ABSORPTION AND METABOLISM OF THE ABSORPTION ENHANCER 
DIDECANOYLPHOSPHATIDYLCHOLINE IN RABBIT NASAL EPITHELIUM IN VIVO

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ABSTRACT:
The absorption enhancer, didecanoylphosphatidylcholine (DDPC), improves the nasal absorption of human growth hormone in rabbits. We elucidated the uptake and the metabolism of 1,2-di[1,14C]decanoyl-L-3-phosphatidylcholine and 1,2-didecanoyl-L-3-phosphatidy[1H-methyl]choline in rabbit nasal mucosa in vivo. One minute after nasal application of DDPC, 4.4–7.5% of the applied dose was found absorbed into the mucosa. The retained radioactivity left the tissue in <2 hr. The lipophilic tissue extract revealed that, at t = 1 min, only 1.1–1.4% of the applied dose was found as intact DDPC in the nasal mucosa. The other labeled compounds were decanoic acid, DDPC reacylated with endogenous fatty acids, a neutral lipid, and very small amounts of lyso-DDPC and phosphatidylethanolamine. The water-soluble metabolites revealed formation of phosphorylcholine, glycerophosphorylcholine, cytidinediphosphatecholine, and a slight amount of choline. The detection of these metabolites suggests that DDPC was rapidly cleared from the nasal mucosa, partly by degradation by phospholipases. In addition, data illustrated reutilization of DDPC metabolites in the formation of cytidinediphosphatecholine and phosphatidylethanolamine, together with DDPC being reacylated with endogenous fatty acids. The rapid formation of decaonic acid raises the possibility that this acid may contribute significantly to the drug-enhancing properties of DDPC.

Due to improved biotechnology, the accessibility of biologically active peptides to the pharmaceutical industry has increased considerably. However, a limiting factor in the development of peptide drugs is the relative ineffectiveness when given parenterally. Almost all peptide drugs are parenterally administered, although parenterally administered peptide drugs are often connected with low patient compliance. As alternatives to these routes, several nonparenteral routes have been investigated for peptide drug delivery (e.g. nasal, buccal, rectal, vaginal, pulmonal, and transdermal). Among these routes, Aungst et al. (1) considered the nasal route to be the most promising. The i.n.1 delivering of peptide drugs offers several advantages (e.g. easy application, fast absorption, avoidance of hepatic first-pass metabolism, and destruction of the peptide in the gastrointestinal tract) (2–4). A common important drawback of this route is, however, that the mucosa permeability decreases for higher molecular weight compounds (5). Thus, i.e. application of large, hydrophobic drugs (such as polypeptides and proteins) shows a very low bioavailability (5), and coadministration of absorption enhancers is often required to achieve therapeutic blood levels (6, 7). Different types of absorption enhancers have been investigated, such as bile salts, chelating agents, and

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1 Abbreviations used are: i.n., intranasal; DDPC, didecanoylphosphatidylcholine; α-CD, α-cyclodextrin; 14C-DDPC, 1,2-di[1,14C]decanoyl-L-3-phosphatidylcholine; 3H-DDPC, 1,2-didecanoyl-L-3-phosphatidy[1H-methyl]choline; MAG, monoacylglycerol; DAG, didecanoylglycerol; CHO, choline; P-CHO, phosphorylcholine; CDP-CHO, cytidinediphosphatecholine; GPC, glycerophosphorylcholine; PC, phosphatidylcholine; PLα, phospholipase A2; PLβ, phospholipase A2; TAG, triacylglycerol; PLC, phospholipase C; PLD, phospholipase D; DPPC, dipalmitoylphosphatidylcholine.

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Materials and Methods

Radiolabeled DDPC (14C-DDPC) (specific activity: 67 mCi/mmol) and 3H-DDPC (specific activity: 85 Ci/mmol) were from Amersham International (Buckinghamshire, UK). The initial radiochemical purity was ≥95%. Novo Nordisk A/S (Bagsvaerd, Denmark) delivered the unlabeled DDPC powder. Lipid standards were obtained from Sigma Chemical Co. (St. Louis, MO) TLC plates (silica gel 60 and silica gel 60 W) and organic solvents were obtained from Merck (Darmstadt, Germany). For autoradiography, Kodak Safety Film was used.

