Effects of recombinant human keratinocyte growth factor on surfactant, plasma, and liver phospholipid homeostasis in hyperoxic neonatal rats

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Departments of 1Neonatology and 2Internal Medicine, Faculty of Medicine, Eberhard-Karls-University, Tübingen; 3Institute of Physiological Chemistry, Faculty of Medicine, University of Technology, Dresden; and 4Section of Experimental Pneumology, Research Center Borstel, Leibniz Center for Medicine and Biosciences, Borstel, Germany

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Raith M, Schaal K, Koslowski R, Fehrenbach H, Poets CF, Schleicher E, Bernhard W. Effects of recombinant human keratinocyte growth factor on surfactant, plasma, and liver phospholipid homeostasis in hyperoxic neonatal rats. J Appl Physiol 112: 1317–1328, 2012. First published February 9, 2012; doi:10.1152/japplphysiol.00887.2011.—Respiratory distress and bronchopulmonary dysplasia (BPD) are major problems in preterm infants that are often addressed by glucocorticoid treatment and increased oxygen supply, causing catabolic and injurious side effects. Recombinant human keratinocyte growth factor (rhKGF) is noncatabolic and antiapoptotic and increases surfactant pools in immature lungs. Despite its usefulness in injured neonatal lungs, the mechanisms of improved surfactant homeostasis in vivo and systemic effects on lipid homeostasis are unknown. We therefore exposed newborn rats to 85% vs. 21% oxygen and treated them systemically with rhKGF for 48 h before death at 7 days. We determined type II pneumocyte (PN-II) proliferation, surfactant protein (SP) mRNA expression, and the pulmonary metabolism of individual phosphatidylcholine (PC) species using [D9-methyl]choline and tandem mass spectrometry. In addition, we assessed liver and plasma lipid metabolism, addressing PC synthesis de novo, the liver-specific phosphatidylethanolamine methyl transferase (PEMT) pathway, and triglyceride concentrations. rhKGF was found to maintain PN-II proliferation and increased SP-B/C expression and surfactant PC in both normoxic and hyperoxic lungs. We found increased total PC together with decreased [D9-methyl]choline enrichment, suggesting decreased turnover rather than increased secretion and synthesis as the underlying mechanism. In the liver, rhKGF increased PC synthesis, both de novo and via PEMT, underlining the organotypic differences of rhKGF actions on lipid metabolism. rhKGF increased the hepatic secretion of newly synthesized polyunsaturated PC, indicating improved systemic supply with choline and essential fatty acids. We suggest that rhKGF has potential as a therapeutic agent in neonates by improving pulmonary and systemic PC homeostasis.

LUNG IMMATURITY AND SURFACTANT DEFICIENCY, the major causes for respiratory morbidity in preterm infants, often are followed by bronchopulmonary dysplasia (BPD) (1, 31). The preconditions for BPD development are the noxious effects of high oxygen pressure together with underdeveloped antioxidative capacity, inflammation, and inhibition of alveolarization (12, 35). Glucocorticoids are used not only systemically before birth to improve surfactant homeostasis and lung function but also postnatally by inhalation to decrease inflammation and prevent BPD (4). High-dose glucocorticoids, however, may have systemic side effects despite their topical application (2), and the effects on long-term outcome are unknown. Thus their inhibitory effects on lung development and alveolarization, together with their well-known systemic catabolic side effects on growth and metabolic homeostasis (11), have led to a quest for developing new therapeutic and prophylactic strategies (27, 34). Here, the use of recombinant human KGF (rhKGF) may present a better alternative, because it protects lung epithelia from many injuries (55). Importantly, low KGF concentrations in lung secretions are associated with BPD development, and mechanical ventilation as a potentially injurious factor is known to decrease KGF expression in lung tissue (16, 17). KGF is an anabolic growth factor mainly expressed in interstitial cells and acting on epithelia via specific receptors on type II pneumocytes (PN-II).

KGF belongs to the family of fibroblast growth factors, partially acting via phosphatidylinositol trisphosphate and MAP kinases protecting epithelia from apoptosis and facilitating differentiation (43). The current clinical use of rhKGF is restricted to the treatment of mucositis, a frequent and severe complication during radiation and chemotherapy (52). In the lungs, rhKGF enhances PN-II differentiation and surfactant protein (SP) expression and improves survival even under extreme conditions of 95% oxygen treatment (3, 13, 23, 28, 50). In adult lungs, rhKGF may be effective after onset of injury, one important precondition of clinical usefulness, because it dampens inflammation and epithelial damage and improves DNA repair and alveolar epithelial barrier function (36, 42, 44, 52, 58). Moreover, whereas glucocorticoids blunt PN-II proliferation, the latter is maintained after systemic rhKGF treatment of healthy neonatal rats (25). Furthermore, although rhKGF increases secreted surfactant in immature neonatal rats, this increase was not accompanied by decreased tissue phospholipids as it is with glucocorticoid treatment (25, 32). However, the basic mechanisms of rhKGF effects on surfactant homeostasis and its efficacy in immature lungs injured by hyperoxia in vivo are as yet unknown, which suggests that further investigation is required into rhKGF as a therapeutic option in preterm infants.

KGF is expressed, and its receptor is present, in many developing parenchymal organs in addition to the lungs, such as the liver; this characterizes KGF as a pleiotropic growth factor integrating organ functions during development (20, 21, 23, 25). Systemic use of rhKGF for treating injured lungs must therefore be investigated in relation to its effects on other organs. This applies particularly to the liver as the central

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organ of metabolism and to plasma as the carrier of hepaticogenic nutrients for the developing peripheral organs.

