Diphosphatidylglycerol in experimental acute alveolar injury in the dog[1]

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Abstract Acute alveolar injury closely resembling that seen in humans was induced in dogs by subcutaneous injection of N-nitroso-N-methylurethane. Necrosis of alveolar epithelial cells was observed during early injury. Proliferation of immature epithelial cells which began during early injury and became massive after peak injury was followed by their differentiation to mature type II cells during recovery. Quantities of diphosphatidylglycerol (DPG) and of phosphatidylglycerol (PG) in alveolar lavage and in post-lavage lung tissue were measured. An increase in tissue DPG coincided with a sharp decrease in tissue and lavage PG during early injury. DPG was not detectable in the lavage. During late recovery, tissue DPG increased threefold over controls. This increase was accompanied by persistence of a 50% decrease in tissue PG and 83% decrease in lavage PG. Biosynthesis of DPG and PG in isolated lung mitochondria demonstrated that DPG was formed from PG in the presence of CDP-diglyceride. These findings suggest that the low level of PG in the surfactant complex during acute alveolar injury is due to increased turnover of PG to DPG in the lung.—**Liau, D. F., C. R. Barrett, A. L. L. Bell, G. Cernansky, and S. F. Ryan.** Diphosphatidylglycerol in experimental acute alveolar injury in the dog. *J. Lipid Res.* 1984. **25:** 678–683.

Supplementary key words phosphatidylglycerol • pulmonary surfactant

Diphosphatidylglycerol (DPG) has been identified in microorganisms and in many mammalian tissues including heart, brain, liver, kidney, spleen, and lung (1, 2). It is a characteristic phospholipid of mitochondrial membranes acting as a structural component in the bilayer region (3–5). The functional role of this phospholipid is uncertain, but it has been proposed that it may be bonded to a membrane enzyme to alter the enzyme structure and function (5–8).

Considerable evidence has established that mitochondria are capable of catalyzing the autonomous synthesis of DPG (9–12). Studies on the biosynthesis of DPG in vitro have shown that, in liver mitochondria, DPG is formed from phosphatidylglycerol (PG) and CDP-diglyceride (9–13). However, in a particular membrane-containing fraction in micororganisms, DPG is formed mainly from two molecules of PG (12, 14–16). An increase in the level of DPG and a corresponding decrease in the level of PG has been found in microorganisms during transition from the exponential to the stationary growth phase (17, 18). These findings have been interpreted to suggest that following rapid cell division, PG is utilized to synthesize DPG for membrane construction.

In this report we measured the quantity of PG and DPG in the surfactant complex and residual lung of dogs with acute alveolar injury induced by N-nitroso-N-methylurethane and studied the biosynthesis of these two components in a preparation of mitochondria from normal lung. The results suggest that the low level of PG in the surfactant complex during injury and its failure to increase during recovery are due to increased turnover of PG to DPG in the lung.

MATERIALS AND METHODS

Adult male mongrel dogs weighing 14–28 kg were used for the studies. Acute alveolar injury was induced in 16 dogs by the subcutaneous injection of 7 mg/kg of N-nitroso-N-methylurethane (19). After the animal was killed with intravenous Pentobarbital, the lung was excised quickly, lavaged, and perfused with cold TN buffer (0.01 M Tris–HCl, pH 7.4, 0.15 M NaCl).

Detailed procedures used for the alveolar lavage, perfusion, and the analyses of the quantities and qualities of lipids from alveolar lavage and lavaged lung tissue have

Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine; TLC, thin-layer chromatography; PLW, predicted normal lung weight.

[1] Parts of this study were presented at the 66th Annual Meeting of the Federation of American Societies for Experimental Biology (New Orleans, LA, April 15–23, 1982). *Federation Proc.* 1982. **41:** 618 (Abstract).

been reported from this laboratory (20–22). In brief, the bronchus, pulmonary artery, and pulmonary veins were cannulated. The lung was degassed in a vacuum and then was lavaged six times, each time with 10 ml of TN buffer per g of predicted normal lung weight (PLW). PLW was calculated from preinjection body weight using the data of Frank (23). After lavage, the pulmonary artery was perfused at a pressure of 30 cm of water with 1500 ml of TN buffer. The lung was then minced and homogenized in 4 volumes of its PLW of TNES buffer (0.01 M Tris–HCl, pH 7.4/0.15 M NaCl/0.01 M EDTA/0.25 M sucrose) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Two 600-ml volumes of the combined alveolar lavages and two 60-ml volumes of the homogenized lung tissue were lyophilized and extracted for lipid analysis according to the method of Folch, Lees, and Sloane Stanley (24). Phospholipids were separated by thin-layer chromatography (TLC) on silica gel 60 and quantitated by fluorometry after spraying with Rhodamine 6G (20). The quantification of disaturated phosphatidylcholine (DSPC) was carried out by the method of Mason, Nellenbogen, and Clements (25) except that the isolation of DSPC from the reaction mixture was accomplished by TLC rather than by a column.

