

Serum cholestenic acid as a potential marker of pulmonary cholesterol homeostasis: increased levels in patients with pulmonary alveolar proteinosis

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Abstract The conversion of cholesterol into the more polar metabolites 27-hydroxycholesterol (27-OH) and cholestenic acid by the cytochrome P450 sterol 27-hydroxylase is a cholesterol-removal mechanism used by almost all cells. Most of the cholestenic acid present in the circulation originates from the lung, and it has been suggested that sterol 27-hydroxylase is of particular importance for cholesterol homeostasis in this organ. As an example of pulmonary cholesterol accumulation, a known disorder of surfactant homeostasis, pulmonary alveolar proteinosis (PAP), was studied. Analysis of bronchoalveolar lavage fluid from PAP patients revealed a significant accumulation of the cholesterol metabolites cholestenic acid and 27-OH. This pattern was recapitulated in serum, with a significant increase in the levels of both cholestenic acid ($P = 0.003$) and 27-OH ($P = 0.017$) in PAP patients compared with healthy controls. Analysis of PAP alveolar macrophages did not reveal a significant change in mRNA expression levels of either sterol 27-hydroxylase or the cholesterol-esterifying enzyme acyl-CoA:cholesterol acyltransferase-1. These results are consistent with the contention that substrate availability, rather than enzyme expression, is the key factor in regulating the production of cholestenic acid by the lung and that serum cholestenic acid may be a marker of pulmonary cholesterol homeostasis.—Meaney, S., T. L. Bonfield, M. Hansson, A. Babiker, M. S. Kavuru, and M. J. Thomassen. Serum cholestenic acid as a potential marker of pulmonary cholesterol homeostasis: increased levels in patients with pulmonary alveolar proteinosis. *J. Lipid Res.* 2004. 45: 2354–2360.

Supplementary key words pulmonary surfactant • cytochrome P450 sterol 27-hydroxylase • 27-hydroxycholesterol • oxysterol

The conversion of cholesterol into the more polar metabolites 27-hydroxycholesterol (27-OH) and 3 β -hydroxy-5-cholestenic acid (cholestenic acid) is a widespread oc-

currence in vertebrate tissues. In addition to having an essential role in bile acid biosynthesis, this conversion is also believed to be an antiatherogenic mechanism (1). This transformation is mediated by the activity of the cytochrome P450 sterol 27-hydroxylase (CYP27A1), which, unusual for a cytochrome P450, is able to further hydroxylate 27-OH at the C-27 position, leading to the production of the carboxylic acid cholestenic acid (2). Cells of monocytic lineage are highly active in this reaction, with alveolar macrophages being the most active cell type tested to date (3). It has been demonstrated that the majority of cholestenic acid present in the circulation is derived from the lung (4), suggesting that CYP27A1-mediated reverse cholesterol transport is important in lung cholesterol homeostasis.

Cholesterol is the most abundant neutral lipid of pulmonary surfactant, constituting up to 90% of the neutral lipid material (5). At least 80% of the cholesterol present in the lung, and virtually all that in surfactant, is derived from circulating lipoproteins (6), with very low density lipoprotein believed to be the major vehicle for this delivery. Type II pneumocytes are believed to be responsible for the majority of uptake, packaging, and secretion of the surfactant components (7). Moreover, surfactant lipids are efficiently recycled by the type II pneumocytes, with up to half of the material taken up resecreted into the surfactant compartment (8). The remaining material is believed to be catabolized by alveolar macrophages and eliminated from the lung (8). However, the exact mechanisms by which surfactant cholesterol is removed are poorly understood.

Pulmonary alveolar proteinosis (PAP), first described in humans in 1958, is associated with the accumulation of surfactant lipids and proteins in the airspaces that impair gas exchange, causing dyspnea, fatigue, and exercise intolerance (9). A milk-colored bronchoalveolar lavage (BAL)

Manuscript received 9 August 2004 and in revised form 16 September 2004.