Study Design. A radiolabeled DDPC solution (14C-DDPC and 3H-DDPC), including 0.1% unlabeled DDPC in Krebs-Ringer buffer, was prepared by sonication. After sonication, a sample of the DDPC solution was incubated for 2 hr in Krebs-Ringer buffer at 37°C. Subsequently, the stability of this DDPC was
evaluated by TLC and found 98–99% intact. Thus, a dose of 50 μl contained 50 μg unlabeled DDPC, 2,727,300 dpm 3H-DDPC (final specific activity: 10 mCi/mmole), and 4,284,000 dpm 14C-DDPC (final specific activity: 22 mCi/mmole).

Twenty-five New Zealand White rabbits weighing 2.5–3.5 kg (Novo Nordisk A/S) were divided into five test groups, with five rabbits in each group. Animals were anesthetized with pentobarbital (30 mg/kg iv) immediately before the experiment started. At \( t = 0 \), groups 1–5 were dosed intranasally with 50 μl test preparation in one nostril. At \( t = 1 \), 15, 30, 60, and 120 min, the animals were killed by 200 mg pentobarbital (intravenously; five animals at each time point), and the nasal mucosas were isolated as described by Carstens et al. (14). The isolation procedure took 5 min before the mucosas were placed on ice. This period may have influence on the metabolite profile. The isolated mucosas were washed 5 times in a total of 5 ml of ice-cold 0.9% NaCl. Autoradiography of the intact mucosas was done to evaluate the localization of the radioactivity. The mucosas were placed on a board. The film was fixed firmly on the mucosas and exposed at −80°C in the dark for 1 week. The mucosas and rinsing water from the first wash of each mucosa were analyzed for DDPC and DDPC metabolites.

Lipid Analysis. The nasal mucosas were homogenized and extracted with chloroform/methanol (2:1) and 0.92% CaCl solution to derive lipid and aqueous fractions. Radioactivity in both fractions was determined.

Internal standards were added to the lipid fraction: 1-decanoylglycerophosphorylcholine, decanoic acid, MAG, DAG, PE, cholesteryl ester, and DDPC. The metabolites in the lipid fraction were separated by TLC on silica gel 60, and the plates were developed for 72 min in a solvent system of chloroform/methanol/ammonia/water (120:80:10:5). The plates were dried and then rotated 90° and developed for 90 min in a solvent system of chloroform/acetone/methanol/acetic acid/water (100:40:30:20:12). After the second development, the TLC plates were exposed for autoradiography for 1 week at −80°C.

Internal standards were added to the aqueous fraction: CHO, P-CHO, CDP-CHO, and GPC. Metabolites in the aqueous fraction were separated by TLC on silica gel 60 W. Plates were developed for 3–4 hr in a solvent system of 0.5% NaCl ethanol/methanol/concentrated ammonium hydroxide (50:30:25:20-5) and then exposed for autoradiography for 1 week at −80°C. Radioactivity was visualized by autoradiography, and the silica gel of these areas were scraped for quantitative measurement of the radioactivity by liquid scintillation counting (Packard 1900 TR liquid scintillation analyzer).

Data are presented as means ± SE (N = 5).

Results

Autoradiography of intact mucosas showed that the amount of total radioactivity associated with the tissue was largest at \( t = 1 \) min and smallest at \( t = 120 \) min, and that the dosing was reproducible (data not shown). 203,049 3H-dpm ± 10,883 3H-dpm and 187,861 14C-dpm ± 15,201 14C-dpm were absorbed at \( t = 1 \) min (fig. 1), which corresponded to an absorption of 7.5 ± 0.4% and 4.4 ± 0.4% of the applied dose, respectively (fig. 2). At \( t = 1 \) min, 9.1 ± 2.0% of the applied 3H-dose and 6.1 ± 1.3% of the applied 14C-dose was found in the mucosal wash water (N = 5, data not shown). Preliminary studies of dosing-colored DDPC solutions showed pronounced accumulation of color in the nasal conchae. Besides this, a majority of the dose was probably swallowed or to a minor degree lost by sneezing. At \( t = 1 \) min, only 1.4 ± 0.3% 3H-dpm and 1.1 ± 0.2% 14C-dpm of the applied dose corresponded to intact DDPC (fig. 2). This indicated that only a small part of the applied DDPC was associated with the nasal epithelium as intact DDPC. Total radioactivity and radioactivity of intact DDPC in the tissue decreased with time (figs. 1 and 2). Two hours after dosing, only 4.1 ± 1.1% 3H-radioactivity and 0.2 ± 0.0% 14C-radioactivity of the applied dose was tissue-associated, and only 0.02 ± 0.00% 3H- and 14C-radioactivity corresponded to intact DDPC (fig. 2). This means that the DDPC taken up at \( t = 1 \) min was nearly 100% decomposed or disappeared within 2 hr.