Surfactant is a unique lipid and protein complex whose presence is essential in producing low surface tension at the pulmonary air:liquid interface. It comprises 80–85% phospholipids, 10% neutral lipids, and about 2–5% specific proteins SP-A to SP-D, which are all synthesized, stored, and secreted by PN-II. Given that PN-II extensively proliferate during alveolarization (25), pathological and therapeutic effects on surfactant homeostasis may be due to altered PN-II proliferation, differentiation, and/or metabolic activity of these cells (19). The main phospholipid of surfactant is phosphatidylcholine (PC), which is dominated by dipalmitoyl-PC (PC16:0/16:0), palmitoyl-lysoprostacyclin-PC (PC16:0/14:0), and palmitoyl-palmityl-PC (PC16:0/16:1). These are preferentially sequestered into the lamellar bodies of PN-II via the ATP binding cassette (ABC) transporter A3 (ABC-A3), whereas other important components of lung PC, containing oleic, linoleic, or polyunsaturated fatty acids, are preferentially retained in lung tissue (6, 14). The hydrophobic surfactant proteins SP-B and SP-C, along with lipids, contribute to the spreading, organization, and dynamic behavior of surfactant, whereas SP-A and SP-D determine innate immune functions and surfactant homeostasis (57). Consequently, SP are established markers of PN-II differentiation and reactivity to noxious or therapeutic challenges (23, 25).

Previous investigations have demonstrated that rhKGF improves the surfactant status in both healthy, preterm lungs and in term but immature lungs. This improvement is found at the levels of both secreted SP-B/C and surfactant PC. In contrast to glucocorticoid treatment, the improvement is not at the expense of whole tissue PC homeostasis (25, 32). Taking everything into account, surfactant homeostasis has to be viewed in the context of PN-II proliferation and differentiation, SP expression, and the highly variable metabolism of individual PC species; these comprise intracellular synthesis, organized cellular trafficking, secretion, and reuptake into PN-II (33). Hence, due to the individual effects of therapeutics on these parameters, PN-II proliferation and gene expression, as well as synthesis, secretion, and turnover of total PC and PC subgroups, may change in very distinctive ways.

Phospholipid metabolism in the liver, which is the central organ of nutrient accretion and supply to peripheral organs, is essentially different from that of the lungs (8, 45, 46, 53). Lung PC is exclusively synthesized de novo by direct transfer of choline phosphate to diacylglycerols via the CDP choline (Kennedy) pathway, and the fatty acid (FA) composition is subsequently modified via the Lands cycle, resulting in a preponderance of saturated and monounsaturated PC. In contrast, the liver predominantly synthesizes di- and polyunsaturated PC. In addition to de novo synthesis, methylation of phosphatidylethanolamine by phosphatidylethanolamine methyltransferase (PEMT) contributes to the formation of liver PC. This pathway requires methionine (5-aminohexanethione, SAMe) as a methyl donor, where the methyl groups can originate from choline catabolism (38, 45, 53). The supply of arachidonic acid (C20:4) and docosahexaenoic acid (C22:6) to peripheral tissues in the form of polyunsaturated PC is a major function of the liver, and the PEMT pathway in particular contributes to this process (15, 45). These metabolic pathways and their substrate availabilities may well be affected by rhKGF, either compromising or improving the overall developmental effects of systemic rhKGF in preterm infants. The investigation of rhKGF effects on (phospho)lipid metabolism and homeostasis of the liver and of plasma are therefore important to comprehensively evaluate the usefulness of systemic rhKGF in neonatal medicine.

We set out to determine the effects of systemic rhKGF on surfactant homeostasis in neonatal rats in vivo in the context of hyperoxic lung injury. We therefore exposed newborn rats, from birth to day 7, to 85% vs. 21% oxygen and treated them with either placebo or rhKGF for 2 days before death at day 7. We first investigated the response of lung tissue to hyperoxia and/or rhKGF by routine histopathology and PN-II proliferation by BrdU incorporation. We then assessed the expression of SP-A, -B, -C, and -D genes as nonlipid components of surfactant to determine the efficacy of rhKGF treatment on PN-II under normal and increased oxygen concentrations. To examine the effects of surfactant homeostasis, we investigated the total amounts of PC in the secreted fraction (lung lavage fluid, LLF) and the amounts of PC and triglycerides (TG) in lung tissue. This was followed by fractionating PC into its major subspecies using electrospray ionization tandem mass spectrometry (ESI-MS/MS). To address the mechanisms involved in the changes found, we investigated synthesis and turnover of PC by spiking the rats with deuterated choline ([D₉]-methylcholine). Finally, we performed a similar analysis of liver and plasma PC and TG to assess the potential effects of rhKGF and hyperoxia on systemic lipid homeostasis.

MATERIALS AND METHODS

Materials. Phospholipid standards, reagents, and solvents were of analytical grade and were obtained from various commercial sources. Medical oxygen (100%) was supplied by Linde (Uterschleissheim, Germany). rhKGF (Kepivance) was a gift from Amgen (Thousand Oaks, CA). Betamethasone (Celestan Soluble, 4 mg/ml) was purchased from Essex Pharma (Munich, Germany). Ketamine (Selectvet) and xylazine (Sedaxylan) were obtained from Dr. Fischer (Weinheim, Germany) and WDT (Garbsen, Germany), respectively.

