Expression and localization of diacylglycerol kinase isozymes and enzymatic features in rat lung

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Diacylglycerol kinase (DGK) catalyzes phosphorylation of diacylglycerol (DG) to generate phosphatidic acid (PA) and plays a major role in controlling the cellular level of DG (9, 33). Furthermore, there is accumulating evidence that PA, a product of DGK, also may serve as a second messenger that modulates the activity of several enzymes including PKC-ζ (20) and PLC-γ1 (15). Therefore, DGK occupies a central position in intracellular signal transduction through these two second messengers. DGK represents a large gene family of isozymes, and, in mammals, nine DGK isozymes have been isolated so far (9, 33). Previous studies have revealed that DGK isoforms show remarkable heterogeneity in structure, tissue expression, and enzymological properties. We have so far reported the detailed cellular expression of mRNAs for the isozymes and their functional implications in the central nervous system and heart (5–8, 12, 32). These observations suggest that each isoform has its own specific function in various biological processes. The PI cycle has been shown in lung cells to mediate a variety of cellular signaling pathways (18, 22). In addition to the functional significance of DGK in signal transduction, it should also be mentioned that DG and PA are important intermediates for the synthesis of various lipids, such as phosphatidylcholine (PC), phosphatidylethanolamine, and triacylglycerol, which are intimately involved in the biosynthetic pathway for surfactant in the lung (27). Therefore, we should take into consideration how DGK is involved in these apparently distinct mechanisms, i.e., surfactant synthesis and signal transduction.

To gain an insight into the functional implication of DGK in the lung, we first attempted to clarify the expression and localization pattern of DGK isozymes together with the developmental changes in their mRNA expressions and enzymatic properties in rat lung. Our results clearly show different localization of each DGK isoform and its characteristic gene expression pattern in developing rat lung, suggesting that each DGK isoform plays a different functional role in a different developing stage of the lung.

MATERIALS AND METHODS

Animals. This study was carried out in accordance with the Guide for Animal Experimentation, Yamagata University School of Medi-

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oxidase complex system (Vector Laboratories) with diaminobenzidine antigen-antibody reaction were visualized by avidin-biotinylated per-goat anti-rabbit IgG (dilution 1:250; Vector Laboratories, Burlingame, a moist chamber. Then, the sections were incubated with biotinylated temperature instead of biotinylated goat anti-rabbit IgG. Some sections were incubated with goat anti-rabbit IgG-Alexa 546 (dilution 1:400; Molecular Probes). The sections were dehydrated in graded alcohols, cleared with xylene, treated with 5% normal goat serum in PBS (NGS/PBS) to block nonspecific binding sites and incubated with anti-rat DGK

RESULTS

Expression and localization of DGK isozymes in the lung. In Northern blot analysis, hybridization signals for the three DGK isozymes were observed in the lung tissue from 1 to 12 weeks old, with the highest expression found in the adult lung (Figure 2). Immunohistochemistry also confirmed the expression of DGK isozymes in specific lung cell types (Figure 3).

The expression of DGKα was predominant in the alveolar type II cells and the bronchial epithelial cells, while DGKβ was expressed in the bronchial smooth muscle cells and DGKγ was present in the type I cells and the endothelial cells. The expression pattern of DGKα, β, and γ was consistent with the results of Northern blot analysis.

Immunofluorescence staining also confirmed the localization of DGK isozymes in the lung. DGKα was localized in the cytoplasm and plasma membrane of the alveolar type II cells, while DGKβ was mainly present in the cytoplasm of the bronchial smooth muscle cells. DGKγ was detected in the cytoplasm of the type I cells and the endothelial cells.

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Expression of DGKα and -ζ in isolated alveolar type II cells and macrophages. We further examined the expression of DGKα and -ζ using isolated alveolar type II cells and macrophages. The isolation procedures yielded ~98% purity for both cell types, which was determined by immunocytochemical staining for anti-SP-C or anti-ED-1 antibody (data not shown). Immunoreactivities for DGKα and -ζ were clearly detected in both cell types, with the former fine dotted in the cytoplasm and the latter speckled in the nucleus (Fig. 5). These results were the same as those of the immunostaining in the lung, further confirming that DGKα and -ζ are expressed in alveolar type II cells and macrophages.