Published, JLR Papers in Press, October 1, 2004.
DOI 10.1194/jlr.M400302.JLR200

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fluid and foamy macrophages are observed. Although accumulation of surfactant phospholipids is a common finding, cholesterol crystals are also sometimes present upon cytological examination (10). Studies in granulocyte/macrophage colony-stimulating factor (GM-CSF) knockout mice have revealed a critical role for GM-CSF in lung homeostasis (reviewed in Ref. 11). Human idiopathic PAP has come to be recognized as an autoimmune condition in which neutralizing antibodies to GM-CSF limit the availability of biologically active GM-CSF. Indeed the presence of autoantibodies to GM-CSF is considered to be diagnostic of PAP (12).

It has been reported that the addition of CSFs to peripheral blood monocytes *in vitro* can induce the expression of an alveolar macrophage phenotype, as defined by a variety of functional parameters, including surfactant catabolism and phagocytosis (13). Transcriptional analysis of more than 50,000 genes using a Serial Analysis of Gene Expression (SAGE) approach identified a set of genes highly regulated after *in vitro* differentiation by GM-CSF (14). Interestingly, several genes involved in lipid homeostasis were highly induced compared with control cells, including apolipoprotein E (>150-fold increase) and CYP27A1 (30-fold increase), two genes involved in reverse cholesterol transport. Similar, although less pronounced, increases were observed after differentiation in the presence of M-CSF (100- and 6-fold, respectively).

These findings prompted us to investigate if the accumulation of cholesterol in PAP alveolar macrophages is attributable to impaired induction of CYP27A1 and a loss of this cholesterol-removal pathway.

MATERIALS AND METHODS

Materials

All organic solvents used were of gas chromatography or high performance liquid chromatography grade. Internal standards for gas chromatography-mass spectrometry were as previously described (15).

Patients

This study was approved by the Cleveland Clinic Institutional Review Board, and informed consent was obtained from all subjects. The diagnosis of PAP was established by open lung or transbronchial biopsy. The characteristics of the patients with PAP were as follows: age, 42 ± 7 years (mean \pm SD); male-to-female ratio, 10:4. None of the subjects was on medication at the times the samples were obtained. None of the PAP patients gave a history suggestive of underlying liver disease. On examination, there were no stigmata of chronic liver disease (i.e., no hepatomegaly). Baseline liver function studies were available for nine PAP patients, seven of which were completely normal, with two patients displaying mild increases of transaminases (i.e., <2-fold increase above normal). None of the patients had increased bilirubin. The PAP in these patients was felt to be idiopathic in all cases based on the exclusion of known secondary causes of PAP. Healthy volunteers ($n = 13$) were identified by local advertising and through the employee pool.

BAL fluid and serum

BAL was performed as previously described (16). Fluid was separated from cells, divided into aliquots, and stored at -80°C

until assayed. Serum samples were obtained from all patients with PAP and control subjects at the time of BAL. Briefly, peripheral blood was collected in serum separator tubes, centrifuged, divided into aliquots, and stored at -80°C until assayed.

Determination of albumin levels

Albumin levels were estimated by means of a routine enzymatic colorimetric assay using a Roche/Hitachi modular analyzer (17).

Determination of levels of cholestenic acid and 27-OH

27-OH levels were determined using sensitive isotope dilution mass spectrometry as previously described (15, 18), with the exception that 50 ng of internal standard ($[^2\text{H}_6]$ 27-OH) was used. The use of a smaller amount of internal standard was necessitated by the limited amount of sample material available for analysis.

Cholestenic acid was determined essentially as previously described. Briefly, 200 ng of the internal standard *nor*-cholestenic acid was added to an aliquot of serum, and the volume was adjusted to 1 ml using physiological saline. After the addition of 6 ml of chloroform-methanol (2:1, v/v), the sample was vigorously mixed and centrifuged at 1,600 *g* for 5 min. The organic phase was transferred to a new tube, dried under a gentle stream of nitrogen, and redissolved in 0.5 ml of chloroform. This extract was then applied to an BondElut[®] NH₂ column (Varian, Harbor City, CA), which had been previously conditioned with 4 ml of *n*-hexane. Neutral and acidic lipids were eluted sequentially with 4 ml of chloroform-isopropanol (2:1, v/v) and acetic acid-diethyl ether (1:49, v/v), respectively. Samples were dried under nitrogen, methylated using diazomethane, and finally trimethylsilylated with a mixture of pyridine-hexamethyldisilazane-trichloromethylsilane (3:2:1, v/v/v) as previously described (18). Samples were analyzed by GC-MS according to previously published conditions.