Animal experiments. Experiments were approved by the local authorities and met the NIH Guidelines for the Care and Use of Laboratory Animals. Female Sprague-Dawley rats were kept under standardized specific pathogen-free conditions, and their pups of either sex were kept with their mothers until death. Animals were kept for 7 days with a constant flow of a mixture of pure oxygen and synthetic air to reach final concentrations of 21% or 85% oxygen (18). The animals had free access to animal chow and tap water. Every 24 h the dams were exchanged to avoid any bias by differences in the oxygen exposure of lactating dams. Pups were subcutaneously injected 48 and 24 h before death at day 7 with either 5 mg/kg body wt rhKGF dissolved in phosphate-buffered saline (PBS), to induce maximal treatment effects, or with PBS alone, as has been described previously (25). [D₉]-methylcholine chloride (50 mg/kg body wt = 336μmol/kg) was injected intraperitoneally 6 h before death, which in small laboratory rodents in vivo is near maximum for precursor incorporation into lung and liver PC and in the linear phase of secretion of labeled PC by these organs (Refs. 5, 7, and unpublished data). Body weight was examined daily during treatment (days 5–7) using an electronic balance (model 440-45; Kern & Sohn, Balingen, Germany). Animals received 100 mg/kg body wt ketamine and 4 mg/kg body wt xylazine intraperitoneally 10 min before death. After death, all animals were controlled for the presence of milk in their stomachs to ensure regular food intake.
Histopathology and immunohistochemistry. Routine histopathological assessment was performed on paraffin sections stained with hematoxylin and eosin (H&E) using an Olympus BX51 microscope (Olympus Deutschland, Hamburg, Germany). Lung cell proliferation was determined at day 7 by uptake of 8-bromo-2’-deoxyuridine (8-BrdU), which was administered intraperitoneally 1.5 h before death (50 μg/kg body wt). Lungs were perfusion-fixed, embedded, and analyzed as described previously (48). Proliferating cells were detected by indirect immunohistochemistry using mouse anti-BrdU antibody clone IIB5 (BioGenex, San Ramon, CA) diluted 1:100 for 1 h at 37°C after treatment with protease (Sigma P5147; Sigma, Steinheim, Germany) and hydrolysis. Design-based stereology was used to quantify the fraction of alveolar surface area that was covered by BrdU-positive alveolar septal cells. Intersection counting was performed by means of the computer-assisted newCAST system (Visiopharm, Hoersholm, Denmark) using a line segment test system laid over fields of view, which was collected according to a systematic uniform random sampling procedure (20).

RNA isolation and RT-PCR. Total RNA was prepared from lung tissue samples using PeqGold solution (Peqlab Biotechnologie, Erlangen, Germany) according to the manufacturer’s instructions. Total RNA (1 μg) was reverse transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany) with oligo-(dT)15 primers following the manufacturer’s protocol in a final volume of 25 μl. RT-PCR was performed with primers for SP and β-actin as described previously (25) using Taq-polymerase (Master Mix kit; Eppendorf, Hamburg, Germany). β-Actin was used as housekeeping gene. In separate experiments, one further housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, was included. We tested that the expression of the two potential housekeeping genes chosen was not regulated in response to hyperoxia exposure. Although these results showed identical expression of the two housekeeping genes, with no change upon treatments, regulation by rhKGF cannot, however, be finally excluded. Reactions were run on a PCR Mastercycler (Eppendorf, Hamburg, Germany). PCR conditions were optimized for every pair of primers to fix an optimum annealing temperature and to make sure that the cycle number allows a quantification of mRNA concentrations during the exponential phase of the amplification process. The optimization process included primers for the housekeeping gene β-actin and a set of conditions to the abundance of the specific mRNA species using cDNA samples reverse transcribed from rat lung total RNA. After separation of PCR products by electrophoresis in 1.5% agarose gels containing ethidium bromide, products were visualized (UV-GelDoc SynGene system with GeneSnap 3.00.27 software, Cambridge, UK) and evaluated densitometrically using ImageQuant software. For semiquantitative evaluation of mRNA expression, specific signals were adjusted to the signals of the housekeeping gene.

Harvesting of samples for lipid analysis. Blood (0.4–0.6 ml) was drawn into EDTA containers by right ventricle puncture, and the lung vessels were then flushed with ice-cold saline. LLF was harvested as described elsewhere (6, 25). Cells were removed from LLF by centrifugation at 200 g for 10 min at 4°C. Blood was spun at 1,000 g for 10 min and plasma aspirated for further analysis. Lungs and livers were excised, and all samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Analysis of PC and TG. Lipids were extracted from LLF and from plasma according to the method of Bligh and Dyer (9) and from lung and liver tissue according to Folch et al. (22). Phospholipids were quantified according to the method of Bartlett (6) after digestion of the organic compounds. For mass spectrometry of PC, total lipid extracts were spiked with dieicosanoyl-PC (PC20:0/20:0) as an internal standard. TG were isolated from lipid extracts using 100-mg Strata NH2 disposable cartridges (Phenomenex) and eicosahexatrienoic acid (C20:3) as an internal standard for subsequent gas chromatography (46).

Mass spectrometry of endogenous and newly synthesized PC molecular species. PC species were quantified using a Thermo TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo, Dreieich, Germany) equipped with a heated electrospray ionization (H-ESI) interface. Samples were dissolved in butanol-methanol-water (75:23:2 vol/vol) at a concentration of 3 (LLF) or 6 μmol/l (plasma, liver, and lung tissue). Samples (25 μl) were introduced into the mass spectrometer via loop injection using a Finnigan Surveyor Autosampler Plus and MS Pump Plus (Thermo). The mobile phase comprised butanol-methanol-water-25% NH4OH (75:23:1.7:0.3 vol/vol), which was delivered at rates increasing from 10–30 μl/min (0–1.5 min) to 200 μl/min (3–3.49 min) and back to 10 μl/min (3.5 min). PC molecular species were scanned over their elution times from 0.6 to 2.5 min and quantified from diagnostic precursor ion scans of the respective fragment ions in the selective reaction monitoring (SRM) mode. Defined PC species comprising >95% of total PC in rat tissues and their labeled derivatives were selected as described elsewhere (45). Phosphocholine [mass/charge (m/z) = +184] was used for endogenous PC. For direct incorporation of [D6-methyl]choline from de novo synthesis (M+9), D6-phosphocholine (m/z = +193) served as the diagnostic fragment. For the PEMT pathway, the incorporation of one or two deuterated methyl groups (D3-H-) from [D6-methyl]choline catabolism and transfer to form D3-SAME results in mass shifts of M+3 and M+6 for all PC species. Hence, D3- and D6-phosphocholine (m/z = +187 and +190, respectively) were used as diagnostic fragments. Absolute values of PC synthesis via the PEMT pathway were calculated by dividing the concentrations of D3/D6-labeled PC by the D3-methyl enrichment (E) of SAME as the PEMT substrate, according to the equation E = D6/(D6+D3) (44).