DSPC from the alveolar lavage or lavaged lung tissue was separated in chloroform–methanol–water 65:25:4 (v/v/v). This solvent also was used for the separation of PG from the lavage (22). However, DPG and PG from the lavaged lung tissue were quantified by two-dimensional TLC using the above solvent for the first dimension. The second dimension was developed in tetrahydrofuran–methylal–methanol–2 M ammonium hydroxide 20:16:4:2.2 (v/v/v/v) (26).

Mitochondria were isolated from the homogenized lung tissue as described previously (27). The mitochondrial suspension was frozen in small aliquots and stored at −80°C until assayed. Protein content was determined by the method of Lowry et al. (28).

The incubation mixture used for the assay of DPG and PG synthesis was adopted from those described by Hostetler and van den Bosch (11) and by Hallman and Gluck (29). The 0.5-ml mixture consisted of the following components: 100 mM Tris-HCl containing 1 mg/ml Triton X-100 (pH 7.4); 0.05 mM CDP-diglyceride; 0.08 mM *sn*-[^{14}C(U)]glycerol-3-phosphate (32 mCi/mM); 5 mM reduced glutathione; 2 mM EDTA; 10 mM $MgCl_2$; 75 mM sucrose; and 1.5 mg mitochondrial protein. The mixture was sonicated for 30 sec at 0°C using a microtip (Cell Disruptor, Heat Systems-Ultrasonics Inc., Plainview, NY) before adding the mitochondria, and was incubated with shaking at 37°C for various times. After incubation the reaction was stopped by the addition of 0.5 ml of 0.01 N HCl in chloroform–methanol 1:4 (v/v) and lipids were extracted by the successive addition of 2.5 ml of chloroform–methanol 4:1 (v/v) containing 50 μg of carrier PG and 50 μg of carrier DPG. The chloroform-soluble lipids were washed twice with 3 ml of 0.5 M KCl and centrifuged at 3000 *g* for 10 min. The lower layer was aspirated and evaporated to a small volume (300 μl) under nitrogen. DPG and PG were isolated by TLC on silica gel 60 in chloroform–methanol–acetic acid–water 65:25:4:4 (v/v/v/v) modified from that used by Hostetler, van den Bosch, and van Deenen (10). The DPG and PG spots corresponding to pure references were visualized in iodine vapor and scraped into scintillation vials containing Aquasol for radioactivity counting.

The incubation mixture used for the assay of the synthesis of DPG from PG was carried out using ^{14}C-labeled PG. ^{14}C-Labeled PG was prepared by the incubation of lung mitochondria with *sn*-[^{14}C(U)]glycerol-3-phosphate and CDP-diglyceride for 2 hr at 37°C as described above. The ^{14}C-labeled PG scraped from the TLC plates was eluted twice with 10 ml of chloroform–methanol 2:1 (v/v). After removal of the solvent in vacuo, this radioactive PG was suspended in Tris-HCl (100 mM, 1 mg/ml Triton X-100, pH 7.4) by sonication as above. The incubation mixture contained 0.2 mM ^{14}C-labeled PG (6 mCi/mM) in place of *sn*-[^{14}C(U)]glycerol-3-phosphate. Other conditions were the same as those described above.

Differences between mean values in the control dogs and in the experimental dogs were evaluated using a conventional unpaired *t* test (30).

sn-[^{14}C(U)]Glycerol-3-phosphate (144 mCi/mM) and Aquasol were purchased from New England Nuclear. CDP-diglyceride (dipalmitoyl), *sn*-glycerol-3-phosphate, diphosphatidylglycerol (source: bovine heart), and phosphatidylglycerol (source: egg lecithin) were obtained from Sigma Chemical Co. Silica gel 60 (Merck) and other chemicals were obtained from American Hospital Supply Corp., Edison, NJ.