Quantification of mRNA levels by real-time PCR

Levels of mRNA of CYP27A1 and ACAT-1 were determined with real-time quantitative PCR. Alveolar macrophage cDNA from

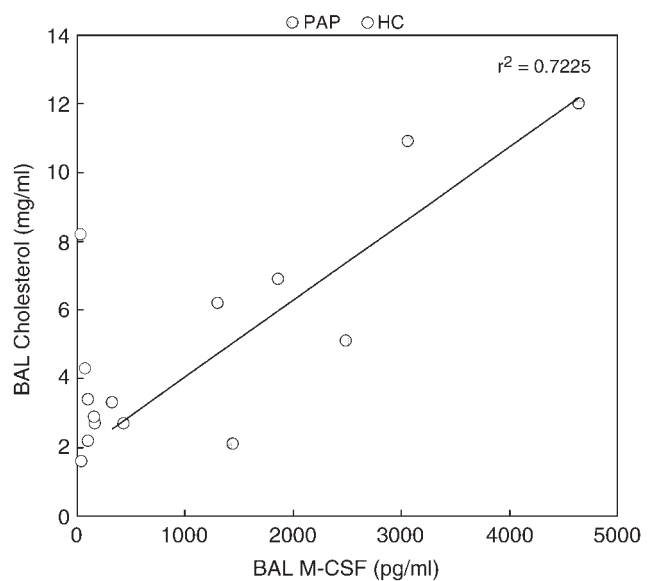


Fig. 1. Correlation between bronchoalveolar lavage (BAL) macrophage colony-stimulating factor (M-CSF) and cholesterol in pulmonary alveolar proteinosis (PAP) patients ($n = 7$; $r^2 = 0.72$). No significant correlation was observed for healthy controls (HC; $n = 8$; $r^2 = 0.1$).

healthy controls and PAP patients was synthesized according to standard procedures. Analysis of the expression levels was performed using single-plex real-time PCR analysis and FAM-labeled probe mixtures on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Probe mixtures, from Applied Biosystem's Assay-on-demand™ service, were as follows: CYP27A1 (Assay ID: Hs00168003_ml) and ACAT-1 (Assay ID: Hs00162077_ml). Cyclophilin A (Assay ID: Hs99999904_ml) was used as an internal standard. All samples were analyzed in triplicate, and data were analyzed by the comparative threshold cycle ($\Delta\Delta C_T$) method as described by the manufacturer (19). Results from PAP patients are expressed as means \pm range relative to healthy controls, which were set at a reference value of 1.

Ethical aspects

All experiments were reviewed and approved by The Cleveland Clinic Foundation Institutional Review Board and by the Hudding University Hospital Ethics Committee.

RESULTS

CYP27A1 and ACAT-1 mRNA expression is unchanged in PAP alveolar macrophages

To determine if increased cholestenic acid in the circulation was attributable to altered expression of CYP27A1, mRNA expression levels were quantified by real-time PCR. The mRNA expression of CYP27A1 in alveolar macrophages of PAP patients was not significantly different compared with that of healthy controls (mean and range relative to controls: 0.61, 0.21–1.8; $n = 4$). To evaluate the possibility of altered cholesterol esterification in PAP alveolar macrophages, expression levels of ACAT-1 were determined. ACAT-1 expression was not significantly different from that of healthy controls (mean and range relative to controls: 0.72, 0.31–1.7; $n = 4$).