DGK activity in the whole lung, alveolar type II cells, and macrophages. In the previous study, DGK activity has been detected in rat lung (13). However, recent molecular cloning studies of several DGK isozymes have revealed different enzymological properties of the isozymes, i.e., DGKα, -β, and -γ are activated by Ca²⁺, and DGKɛ specifically phosphorylates arachidonoyl-containing DG (1-stearyl-2-arachidonoyl-sn-glycerol) (33). Therefore, we reexamined DGK activity in the whole lung with attention to Ca²⁺ dependency and substrate specificity. We also examined the enzymatic activity of the isolated alveolar type II cells and macrophages. An octylglucoside-mixed micelle assay was used to evaluate substrate specificity toward 1-stearyl-2-linoleoyl-sn-glycerol (18:0/18:2 DG) and 1-stearyl-2-arachidonoyl-sn-glycerol (18:0/20:4 DG) in the presence of 0.1 mM Ca²⁺ (21). As shown in Fig. 6A, DGK activity was detected in the whole lung at 10-fold lower levels in the brain where most of the isozymes are abundantly expressed. The activities toward 18:0/18:2 DG and 18:0/20:4 DG were almost comparable in the lung, showing no apparent substrate specificity. DGK activities in the isolated alveolar type II cells and macrophages were ~20% and 150% compared with that of the whole lung, respectively, and almost equal toward 18:0/18:2 DG and 18:0/20:4 DG. In the presence (0.1 mM) or absence of Ca²⁺, the whole lung and the isolated cells showed similar enzymatic activities (Fig. 6B).

Double-immunofluorescent staining of alveolar type II cells and macrophages. To confirm the expression of DGKα and -ζ in alveolar type II cells and macrophages, we performed double-immunofluorescent analysis using specific markers for those cells, i.e., anti-SP-C antibody and anti-ED-1 antibody for alveolar type II cells and macrophages, respectively. As shown in Fig. 3A, most of the DGKα-immunoreactive cells were also SP-C immunoreactive, indicating that DGKα is expressed in alveolar type II cells. On the other hand, DGKζ-immunoreactive cells outnumbered SP-C-immunoreactive cells in the alveoli (Fig. 3B), suggesting that DGKζ is expressed not only in type II cells but also in type I cells, although at low levels. With regard to alveolar macrophages, ED-1-immunoreactive macrophages were also immunoreactive for both DGKα and -ζ, showing that these isozymes are expressed in these cells (Fig. 4).
Expression of DGK isozymes in the developing lung. Development of the lung begins on approximately E11 in rats as a ventral foregut outpouching. Subsequently, repetitive branching of the lung bud occurs with eventual formation and maturation of the alveolar acinar units, together with specialized epithelial cell differentiation and surfactant production. In the perinatal period, the sudden exposure of the air-blood interface of the lung to increased oxygen tension must cause drastic changes to lung cells. To understand the functional implications of DGK isozymes during lung development, we examined temporal expression patterns of mRNA for DGK isozymes in the developing lung by Northern blot analysis using 18S ribosomal RNA as a control (Fig. 7A). Hybridization signals for DGKα showed a constant level during the embryonic stage but gradually increased after birth. At adult stage, the signals were approximately fourfold higher than those of the embryonic stage and almost equivalent to those of adult brain. On the other hand, the hybridization signals for DGKε were nearly constant during embryonic and postnatal stages. A similar expression pattern was found in DGKζ except that the signals increased slightly after birth.

It should be noted, however, that during the perinatal period, distinct expression patterns were observed for DGKα, -ε, and -ζ (Fig. 7A): the expression of DGKε was transiently elevated immediately after birth (P0) compared with that on E21. On the other hand, the expression for DGKα and -ζ was inversely elevated on E21 and then decreased on P0. To further examine these changes during the perinatal period, we used semiquantitative RT-PCR with GAPDH as a control (Fig. 7B). The data clearly showed that the expression for DGKε was transiently elevated approximately fourfold on P0 compared with that on E21 and then decreased on P1, although changes in the expression for DGKα and -ζ during the perinatal period were not statistically significant. These data suggest that DGK isozymes play distinct roles in developing lung, especially during the perinatal period.

Fig. 3. Immunofluorescent localization of DGK isozymes and surfactant protein (SP)-C in the alveolus. Adult rats were fixed and double immunostained as described in MATERIALS AND METHODS. Images of the 2 fluorophores were colorized and combined (merge). **A**: immunofluorescent localization of DGKα and SP-C (a marker for alveolar type II cells). Red indicates Alexa 546 staining of DGKα, and green indicates Alexa 488 staining of SP-C. **B**: immunofluorescent localization of DGKζ and SP-C. Red indicates Alexa 546 staining of DGKζ, and green indicates Alexa 488 staining of SP-C. DGKα immunoreactivity is detected in the cytoplasm of round alveolar cells located near the angles between neighboring septa (arrowheads), where it colocalizes with SP-C immunoreactivity in one and the same cells. In addition, DGKζ immunoreactivity is also detected moderately in the nucleus of squamous alveolar cells that are not immunoreactive for SP-C (arrows). Bar = 10 μm.