Statistics. Data are means ± SE. Two-way and one-way analysis of variance was performed for testing treatment effects. For multiple group comparisons, data were analyzed post hoc using the method of Bonferroni-Holm with SPSS version 17.0 (SPSS, Chicago, IL) or InStat 3.0 (GraphPad Software, La Jolla, CA). Significance was accepted at P < 0.05.

RESULTS

Effects of hyperoxia and rhKGF treatment on growth parameters, lung histopathology, proliferation, and the expression of surfactant protein mRNA. The body weights were 13.45 ± 0.33, 11.76 ± 0.24, 15.24 ± 0.32, and 12.48 ± 0.54 g at day 7 for 21% oxygen, 21% oxygen + rhKGF, 85% oxygen, and 85% oxygen + rhKGF, respectively (P < 0.05). Likewise, growth rates from day 5 to day 7 were not significantly different, being 26.1 ± 2.1, 19.7 ± 3.1, 26.8 ± 2.6, and 20.3 ± 0.5%, respectively (P > 0.05). Histopathologically, alveolar septa appeared to be thickened in response to hyperoxia (Fig. 1B) compared with 21% O2 (Fig. 1A and C), an effect that was prevented by treatment with rhKGF (Fig. 1D). In normoxic lungs, however, rhKGF showed no visible effect (Fig. 1C). BrdU-positive cells relative to alveolar surface area were present in lung tissue of all treatment groups, comprising 0.89 ± 0.18 (n = 6), 0.72 ± 0.10 (n = 5), 0.52 ± 0.17 (n = 2), and 0.89 ± 0.24% (n = 3), respectively (P > 0.05), and indicating that with rhKGF treatment, cell proliferation is maintained in both normoxic and hyperoxic neonatal lungs.

As demonstrated in Fig. 2, hyperoxia, but not rhKGF, increased SP-A mRNA expression, whereas expression of SP-D mRNA was not altered by any treatment tested. In contrast, rhKGF increased the expression of SP-B mRNA under both normoxic and hyperoxic conditions. However, this increase was attenuated in hyperoxic lungs. The expression of
SP-C mRNA was significantly increased by hyperoxia, but a combination of hyperoxia with rhKGF produced the strongest effect. In summary, these data indicate that rhKGF counteracted the effect of hyperoxia on lung morphology. Apart from its highly differential effects on SP gene expression, rhKGF noticeably increased SP gene expression in both normoxic and hyperoxic lungs.

Effects of rhKGF and hyperoxia on PL and PC homeostasis of LLF and lung tissue. In LLF, hyperoxia decreased the amount of total PL (Fig. 3A) and its major component, PC (Fig. 3B), by about 20%. Treatment with rhKGF, however, increased total PL and PC by more than twofold in both normoxic and hyperoxic rats. In lung tissue, hyperoxia significantly decreased total PL, whereas rhKGF increased its pool size in both normoxic and hyperoxic rats (Fig. 3E). However, with respect to total PC, the only significant effect observed was in normoxic rats treated with rhKGF compared with untreated normoxic animals (Fig. 3F).

Effects of rhKGF and hyperoxia on \([D_9\text{-methyl}]\)choline metabolism of PC in LLF and lung tissue. Whereas hyperoxia decreased total PC pools in LLF, secretion of newly synthesized \([D_9\text{-methyl}]\)choline-labeled PC (Fig. 3C) was unchanged, resulting in a significant increase in the \([D_9\text{-methyl}]\)choline enrichment of PC in LLF (Fig. 3D). In contrast, rhKGF increased \([D_9\text{-methyl}]\)choline-labeled PC in LLF in both normoxic and hyperoxic rats (Fig. 3C). This increase, however, was smaller than that of total PC (Fig. 3B), resulting in decreased \([D_9\text{-methyl}]\)choline enrichment of LLF-PC in response to rhKGF treatment (Fig. 3D). In lung tissue, neither treatment caused significant changes in the pool size of newly synthesized PC (Fig. 3G) or in \([D_9\text{-methyl}]\)choline enrichment (Fig. 3H). Hence, the twofold increase in total PC in response to rhKGF, together with a much lower increase in newly synthesized PC in LLF, indicates that the increase in LLF-PC in response to rhKGF is mainly due to decreased turnover of secreted PC rather than increased secretion. By contrast, the opposite finding in hyperoxia, i.e., decreased total PC together with unchanged amounts of newly synthesized PC in LLF, suggests that hyperoxia-induced surfactant depletion is due to increased turnover of secreted PC rather than impaired secretion. Moreover, the unchanged pools of newly synthesized \((D_9\text{-methyl})\)choline-labeled PC in lung tissue show that increased or decreased synthesis is not involved in the changes.