Relation between BAL M-CSF level, cholesterol, and sterol metabolites

M-CSF is a pleiotropic cytokine that shares many activities with GM-CSF, although both cytokines lead to the development of distinct subsets of mature cells (20). As it

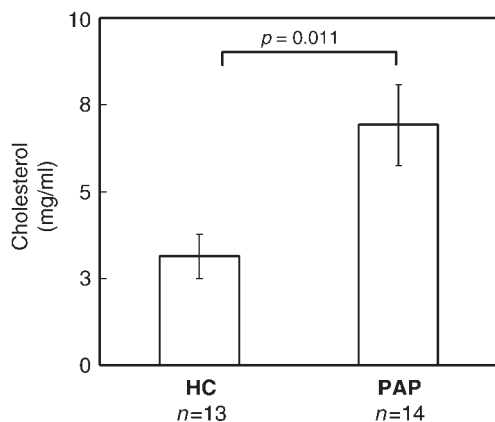


Fig. 2. Cholesterol levels in BAL fluid of healthy controls (HC; $n = 13$) and PAP patients ($n = 14$). Mean cholesterol levels were 3.3 ± 0.64 mg/ml and 6.92 ± 1.14 mg/ml, respectively. Results are shown as mean \pm SEM.

has previously been reported that PAP patients have vastly increased levels of M-CSF (12), it was considered prudent to investigate possible relationships between M-CSF and pulmonary sterols. No correlation was observed between the BAL levels of M-CSF and the corresponding levels of cholestenic acid or 27-OH. This is in agreement with our previous reports that demonstrated that M-CSF is not a critical factor for the induction of cholesterol-removal capacity during the differentiation of human monocytes to macrophages (21). There was, however, a significant correlation between the levels of M-CSF and cholesterol in the BAL of PAP patients ($r^2 = 0.72$) but not in healthy controls ($r^2 = 0.11$) (Fig. 1).

Cholesterol is increased in BAL fluid of PAP patients

Cholesterol is an essential component of the pulmonary surfactant, and cholesterol crystals are occasionally observed in cytological examinations of BAL fluid from PAP patients (9). In accordance with earlier findings (10, 22), total cholesterol levels were found to be significantly increased ($P = 0.011$) in BAL fluid from PAP patients (6.9 ± 1.14 mg/ml; $n = 14$) compared with healthy controls (3.3 ± 0.64 mg/ml; $n = 13$) (Fig. 2). There was no significant dif-

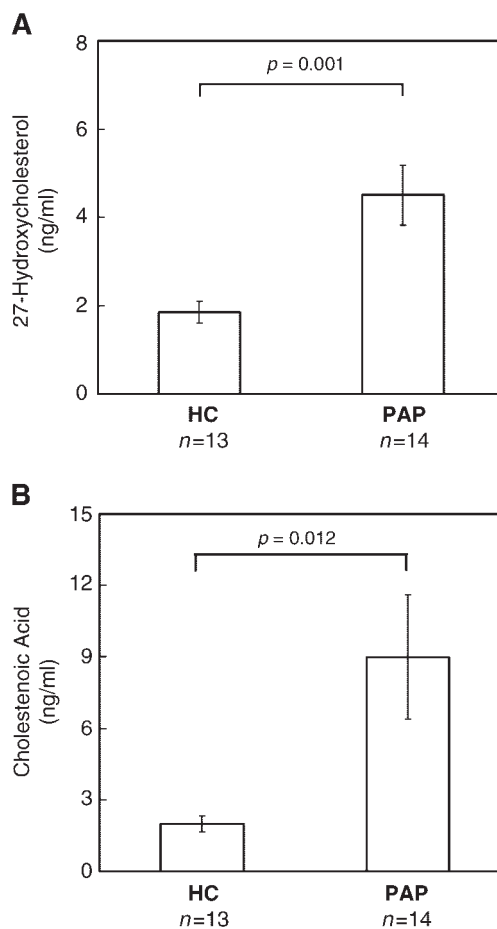


Fig. 3. Levels of 27-oxygenated cholesterol metabolites in BAL fluid of PAP patients and healthy controls (HC). A: Concentration of 27-hydroxycholesterol (27-OH) in BAL fluid. B: Concentration of cholestenic acid in BAL fluid. Results are shown as mean \pm SEM.

ference in serum cholesterol levels between the controls and PAP patients (2.7 ± 0.1 mM and 2.9 ± 0.1 mM, respectively).