RESULTS

Based on morphological and biochemical observations (19–21), the experimental animals were separated into four groups following injection of N-nitroso-N-methylurethane (**Table 1**). The four groups were designated as early injury (day 4), peak injury (day 7), early recovery (day 10), and late recovery (day 15). During early injury, necrosis and early regeneration of type II epithelial cells occurred. Maximal necrosis of cells was seen at peak injury. The number of regenerating epithelial cells increased steadily even as the numbers of necrotic cells increased. During early recovery, regeneration of epithelial cells was massive and some of them were recognizable as type II cells by their content of cytoplasmic

TABLE 1. Acute alveolar injury induced by N-nitroso-N-methylurethane (NNNMU) in the dog

	Days Post NNNMU				
	0	2–4	6–8	10–12	15–20
Phase of injury	NNNMU	Early injury	Peak injury	Early recovery	Late recovery
Epithelial necrosis		++	++++	+	
Epithelial regeneration		++	++	+++++	++++
Epithelial maturation			+	++	+++

The number of necrotic epithelial cells increases until about day 7. Regeneration of epithelial cells begins about day 3 and their numbers increase steadily even as the number of necrotic cells increases. Maturation of these regenerating cells (appearance of cytoplasmic lamellar bodies) is first seen about day 10 and their numbers (and numbers of lamellar bodies per cell) increase until about day 15–20.

lamellar bodies. By late recovery, most of the cells had matured to type II cells and a great number of the alveoli were lined entirely with these cells.

The post-lavage lung tissue and alveolar lavage will be simply referred to as tissue and lavage. **Table 2** presents quantitative data for phospholipids in the tissue and lavage. The quantities of PC and DSPC in the tissue represents the PC and DSPC in type II epithelial cells and in other types of cells in the lung. Tissue PC and DSPC, expressed as mg/g PLW, decreased to the lowest level at peak injury ($P < 0.01$), and increased steadily to twofold that of normal lung during late recovery. Morphological studies revealed that these remarkable increases were very likely derived from massively regenerated type II cells (Table 1). PC and DSPC in the lavage also decreased to the lowest levels at peak injury ($P < 0.001$), but they increased only to near normal level during late recovery.

The quantities of PG in the tissue decreased sharply coincident with an increase in tissue DPG at early and peak injury (days 4 and 7, $P < 0.005$ and $P < 0.01$, respectively). During early and late recovery (days 10 and 15), the tissue PG failed to increase at the same rate as tissue PC or DSPC. At day 15, the PG content was only one-half that of controls, a remarkable difference from the twofold increases found in both tissue PC and DSPC. In contrast, the tissue DPG increased dramatically to twofold and threefold over controls at day 10 and day 15, respectively.

PG in the lavage also decreased significantly with time as did the lavage PC and DSPC during early and peak injury ($P < 0.001$ for each). However, the lavage PG did not increase as did the lavage PC and DSPC during early and late recovery. DPG was not detected in the lavages either of controls or of experimental animals.

On TLC of the in vitro synthesized phospholipids, the radioactivity co-chromatographed with the reference PG (R_f: 0.51) and DPG (R_f: 0.66) used as carrier. **Fig. 1** shows results of PG and DPG sythesis from *sn*-[^{14}C(U)]glycerol-3-phosphate and CDP-diglyceride as a function of time. The rate of PG synthesis was very high. PG synthesis was linear for 1 hr, after which incorporation of radioactivity was low. Synthesis of DPG was linear for 2 hr, but the rate of its synthesis appears to be quite low.

Synthesis of DPG from ^{14}C-labeled PG in the presence

TABLE 2. Phospholipids in lavaged lung tissue and alveolar lavage at intervals after injection of N-nitroso-N-methylurethane (NNNMU)