At the start of the experimental period (\( t = 1 \) min), the 14C radioactivity of the lipid fraction as evaluated by TLC was mainly associated with decanoic acid (35.3 ± 0.4%), intact DDPC (29.6 ±

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**Fig. 1. Catabolism of 3H-DDPC and 14C-DDPC in the nasal mucosa in vivo.**

At \( t = 0 \), 25 rabbits were dosed with 2.5–4 × 10⁶ dpm + 50 μg of unlabeled DDPC in 50 μl. Mean values ± SEM (N = 5). It took 5 min to prepare the mucosas, and these 5 min are not included on the time axes. *, 3H-dpm; †, 14C-dpm.

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**Fig. 2. Catabolism of 3H-total, 14C-total, 3H-DDPC, and 14C-DDPC in the nasal mucosa in percentage of injected dose.**

100% 3H = 2,727,300 dpm and 100% 14C = 4,284,000 dpm. Mean values ± SEM (N = 5). *, 3H-total; †, 3H-DDPC; △, 14C-total; (†), 14C-DDPC.

3.6%, PC (mainly DDPC reacylated with endogenous fatty acids) (18.4 ± 2.5%), and neutral lipid (11.8 ± 1.0%) (fig. 3). Only a negligible amount of 14C-dpm was found in the aqueous fraction. The detection of these metabolites may indicate degradation of DDPC.
mainly by PLA₁/PLA₂, and reacylation. Within 2 hr, the occurrence of decanoic acid and DDPC decreased by 30% and 19%, respectively, whereas the percentage of PC (18.4 ± 2.5%, t = 1 min) was doubled. The increase in DDPC reacylation was supported by an increasing $^{3}$H/$^{14}$C-ratio in the elongated PC spot on the TLC plate (figs. 4 and 5). This ratio increased from 1.8 ± 0.1 to 10.2 ± 2.5 (fig. 4), whereas the $^{3}$H/$^{14}$C-ratio in the DDPC spot held a constant value of 0.64. In the lipid extract, there was initially found a minimal occurrence of lyso-DDPC, which disappeared with time (fig. 3). The percentage of a neutral metabolite—which comigrated with MAG, DAG, TAG, and cholesteryl esters—increased from 11.8 ± 1.0% to 28.6 ± 4.7% during the experimental time (fig. 3). The formation of radiolabeled neutral lipids can be caused by reacylation with decanoic acid and/or the activity of PLC and PLD/phosphatidic acid phosphatase. Figure 6 shows the distribution of the $^{3}$H-labeled metabolites in the nasal tissue as obtained from the lipid and aqueous extracts each analyzed by TLC. At the start of the experiment ($t = 1$ min), P-CHO and GPC constituted 32% and 12%, respectively, of the $^{3}$H-labeled, water-soluble compounds. Both metabolites decreased gradually during the 2-hr period (fig. 6). In addition, CDP-CHO and small amounts of CHO were also detected in the aqueous extract, suggesting reutilization of CHO and P-CHO for PC synthesis.