Effects of hyperoxia and rhKGF on pools and metabolism of PC subgroups in LLF and lung tissue. Examining these findings with respect to PC subgroups (Fig. 4) revealed that, in general, the molecular composition of PC in response to both hyperoxic and rhKGF treatment was maintained (not shown).
Hence, rhKGF caused increases of all PC subgroups in LLF, which applied to both normoxic and hyperoxic lungs (Fig. 4A). However, we found a few exceptions to this principle of lipidomic maintenance for hyperoxic treatment and for lung tissue: in LLF, hyperoxia only caused a significant decrease in surfactant PC ($P < 0.01$) (Fig. 4A). In lung tissue, rhKGF increased the pools in surfactant-PC and in C18:2-PC in normoxic lungs (Fig. 4D). In contrast, polyunsaturated PCs (C20:4-PC, C22:6-PC) of lung tissue were increased by rhKGF not only in normoxic but also in hyperoxic lungs. Newly synthesized PC subgroups in LLF were increased by rhKGF to a lesser degree than their total pools (Fig. 4, A and B), resulting in similar decreases of [D9-methyl]choline enrichment for PC subgroups (Fig. 4C) as demonstrated above for total PC. Although only a minor component in surfactant, polyunsaturated C22:6-PC was an exception here, because the increase in [D9-methyl]choline label in response to rhKGF was 188 ± 54% and 272 ± 90% for normoxia and hyperoxia, respectively.
whereas for surfactant PC it was +49 ± 13% and +36 ± 16%, respectively (P < 0.05). Therefore, [D9-methyl]choline enrichment of C22:6-PC was not decreased by rhKGF in a similar way to that of other PC components (Fig. 4C). In lung tissue, [D9-methyl]choline incorporation was constant for all PC subgroups in response to any treatment (Fig. 4E), whereas enrichment of [D9-methyl]choline was even decreased for surfactant PC in rhKGF-treated normoxic lungs (Fig. 4F). In summary, the rhKGF- and hyperoxia-induced changes in total secreted surfactant PC similarly apply to PC subgroups. In lung tissue, data suggest that rhKGF is effective in increasing polyunsaturated PC in both normoxic and hyperoxic lungs, which only applies to normoxic lungs for surfactant PC. For no PC subgroup, however, were pool changes attributable to increased synthesis.

**Effects of rhKGF and hyperoxia on PL and PC homeostasis of liver and plasma.** The molecular composition of PC in neonatal rat plasma (Fig. 5A) and liver tissue (not shown) was essentially different from that of lung PC (Fig. 4). Whereas lung tissue and surfactant PC mainly comprised saturated and monounsaturated components, plasma and liver PC was dominated by components containing linoleic (C18:2), arachidonic (C20:4), and docosahexaenoic acid (C22:6). The equilibrium distribution of plasma PC showed higher diunsaturated PC compared with that of the liver, as indicated by endogenous (unlabeled) components (P < 0.001) (Fig. 5, B and C, open bars). Nevertheless, most plasma PC came from the liver, because PC synthesized via the liver-specific PEMT pathway (45, 53) was highly enriched not only in liver but also in plasma (Fig. 5, B and C, bars with horizontal stripes). Data also demonstrate the preferential synthesis of polyunsaturated PCs via the PEMT pathway and mono- and diunsaturated PCs via de novo synthesis, which is important for the appreciation of systemic rhKGF effects (see below).

**Effects of rhKGF and hyperoxia on liver PL and PC metabolism.** In contrast to the situation in the lung, absolute concentrations of liver PL and total PC were not significantly altered by hyperoxia or rhKGF. Similarly, the fraction of PC relative to total PL, as an indicator of hepatic PC homeostasis (52), showed no significant differences (Table 1, liver). However, [D9-methyl]choline-labeled PC, as a parameter of de novo synthesis, was increased by rhKGF but not altered by hyperoxia, whereas the PEMT pathway was not significantly affected (Table 1, liver). Fractionating PC into its subgroups showed that in normoxic rats, rhKGF increased the pools of saturated and mono- and diunsaturated PCs, as well as C22:6-PCs, to a significant but small extent, whereas C20:4-PCs were not altered (Fig. 6A). C20:4-PC, however, was increased by hyperoxia treatment alone, whereas C22:6-PC was the only subgroup increased by rhKGF irrespective of oxygen exposure. (Fig. 6A).

In contrast to the total PC pool in liver, PC newly synthesized via direct incorporation of [D9-methyl]choline was increased by more than 50% by rhKGF (Fig. 6B). This applied to both normoxic and hyperoxic livers, whereas hyperoxia alone had no effect. On the basis of this pathway (de novo synthesis), rhKGF showed the strongest effect on C20:4-PC in hyperoxic rats, whereas C22:6-PC synthesis was increased in both normoxic and hyperoxic rats (Fig. 6B). In addition, rhKGF significantly increased C22:6-PC synthesis via the methylation (PEMT) pathway, the major pathway for synthesis of polyunsaturated PC (Fig. 6C). Hence, the effects of rhKGF and hyperoxia on liver PL homeostasis were essentially different from those on the lungs. rhKGF significantly increased the synthesis of PC subgroups in both normoxia and hyperoxia, whereas PL and PC pool sizes showed no major changes, suggesting increased hepatic PC turnover in response to rhKGF.

**Effects of rhKGF and hyperoxia on plasma PL and PC metabolism.** In plasma, PL and total PC concentrations were not significantly altered by hyperoxia or rhKGF. Similarly, the fraction of PC relative to total PL, as an indicator of hepatic PC homeostasis (52), showed no significant differences (Table 1, plasma). However, [D9-methyl]choline-labeled PC, as a parameter of de novo synthesis, was increased by rhKGF but not altered by hyperoxia, whereas the PEMT pathway was not significantly affected (Table 1, plasma). Fractionating PC into its subgroups showed that in normoxic rats, rhKGF increased the pools of saturated and mono- and diunsaturated PCs, as well as C22:6-PCs, to a significant but small extent, whereas C20:4-PCs were not altered (Fig. 6A). C20:4-PC, however, was increased by hyperoxia treatment alone, whereas C22:6-PC was the only subgroup increased by rhKGF irrespective of oxygen exposure. (Fig. 6A).

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in the PC-to-PL ratio (Table 1, plasma). This increase applied similarly to PC subgroups (Fig. 6D). Among the polyunsaturated components, C20:4-PC was independently increased by hyperoxia and rhKGF, and combination treatment doubled this effect. C22:6-PC, however, was increased by rhKGF in both normoxic and hyperoxic rats, with no endogenous hyperoxia effect. Addressing PC metabolism showed that in the total PC of both normoxic and hyperoxic rats, [D9-methyl]choline was only effective in normoxic rats at increasing C22:6-PC via independently from oxygen exposure (Fig. 6A and B), whereas it was only effective in normoxic rats at increasing C22:6-PC via PEMT (Fig. 6F), the major PC subgroup synthesized by this pathway (44). In essence, these data demonstrate the complex effects of rhKGF in increasing plasma PL and, particularly, polyunsaturated PC subgroups. Together with increased PC turnover in liver tissue, this is suggestive for increased secretion of PC components by the liver into plasma.