			Phospholipids							
			Tissue				Lavage			
Days Post NNNMU	N	Predicted Lung Weight (PLW)	PC	DSPC	PG	DPG	PC	DSPC	PG	DPG
		g/lung	*mg/g PLW*							
0	8	199 ± 16	5.25 ± 1.02	1.10 ± 0.25	0.16 ± 0.01	0.11 ± 0.01	1.56 ± 0.31	0.76 ± 0.20	0.13 ± 0.04	nd
4	5	181 ± 20	5.37 ± 0.67	1.08 ± 0.18	0.05 ± 0.01[a]	0.13 ± 0.02[b]	0.94 ± 0.20[b]	0.35 ± 0.05[a]	0.04 ± 0.01[a]	nd
7	4	197 ± 18	4.01 ± 1.15	0.73 ± 0.19[c]	0.03 ± 0.01[a]	0.16 ± 0.05[c]	0.72 ± 0.38[b]	0.27 ± 0.19[a]	0.03 ± 0.02[a]	nd
10	5	185 ± 15	6.49 ± 1.41	1.31 ± 0.29	0.07 ± 0.02[a]	0.21 ± 0.04[a]	0.80 ± 0.54[c]	0.35 ± 0.27[c]	0.02 ± 0.02[a]	nd
15	7	188 ± 21	10.4 ± 3.04[a]	2.22 ± 0.73[a]	0.08 ± 0.03[a]	0.31 ± 0.12[a]	1.37 ± 0.63	0.61 ± 0.21	0.02 ± 0.02[a]	nd

PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol. PC and DSPC from tissue or lavage and PG from lavage were determined by one-dimensional TLC. PG and DPG from tissue were determined by two-dimensional TLC; nd, not detectable.

[a] $P < 0.001$ as compared to the control animals.

[b] $P < 0.005$; [c] $P < 0.01$; [d] $P < 0.05$; values are mean ± SE.

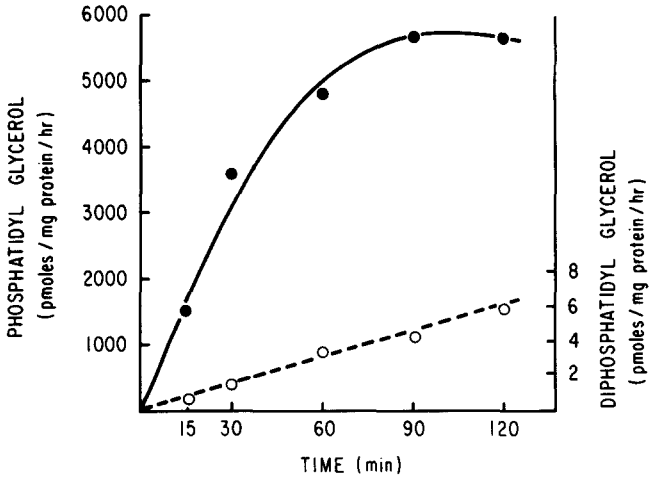

Fig. 1. Synthesis of diphosphatidylglycerol and phosphatidylglycerol in dog lung mitochondria as a function of time. The results are the mean of two independent measurements done in triplicate. The incubation mixture contained 100 mM Tris-HCl containing 1 mg/ml Triton X-100 (pH 7.4); 0.05 mM CDP-diglyceride; 0.08 mM *sn*-[^{14}C(U)]glycerol-3-phosphate (32 mCi/mM); 5 mM reduced glutathione; 2 mM EDTA; 10 mM $MgCl_2$; 75 mM sucrose; 1.5 mg mitochondrial protein in a final volume of 0.5 ml. The mixture was incubated with shaking at 37°C for the times indicated.

and absence of CDP-diglyceride is shown in **Table 3.** Although there is a small amount of DPG synthesis without added CDP-diglyceride, it is apparent that DPG synthesis occurred in the presence of CDP-diglyceride.

DISCUSSION

Surfactant isolated from the lungs of prematurely born rabbits or from patients with adult respiratory distress syndrome has been shown to contain markedly decreased quantities of PG (31, 32). A similar decrease in surfactant PG was also found in the experimental model of acute alveolar injury reported here. As shown in Table 2, the PG content in lavage was remarkably decreased from early injury through recovery. The cause for this decrease of PG, however, is uncertain.

Previous observations (19), as summarized in Table 1, demonstrated that N-nitroso-N-methylurethane selectively injured both types of alveolar epithelial cells. The epithelium is the only population of cells that is seen to undergo injury and repair. Necrosis of epithelial cells occurred during early injury and maximal necrosis was found at peak injury. Regeneration of immature epithelial cells began during early injury and became massive after peak injury. Differentiation of these immature cells to mature type II cells, recognizable by their cytoplasmic lamellar bodies, began during early recovery and their numbers increased with time until about day 15 to 20.