### Discussion

Initially, the nasal mucosa accumulated 7.5 ± 0.4% $^{3}$H-dpm and 4.4 ± 0.4% $^{14}$C-dpm of the applied dose (figs. 1 and 2). The wash water contained 9.1 ± 2.0% and 6.1 ± 1.3% of the applied $^{3}$H- and $^{14}$C-doses, respectively. The dose of DDPC was applied to the nasal mucosa. A minor amount of the dose may also have been lost to the nasal conchae, and the continuous ciliary motion probably transported a major part of the enhancer solution toward the throat. A minor amount of the DDPC solution may also have been lost by sneezing. Within 2 hr, the radioactivity in the tissue decreased to 4.1 ± 1.1% and 0.2 ± 0.0% of the applied dose for $^{3}$H and $^{14}$C, respectively. The decrease of radioactivity with time suggested that DDPC and especially $^{14}$C-labeled DDPC metabolites left the nasal mucosa to enter...
the circulation or the nasal mucus. The pronounced metabolism of DDPC in the tissue and the rich vascularization of the tissue may be a reason for the fast disappearance of tissue-associated DDPC. The removal of 14C-radioactivity (label in the fatty acids) was faster, compared with the removal of 3H-radioactivity (label in CHO) (figs. 1 and 2). This indicated an extensive deacylation of DDPC. Decanoic acid, which is the product of a deacylation process and initially was the predominant 14C-labeled metabolite, is relatively water-soluble and may have left the tissue via the blood or via the nasal mucus. The amount of this metabolite in the wash water (used for washing the mucosa immediately after isolating) supported this, as 90% of 14C-radioactivity in the first milliliter of washing water was decanoic acid (data not shown). From t = 1 min to t = 120 min, the amount of 14C-radioactivity in the wash water decreased by 98%, suggesting that the majority left the mucosa via the blood (data not shown). The more slower decrease in 3H radioactivity may suggest that the choline moiety was reused for PC synthesis by the salvage pathway (15).

When isolating the mucosas 1 min after nasal application, the radioactivity corresponding to intact DDPC was only 18–25% of the total radioactivity in the tissue (fig. 2), suggesting a very rapid catabolism. A rapid catabolism of DDPC was also seen in an in vitro study in rabbit nasal mucosa (16). Like this, the in vivo metabolism products constituted ~59% 14C-dpm and 72% 3H-dpm of the total radioactivity retained in the tissue at t = 1 min, and half of the detected 3H-labeled metabolites were found to be water-soluble. In contrast, lung perfusions with surfactant DPPC for 2 hr in rats resulted in only 13% of water-soluble metabolites (17). This difference may in part be due to internalization of surfactant in endosomes, together with surfactant protein A that may hinder the accessibility of DPPC for the metabolizing enzymes. DDPC is most likely absorbed as monomers into the plasma membrane and may be accessible for the degrading enzymes. Lamellar bodies in the nose (18) indicate the occurrence of surfactant depots. The mechanisms for internalization and reutilization of DPPC seem to be relatively unspecific (19). However, it may seem likely that these mechanisms concern DPPC to a much greater extent than DDPC. DPPC may be taken up by the cells via endocytosis to far greater extent than DDPC, thereby escaping the rapid metabolism seen for DDPC. Short-chain PC will rapidly incorporate into phospholipid membranes, as opposed to PC molecules containing the long-chain fatty acids (20). The predominant occurrence of decanoic acid indicated activity of PLA1, PLA2, or phospholipase B. A number of previous studies indicated metabolism of exogenous PC catalyzed by phospholipase A (21–23). It is well known that short-chain phospholipids are better substrates than long-chain phospholipids for different phospholipases (24, 25). A recent study (26) showed a DDPC-induced increase in prostaglandin synthesis in rabbit nasal mucosa, probably related to a cytotoxic-mediated increase in PLA2 activity.