Cholestenic acid and 27-OH are increased in the BAL fluid and serum of patients with PAP

The conversion of cholesterol into more polar metabolites (such as 27-OH and cholestenic acid) is believed to be a mechanism by which extrahepatic cells eliminate cholesterol (1). To estimate the contribution of this process to pulmonary cholesterol accumulation in PAP, the concentrations of these sterols were measured in matching BAL fluid and serum of patients with PAP.

In the BAL fluid of PAP patients, the absolute levels of 27-OH and cholestenic acid were significantly increased ($P = 0.001$ and $P = 0.012$, respectively) compared with those of healthy controls (Fig. 3). Absolute levels of cholestenic acid and 27-OH were significantly increased ($P = 0.027$ and $P = 0.014$, respectively) in the serum of PAP patients compared with those of controls (Fig. 4A, C). Predictably, the levels of total 27-oxygenated sterols in the serum were significantly increased in the PAP group ($P = 0.015$).

As they are both transported in lipoproteins (15), the serum levels of 27-OH and cholesterol are highly correlated in a normal population. Accordingly, this ratio is used when comparing different groups. As Fig. 4B shows, there was a highly significant increase in the ratio of 27-OH to cholesterol ($P = 0.017$).

In contrast, cholestenic acid is mainly transported in the lipoprotein-free fraction of plasma (15), and albumin levels are believed to reflect the bulk transport capacity of the circulation for cholestenic acid. Albumin levels were measured in conjunction with the cholestenic acid measurements. There was no significant difference in the albumin levels of the healthy controls and the PAP patients (41.8 ± 1 g/l and 39.3 ± 1.4 g/l, respectively). However, the albumin-related levels of cholestenic acid were significantly increased ($P = 0.003$) in serum of PAP patients (Fig. 4D). Under ostensibly normal conditions, there is a close correlation between albumin and cholestenic acid levels in the circulation (A. Babiker, personal communication). In agreement with these findings, a similar correlation was observed in the healthy controls ($r^2 = 0.69$, $P = 0.012$) but not in the PAP patients, in whom there was an uncoupling of the relationship between serum albumin and cholestenic acid (Fig. 5).