Effects of hyperoxia and rhKGF on TG homeostasis of lung tissue, liver, and plasma. With regard to PL and PC homeostasis in the context of TG and lipoprotein metabolism (8), we assessed the pools of total TG and their individual fatty acids (FA). In contrast to PL and PC, total TG pools and TG-to-PL ratios were decreased by rhKGF in lung tissue (Table 2). As demonstrated in Fig. 7A for individual TG-FA, in normoxic lungs rhKGF decreased only unsaturated FA, whereas palmitic acid (C16:0) and myristic acid (C14:0), the saturated FA...
TG, mmol/l 1.078

During postnatal life, which in this species is likely compensated by high-frequency oscillatory respiration in an attempt to prevent lung collapse (6, 25). Therefore, neonatal rat lungs can be considered as a model for human preterm lungs with low surfactant pools; these rat lungs can be used for assessing novel therapeutic approaches.

Effects of hyperoxia and rhKGF on pneumocyte proliferation and SP gene expression. We found no significant changes in growth rates and PN-II proliferation by rhKGF in normoxic and hyperoxic lungs. Although this lack of significance may be attributed to the limited number of histological experiments in this study, our data are consistent with those of Franco-Montoya et al. (23) as well as with our previous observations (25). In addition, lack of significance in decreasing BrdU incorporation in response to 85% oxygen may be due in part to the use of 85% rather than 95% oxygen (23). It must, however, be noted that in neonatal rats, 7 days of 85% oxygen exposure did not increase PN-II proliferation as previously shown for adult rats (18). Nevertheless, hyperoxia resulted in alterations in histopathology, like septal broadening, which were ameliorated by rhKGF. Although the number of animals investigated was limited, the data presented are in line with previous results (25) demonstrating that both growth rate and basal PN-II proliferation in healthy normoxic and hyperoxic neonatal rats are maintained by rhKGF. These findings in 7-day-old rats contrast with those found beyond the first phase of alveolarization (day 21) and in adult rats, where rhKGF strongly increases PN-II proliferation. Moreover, the maintenance or induction of PN-II proliferation in 7- or 21-day-old rats, respectively, by rhKGF contrasts the effect of glucocorticoid treatment, which blunted basal as well as rhKGF-induced PN-II proliferation at either age (25, 55).

In addition, rhKGF was effective in stimulating SP-B gene expression in hyperoxic and normoxic lungs and SP-C expression, particularly, in hyperoxic lungs. Although we did not measure secreted proteins, these data are consistent with previous results showing increased amounts of SP-B and SP-C in LLF in response to rhKGF (25). In contrast, hyperoxia increased SP-A and SP-C expression. This is consistent with previous findings showing that in neonatal lungs, induction of SP gene expression by hyperoxia is small and delayed, compared with a rapid and severalfold increase in adult hyperoxic lungs (41, 54). Since expression of the individual SP genes and reaction to hyperoxia are spatially and temporarily different during neonatal lung development (30), our data only show a snapshot of hyperoxia and rhKGF effects on neonatal lungs.

Table 2. Effects of rhKGF and 85% oxygen on triglyceride concentrations in lung, liver and blood plasma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>21% O₂</th>
<th>21% O₂ + rhKGF</th>
<th>85% O₂</th>
<th>85% O₂ + rhKGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG, µmol/g body wt</td>
<td>0.240 ± 0.006</td>
<td>0.201 ± 0.018&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.224 ± 0.012</td>
<td>0.135 ± 0.012&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG, µmol/µmol PL</td>
<td>0.444 ± 0.009</td>
<td>0.332 ± 0.022&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.487 ± 0.024</td>
<td>0.252 ± 0.043&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG, µmol/g body wt</td>
<td>0.187 ± 0.032</td>
<td>0.364 ± 0.023&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.354 ± 0.028&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.583 ± 0.137&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>TG, µmol/µmol PL</td>
<td>0.144 ± 0.018</td>
<td>0.267 ± 0.014&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.292 ± 0.021&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.421 ± 0.087&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>1.078 ± 0.127</td>
<td>2.475 ± 0.264&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.596 ± 0.201</td>
<td>1.714 ± 0.191</td>
</tr>
<tr>
<td>TG, µmol/µmol PL</td>
<td>0.382 ± 0.032</td>
<td>0.715 ± 0.105&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.491 ± 0.052</td>
<td>0.386 ± 0.032&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4 (rhKGF treatments) or 10–15 (no rhKGF treatments) experiments. PL, phospholipid; TG, triglyceride. <sup>a</sup>P < 0.05; <sup>b</sup>P < 0.01; <sup>c</sup>P < 0.001 vs. 21% O₂. <sup>d</sup>P < 0.05 vs. 21% O₂ + rhKGF. <sup>e</sup>P < 0.05; <sup>f</sup>P < 0.01; <sup>g</sup>P < 0.001 vs. 85% O₂.