A form of PLA2 is activated and secreted by tissue injury (27), and human nasal lavage contain a PLA2, which activity selectively increases in allergic subjects subsequent to nasal challenge with antigen (28). These PLA2’s may thus have been involved in formation of the 14C-labeled decanoic acid. During a PLA2-induced degradation of DDPC, a simultaneous formation of lyso-DDPC was expected. In this study, only extremely small amounts of lyso-DDPC were detected, which is in agreement with other studies on PC metabolism (21, 29). A low content of lyso-DDPC may be due to a high lyso-DDPC turnover. The turnover of lyso-PC has been reported to be 30- to 80-fold faster than the PC turnover (21). The occurrence of GPC at t = 1 min (11.9 ± 3.6%) may be caused by combined PLA1/PLA2 action. Formation of GPC has also been found in other studies of metabolism of extracellular PC (21, 22, 29). The decrease in GPC with time may be due to reacylation or GPC hydrolysis catalyzed by phosphohydrolases generating P-CHO and glycerol (30). At t = 1 min, considerable amounts of DDPC were remodeled with fatty acids of different chain length seen as an elongated PC spot on TLC (23.8 ± 2.4% of total 3H-radioactivity) (figs. 5 and 6). Remodeling increased with time, which was illustrated by the increase in 3H/14C-ratio (fig. 4). Acyltransferases and transacylases may be responsible for this process (31). An increasing percentage of a less polar metabolite was found during the experimental time (fig. 3), and this metabolite cochromatographed with standards of MAG, DAG, TAG, and cholesterol esters. In vitro, only slight amounts of MAG and diacylglycerol were formed from DDPC in nasal mucosa (16) and from DDPC in lung tissue (22). The increase in percentage of neutral 14C-labeled lipids in vivo may be caused by a comparable low formation of these neutral lipids that remain in the tissue along with a large disappearance of 14C-labeled compounds (probably as decanoic acid) from the nasal mucosa. Thereby, the percentage of neutral lipids will increase. Occurrence of CDP-CHO illustrated reutilization of the choline moiety. CDP-CHO may appear by a reversible choline phosphotransferase reaction. However, this enzyme has a low affinity toward saturated PC (32), and it may be more likely that CDP-CHO was generated from P-CHO via a cytidyl transferase reaction (33). Degradation of DDPC by PLC results in DAG and P-CHO formation. At the start of the experiment, P-CHO accounted for 31.6 ± 1.1%, which decreased in time (fig. 6). Decrease in P-CHO may be connected with an increase in CDP-CHO or merely hydrolysis by phosphatases. Alternatively, P-CHO may be a result of PLD activity with subsequent phosphorylation of CHO. CHO could also result from hydrolysis of P-CHO or GPC catalyzed by phosphoesterases. The present study cannot differentiate between these catabolic pathways.

Being relatively water-soluble and possessing short-chain fatty acids, DDPC seemed to have optimum absorption enhancer properties. The rate of incorporation of phospholipids into membranes increases with decreasing fatty acid chain length (20); thus, the

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**Fig. 6. Recovery of 3H-radioactivity in the nasal mucosa in vivo.**

The nasal mucosa was treated with 3H-DDPC for up to 2 hr. 100% = total counts on the TLC plates of the lipid and aqueous fractions. Mean ± SEM (N = 5): ■ Lyso-DDPC; □ DDPC; △ PC; ● CHO; ●, P-CHO; □ GPC; Δ CDP-CHO.
incorporation rate of DDPC was supposed to be high. Ott et al. (34) reported that dimyristoyl-PC after prolonged incubations was absorbed into erythrocyte membranes inducing discocyte to spherocytocye shape changes. Similarly, absorption of DDPC may induce shape changes of the epithelial cells and probably affect the cytoskeleton, as has been found for other PCs (35). Changes in the cytoskeleton may affect the tight junctions (36), resulting in increased paracellular passage. This suggestion that DDPC may affect tight junctions is supported by a decreased resistance of rabbit nasal mucosa in vitro after application of DDPC (14, 16, 37). The i.n. application of a large dose of 8% DDPC (0.960 mg), together with 30% α-CD (3.68 mg) for 15 min in rabbits in vivo resulted in lethally damaged cells (11). Another study showed that the absorption of i.n.-administered growth hormone was not reduced 3 hr after the i.n. dosing of DDPC, thereby also indicating an irreversible absorption enhancement (10). Furthermore, 15 min incubation of isolated nasal mucosas with 2% (35.3 mM) DDPC resulted in irreversible changes of the electrophysiological parameters of the tissue (16, 37). These results may indicate a toxic effect of high doses of DDPC and, with that, DDPC may seem unqualified for use as an absorption enhancer. However, the combination of 8% (0.960 mg) DDPC and 30% (3.68 mg) α-CD as enhancers for the nasal absorption of growth hormone in rabbits in vivo showed a clear reversibility of the enhancing effect after a single application. This suggested that, in this case, the enhancing effect was not caused by irreversible damage of the epithelial barrier, possibly due to formation of inclusion complexes between α-CD and DDPC, thus masking the toxic effect of DDPC (10).

Thus, to explain the exact mechanism and toxicity of DDPC alone or in combination with other enhancers, further and longer studies are required. Thus, the very rapid catabolism of DDPC with the formation of decanoic acid raises the question of whether some of the absorption enhancement properties attributed to DDPC may be mediated by decanoic acid. Medium-chain fatty acid salts have been reported to mask the absorption of insulin (38) and various drugs with a molecular weight of 4,000 Da (39).

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References