During early and peak injury (days 4 and 7, Table 2), PC, DSPC, and PG in both lavage and lung tissue decreased significantly although many regenerating epithelial cells were already present. These decreases were accompanied by a marked increase of DPG in the tissue. During early and late recovery, remarkable increases of PC and DSPC in the lavage and tissue were found but PG remained very low in both. These low levels of PG coincided with a threefold increase in tissue DPG. Available evidence (9–16) and the present study of DPG synthesis in the lung mitochondria shown in Fig. 1 and Table 3 indicate that PG is an essential metabolic precursor of DPG. The marked increase in the tissue DPG thus indicates that active synthesis of PG occurred in the lung. The high rate of PG synthesis found in the mitochondria (Fig. 1) supports this conclusion.

Because the epithelium is the only cell population that appears to be in flux with massive regeneration of epithelial cells occurring after peak injury (Table 1), it seems very likely that regenerating epithelial cells are the major site of the active synthesis of PG and DPG and that the synthesized PG is utilized for the synthesis of DPG. The phenomenon, observed in this experimental model in which massive regeneration of immature alveolar epithelial cells was followed by their maturation to type II cells, is similar to the transition phase from exponential to stationary growth in microorganisms. The transition phase is manifested by an increase in the DPG content and a corresponding decrease in the PG content (17, 18).

PG is contained mostly in the lamellar bodies and extracellular surfactant of the lung (29). Hallman and Gluck (26) demonstrated that surfactant PG is synthesized in the microsomal fraction and transferred subsequently to lamellar bodies. Synthesis of PG in the mitochondrial fraction may also contribute to the formation of surfactant as well as serving as a precursor for DPG synthesis (26). The data we present here suggest that the decreased levels of PG in both lavage and tissue are due to increased turnover of PG into DPG, thus reducing its availability for surfactant in the regenerating type II cells. Presumably the PG thus actively synthesized in the mitochondria and

TABLE 3. Synthesis of diphosphatidylglycerol from phosphatidylglycerol in dog lung mitochondria

	Diphosphatidylglycerol
	pmol / mg per hr
Complete system	6.5
No CDP-diglyceride	0.2
Inactive mitochondria[a]	0

The results are the mean of two independent measurements done in triplicate. Incubation conditions were the same as in Fig. 1, except that *sn*-[^{14}C(U)]glycerol-3-phosphate was replaced by 0.2 mM ^{14}C-labeled PG (6 mCi/mM). The incubation was for 2 hr at 37°C.

[a] Mitochondria was incubated at 60°C for 30 min before adding to the incubation mixture.

microsomes (data are not shown) is utilized preferentially to synthesize DPG for membrane construction in these new cells. Direct evidence concerning the correlation of PG and DPG in isolated type II cells is not available at the present time. Isolation and culture of type II cells from this experimental model with acute alveolar injury is currently being investigated.

The present results for PG and DPG synthesis in the lung mitochondria (Fig. 1 and Table 3) demonstrate that PG is formed from CDP-diglyceride and *sn*-glycerol-3-phosphate and serves as an intermediate in the formation of DPG. The rate of DPG formation appears to be quite low and its formation from ^{14}C-labeled PG was found to occur only in the presence of CDP-diglyceride (Table 3). These findings are similar to those in liver mitochondria (10). The use of dipalmitoyl CDP-diglyceride in the present studies could be partially responsible for the low rate of DPG synthesis. Hostetler et al. (13) found that the fatty acid composition of CDP-diglyceride greatly affected the rates of PG and DPG formation. More rapid DPG synthesis was seen with CDP-diglyceride containing short chain fatty acids or unsaturated fatty acids.

In this model, necrosis of alveolar epithelial cells seems to lead to a process of dedifferentiation in the remaining viable type II cells. These dedifferentiated cells appear to relinquish their surfactant-synthesizing capacity temporarily in order to divide rapidly and repair the lost integrity of the epithelial lining of the alveoli. During recovery, the increasing quantities of PC and DSPC, the return toward normal of PC fatty acid patterns (27), and the persistent low level of PG in lavage suggest a process of biochemical maturation in the regenerating alveolar epithelial cells which coincides with their structural maturation.

The authors are grateful to Charles Dumais and Luba Pustilnik for expert assistance. This work was supported by grants from the National Heart, Lung, and Blood Institute (HL-16592) and by a grant from the Stony Wold-Herbert Fund, Inc. of New York.

Manuscript received 3 May 1983.

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