Fig. 4. Immunofluorescent localization of DGK isozymes and ED-1 in the alveolus. Adult rats were fixed and double immunostained as described in MATERIALS AND METHODS. Images of the 2 fluorophores were colorized and combined (merge) under confocal laser scanning microscopy. **A**: immunofluorescent localization of DGKα and ED-1 (a marker for macrophages). Red indicates Alexa 546 staining of DGKα, and green indicates Alexa 488 staining of ED-1. DGKα immunoreactivity is detected in the cytoplasm of free cell located in the alveolar space, where it colocalizes with ED-1 immunoreactivity in one and the same cells. **B**: immunofluorescent localization of DGKζ and ED-1. Red indicates Alexa 546 staining of DGKζ, and green indicates Alexa 488 staining of ED-1. DGKζ immunoreactivity is detected in the nucleus of free cell in the alveolus, where it colocalizes with ED-1 immunoreactivity in one and the same cells. Bar = 10 μm.
that propagates the IL-2-mediated signal transduction may be shared among relevant cells in general, the expression of DGKζ in these lung cells, as revealed in this study, suggests that this isozyme might be involved in this pathological process in the lung, which warrants further investigation.

Nuclear localization of DGKζ in the lung cells is compatible with our previous study of its immunohistochemical examination in neurons (12), which together with this study, indicates that DGKζ is localized in the nuclei of both proliferating and nonproliferating cells. Although the functional role applicable to all kinds of DGKζ-expressing cells is unclear, our previous study of myocardial infarction model of rats shows that the mRNA expression for DGKζ is enhanced in macrophages that are infiltrated into the necrotic area, suggesting the possible role of DGKζ in phagocytosis of these cells (32). It remains to be elucidated whether macrophages intrinsically express DGKζ at a high level or whether the increased expression is induced by the activation of the phagocytic reaction.

One of the best-characterized functional roles of DGK is in the regulation of PKC, for which DG acts as an allosteric activator (23). In this regard, there is evidence that PKC may...
play an important role in the regulation of surfactant secretion in type II cells (28, 29). Agonists that activate PKC, such as 12-O-tetradecanoylphorbol-13-acetate and cell-permeable DGs, are shown to serve as the most effective surfactant secretagogues in isolated type II cells (29). Rooney et al. (28) describes that ATP triggers the PI cycle via P2Y2 purinoceptors, which results in the activation of PKC and subsequent stimulation of surfactant secretion. Furthermore, calphostin C, a potent inhibitor of PKC, blocked PC secretion stimulated by glucagon-like peptide 1, the truncated and amidated form of glucagon-like peptide 1, in human alveolar type II cells (34). Thus it is highly plausible that DGK may play a role in regulating surfactant secretion, although which one is responsible for the regulation among the three isoforms identified in this study remains to be elucidated.

Enzymatic assays for the whole lung, isolated alveolar type II cells, and macrophages reveal that total DGK activities in these samples show neither Ca\(^{2+}\) dependency nor substrate specificity. To date, enzymatic property has been well characterized for DGK isozymes: Ca\(^{2+}\)-dependent activation for DGK\(\alpha\), -\(\beta\), and -\(\gamma\), and specific phosphorylation toward arachidonoyl-containing DG for DGK\(\varepsilon\) (33). Considering the present Northern blot and RT-PCR data showing that DGK\(\alpha\), -\(\varepsilon\), and -\(\zeta\) are responsible for the expression in the lung, DGK\(\varepsilon\), a Ca\(^{2+}\)-independent isozyme with no substrate specificity, may be dominant among those in terms of the activity.
It should also be mentioned that DGK isozymes show unique patterns of expression during the perinatal period. Using semiquantitative RT-PCR, we found that the expression for DGKε was transiently elevated at approximately fourfold immediately after birth (P0) during the perinatal period. Lung is one of the organs exposed to drastic changes before and after birth. In this regard, the biochemical adaptation of the lung to air or oxygen breathing at birth is incompletely understood. A sudden exposure of the air-blood interface of the lung to increased oxygen tension must pose an acute oxidative stress compared with the relatively anaerobic fetal environment. Indeed, the pulmonary epithelium is usually exposed to the highest oxygen tension present in the organism (2). Our data show that DGKε is very unique in that its expression is upregulated by increased oxygen tension, whereas expression for DGKδ and -ζ is downregulated. It is known that PI has a characteristic fatty acid composition of 1-stearoyl-2-arachidonoyl (11). Considering the substrate specificity of DGK towards arachidonoyl-DG, a possible link might be suggested for DGKδ upregulated by increased oxygen tension, whereas expression is downregulated.

In conclusion, our results reveal for the first time the gene expression of DGKα, -ζ, and -ε in the lung. Furthermore, immunohistochemical analysis shows that DGKα and -ζ are coexpressed in alveolar type II cells and macrophages and that these isozymes are localized at distinct subcellular locations. In the developing lung, distinct expression patterns for DGKα, -ε, and -ζ are observed during the perinatal period. All these data suggest that each isozyme plays a different role in lung functions such as surfactant production and secretion, phagocytic reaction, and adaptation to oxygen breathing. Identification of specific isozymes of DGK in the lung would help us further investigate detailed physiological and pathological roles for each molecule.

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