DISCUSSION

The need for increased oxygen supply is a major threat to immature lungs because of the increased generation of oxygen radicals at a time when radical scavenger pools and antioxidative enzymes are still insufficient (11, 27, 34). Concepts of improving acute therapy and optimizing alveolar formation and lung development in preterm infants are long-standing issues (27, 34). Here, rhKGF may offer progress in neonatal intensive care (3, 13, 16, 25). Its clinical potential is supported by the finding that it is decreased in lungs that subsequently develop BPD and in response to ventilation injury, and that it reduces mortality from high oxygen exposure in mice (12, 17, 47, 55, 56). Although systemically used in adult patients to treat mucositis (40, 52), the route of administration and dosage may influence the magnitude and local control of effects. In the present study, rhKGF was used subcutaneously, at a standard dose of 5mg/kg body wt, to assess pulmonary and extrapulmonary effects in newborn rats exposed to 85% oxygen. Because alveolarization in the rat starts at about postnatal day 4, lungs of neonatal rats are structurally immature, like those of preterm infants. On the other hand, the respiratory system of term rats is sufficiently developed to allow for spontaneous respiration. However, in a similar way to the situation in which a preterm infant is suffering from surfactant deficiency, neonatal rats at day 7 show the lowest level of secreted surfactant during postnatal life, which in this species is likely compensated for by high-frequency oscillatory respiration in an attempt to prevent lung collapse (6, 25). Therefore, neonatal rat lungs can be considered as a model for human preterm lungs with low surfactant pools; these rat lungs can be used for assessing novel therapeutic approaches.

rhKGF and Systemic Phosphatidylcholine Homeostasis • Raith M et al.
neonatal lungs, where oxygen treatment further decreases secreted surfactant, rhKGF increased the amount of secreted surfactant PC. Furthermore, rhKGF increased (normoxia) or at least maintained (hyperoxia) PC in lung tissue, which is contrary to systemic glucocorticoid treatment, which decreases lung tissue PC (25).

The data presented in this report shed new light on the mechanism involved in decreasing secreted surfactant in neonatal hyperoxia and in increasing it in response to systemic rhKGF treatment. The use of stable isotope-labeled choline ([D9-methyl]choline) combined with ESI-MS/MS is key to understanding these mechanisms. In hyperoxic newborn rat lungs not treated with rhKGF, the amounts of newly synthesized [D9-methyl]choline labeled PC secreted into the air spaces were identical to those of controls. However, the amounts of total PC in LLF were decreased, suggesting increased alveolar surfactant turnover in response to hyperoxia, rather than decreased secretion. In contrast, rhKGF had just the opposite consequences in both normoxic and hyperoxic lungs: total PC of LLF and the secreted fraction of total lung tissue PC pools were increased by more than twofold in response to rhKGF, whereas the secreted amounts of newly synthesized PCs were increased by <50%, resulting in decreased [D9-methyl]choline enrichment in PC components of LLF. It is important to note that alterations in surfactant pools and metabolism are independent from PN-II proliferation, which was demonstrated in studies involving glucocorticoids and rhKGF in neonatal, fully alveolarized, and adult rats (19, 25), suggesting effects of rhKGF on cell function rather than proliferation. In neonatal rats with 95% oxygen as a lethal concentration, rhKGF improved wound healing, repair, and cell survival. In contrast to our data, PL and disaturated PC were increased in response to hyperoxia but not in response to rhKGF (23). The reasons underlying these differences are speculative: we used 85% oxygen as an adaptive concentration (18) and twice injected rhKGF (5 mg·kg·day) subcutaneously for 48 h before death (25), rather than lower repetitive doses of intraperitoneal rhKGF application from the beginning of 95% oxygen onward. Exposure to 85% oxygen showed survival for at least 14 days (not shown), suggesting that cell damage may be lower and surfactant changes may be different. Furthermore, differences in application route, dosage, and duration of treatment may explain the different effects. In essence, our data suggest that nonlethal hyperoxia impairs surfactant homeostasis by increasing surfactant turnover, whereas short-term systemic rhKGF administration improves surfactant homeostasis primarily by decreasing its turnover, rather than by increasing synthesis and secretion.

This conclusion is in line with our data on total and newly synthesized PC in lung tissue: rhKGF increased (normoxia) or maintained (hyperoxia) the amounts of PC. Differential molecular analysis of PC molecular species showed that this applied to all PC components with no preferential effects on surfactant PC, because di- and polysaturated PCs were changed in a similar way. rhKGF, however, had no effect on pools of [D9-methyl]choline labeled PC. Elevated amounts of surfactant PC with no increase in synthesis and decreased surfactant turnover are compatible with increased PN-II differentiation in response to rhKGF (13, 43); this is also consistent with previous findings that increased surfactant PC during lung maturation is due to decreased turnover rather than increased synthesis (10). Hence, our [D9-methyl]choline labeling experiments explain the effects of hyperoxia and rhKGF on neonatal

![Image of Figure 7](https://example.com/image.png)

Fig. 7. Effects of rhKGF and 85% oxygen on lung tissue (A), liver tissue (B), and blood plasma (C) triglyceride fatty acid (TG-FA) concentrations. TG-FA of tissue and plasma extracts were quantified with gas chromatography as indicated in MATERIALS AND METHODS. Data are means ± SE of 4–10 experiments. C12:0, lauric acid; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic (Δ7) acid; C18:0, stearic acid; C18:1, oleic (Δ9-) acid; C18:2, linoleic (Δ9,12) acid; C20:4, arachidonic (Δ5,8,11,14) acid; C22:6, docosahexaenoic (Δ4,7,10,13,16,19) acid.

Nevertheless, our data show that rhKGF is not only effective in vitro and in injured adult lungs (13, 20, 28) but also in vivo in neonatal lungs, and without the catabolic side effects of glucocorticoids.

Effects of hyperoxia and rhKGF on surfactant PC metabolism. In neonatal rats exposed for 1 wk to 85% oxygen, rhKGF caused an increase in secreted surfactant that was as high as the increase found in normoxic rats. However, in hyperoxic rats with no rhKGF treatment, secreted surfactant was decreased relative to that in normoxic controls. This applied to total PC as well as to those components specifically enriched in surfactant over lung tissue. Interestingly, this is opposite to the situation in adult rats, where 1 wk of 85% oxygen exposure increased secreted surfactant, accompanied by broadening of alveolar septa and massively increased, rather than maintained, PN-II proliferation (18). Hence, although not efficient in mature rat lungs (19, 25), in immature, neonatal lungs, where oxygen treatment further decreases secreted surfactant, rhKGF increased the amount of secreted surfactant PC. Furthermore, rhKGF increased (normoxia) or at least maintained (hyperoxia) PC in lung tissue, which is contrary to systemic glucocorticoid treatment, which decreases lung tissue PC (25).

