. J. Pharm.* **149**:123–129 (1997).
27. C. Damge, C. Michel, M. Aprahamian, and P. Couvreur. New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier. *Diabetes* **37**(2):246–251 (1988).
28. A. B. Anwana and H. O. Garland. Intracellular dehydration in the rat made diabetic with streptozotocin: effects of infusion. *J. Endocrinol.* **128**(3):333–337 (1991).
29. T. Yoshida, H. Nishioka, Y. Nakamura, and M. Kondo. Reduced noradrenalin turnover in streptozotocin-induced diabetic rats. *Diabetologia* **28**(9):692–696 (1985).
30. L. L. Bellush and W. N. Henley. Altered responses to environmental stress in streptozotocin-diabetic rats. *Physiol. Behav.* **47**(2):231–238 (1990).
31. A. M. Dyer, M. Hinchcliffe, P. Watts, J. Castille, I. Jabbal-Gill, R. Nankervis, A. Smith, and L. Illum. Nasal delivery of insulin using novel chitosan based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. *Pharm. Res.* **19**(7):998–1008 (2002).
32. N. G. Schipper, S. Olsson, J. A. Hoogstraate, A. G. de Boer, K. M. Varum, and P. Artursson. Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement. *Pharm. Res.* **14**(7):923–929 (1997).