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surfactant homeostasis as due in the main to alterations in alveolar and PN-II “economy” of surfactant turnover; this is because of an impairment of turnover in response to hyperoxic injury, which is counteracted by systemic rhKGF treatment. These data must be regarded in the context of contemporary clinical approaches used for treatment of preterm infants. Although alternative approaches to treat neonatal lungs were suggested long ago, glucocorticoids are still used to cope with lung immaturity and perinatal inflammatory processes. Whereas glucocorticoids improve the amounts of secreted surfactant, they inhibit PN-II proliferation, alveolarization, and, if delivered systemically, neurodevelopment (11, 25, 27, 34). Although rhKGF exerts similar beneficial effects on SP gene expression and secreted surfactant, it neither exerts catabolic side effects nor impairs PN-II proliferation or lung tissue PC homeostasis, suggesting that systemic treatment with rhKGF is superior to treatment with glucocorticoids. This latter suggestion, however, must be addressed by detailed investigation of rhKGF effects in the systemic context.

Liver and plasma homeostasis of lipids in response to rhKGF treatment. The liver is the central organ for distributing choline and polyunsaturated PC to peripheral organs, thereby contributing to normal development (15, 38, 53). The kinetics and molecular pattern of liver and plasma PC are essentially different from those of the lungs, with preferential enrichment and secretion of polyunsaturated PC. Importantly, docosahexaenoic acid (C22:6) and arachidonic acid (C20:4) are primarily transported as PC and preferentially used for the supply of the developing brain (37, 46). Although our data only show minor changes in total liver PC concentrations, plasma PC and, particularly, its polyunsaturated subgroups were increased by rhKGF. Labeling with [D9-methyl]choline combined with ESI-MS/MS revealed that rhKGF increased synthesis and metabolism in liver and plasma PC, particularly for the polyunsaturated components (15). In the liver, rhKGF increased the synthesis of PC via both de novo synthesis and the PEMT pathway. Importantly, the calculated hepatic concentration of C20:4-PC species newly synthesized via the PEMT pathway after 6 h of labeling was about 50% of that of the total C20:4-PC pool, demonstrating the high turnover rate of polyunsaturated PC in the liver. For C22:6-PCs, a similarly important nutrient for neurodevelopment (15), calculated amounts of newly synthesized components 6 h after [D9-methyl]choline injection definitely exceeded the endogenous pool, indicating a fast and differential turnover of individual PC components, which are the main carriers of C22:6 to peripheral organs (37, 46). Here, rhKGF further increased C22:6-PC synthesis via PEMT, which may be advantageous for overall and neuronal development, which is critical for preterm infants. Similarly, polyunsaturated PC synthesized de novo were increased by rhKGF, indicating that rhKGF increases hepatic PC synthesis, turnover, and secretion in both normoxic and hyperoxic neonatal rats. This conclusion is consistent with the effects of rhKGF on plasma PL homeostasis, demonstrating that rhKGF increased the concentrations of total PL and total PC. Both total and newly synthesized PC subgroups were increased. Furthermore, the most significant effect of rhKGF was on the increased plasma concentration of C22:6-PC, providing further evidence that rhKGF improves the peripheral supply with essential lipid nutrients (15). C20:4-PCs generated via PEMT comprised one-third of its endogenous analogs, whereas C22:6-PCs from this pathway were even higher than its endogenous analogs. This is in line with the high turnover of polyunsaturated plasma PC, particularly C22:6-PCs, in humans (37, 45). As a consequence, it can be regarded as a desired side effect of systemic rhKGF treatment, because choline, C20:4, and C22:6 are essential for brain development (15, 37).

Interestingly, TG that are regularly exported from the liver as very low density lipoprotein (VLDL) components were increased by rhKGF in the liver of normoxic and hyperoxic rats. That is opposite to what takes place in lung tissue, where TG and the TG-to-PL ratio were decreased after rhKGF treatment. Whereas the loss of TG in the lung may relate to increased TG degradation in lung tissue in response to rhKGF (not shown), the increase in liver and plasma is likely to be associated with the high-fat (11–14%) milk diet of newborn rats (26), highlighting the differences between pulmonary and systemic effects of rhKGF treatment. In normoxic, rhKGF-treated rats, TG were increased most in plasma, whereas in hyperoxic rats, TG were increased most in liver tissue. Because the liver secretes TG via VLDL, this may be due to decreased hepatic TG secretion. Systemic hyperoxia impairs liver perfusion, and is a characteristic of spontaneously breathing animals exposed to hyperoxia (18, 23, 42, 51). This, however, does not apply to the clinical situation of preterm infants with respiratory insufficiency. Here, high inspiratory oxygen is used to achieve normal oxygenation of peripheral organs, rather than increasing oxygen pressure to nonphysiological values higher than 400 mmHg (51). Hence, further investigation of VLDL metabolism will be necessary to address systemic hyperoxia effects with and without rhKGF treatment.

Conclusions and perspectives of rhKGF treatment. rhKGF is currently used for mucositis treatment only (40, 52), whereas a considerable body of experimental evidence suggests that it might be developed into a therapeutic option for preterm infants with or at risk of lung injury. In a former study, we demonstrated improved differentiation markers of the surfactant system and increased secreted surfactant pools in normoxic neonatal rats. Contrary to glucocorticoid treatment, this went along with maintained lung PC homeostasis and PN-II proliferation (25). Our present data on improved surfactant homeostasis in hyperoxic neonatal rats point to its usefulness in the clinical setting. In addition to its pulmonary effects, hepatic and plasma kinetics of essential polyunsaturated lipids in response to systemic rhKGF treatment appear to be improved. Such pleiotropic anabolic effects may be superior to those of systemic or even topical glucocorticoid treatments, where improved surfactant homeostasis is achieved at the expense of local and overall catabolic side effects (11, 25, 27, 34).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