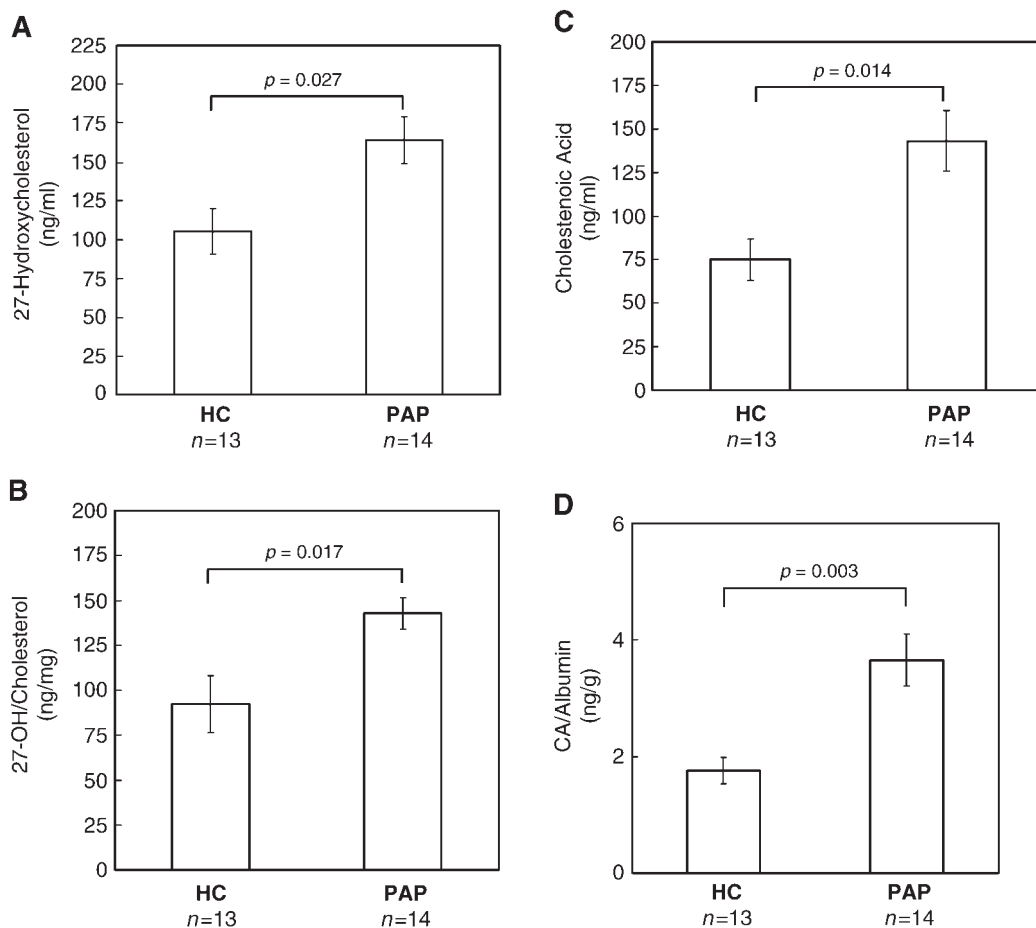


Fig. 4. Levels of 27-oxygenated cholesterol metabolites in serum of PAP patients and healthy controls (HC). A: Absolute level of 27-OH in serum. B: Cholesterol-related concentration of 27-OH in serum. C: Absolute concentration of cholestenic acid in serum. D: Albumin-related cholestenic acid concentration in serum. Results are shown as mean \pm SEM.

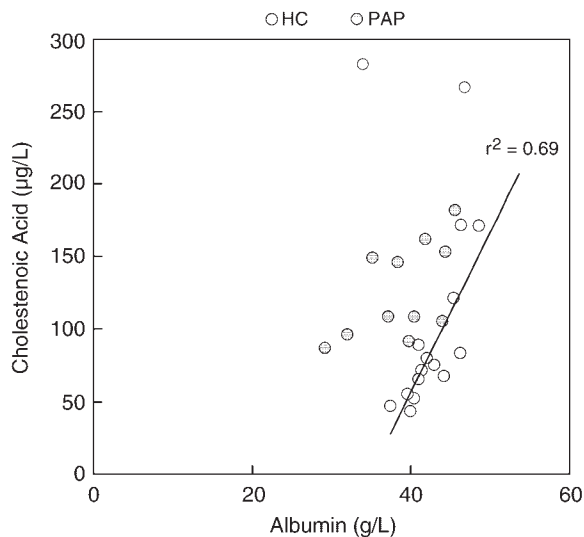


Fig. 5. Correlation between serum albumin and cholestenic acid in healthy controls ($n = 13$) and PAP patients ($n = 14$). Only the correlation line for the healthy controls is shown (r^2 for PAP group = 0.06).

DISCUSSION

The previous demonstrations that CYP27A1 expression is to some extent regulated by GM-CSF, and the predominantly pulmonary origin of circulating cholestenic acid, led us to hypothesize that PAP patients with high-titer neutralizing antibodies to GM-CSF may have decreased levels of cholestenic acid. In contrast to these expectations, we observed a significant increase in the levels of 27-oxygenated metabolites in the serum and BAL fluid of PAP patients. According to the present data, this difference does not appear to be the result of altered expression of CYP27A1 in the alveolar macrophages of PAP patients but rather is attributable to an increase in substrate supply.

The absence of a difference in ACAT-1 expression is consistent with previous data that demonstrated that ACAT-1 activity is mainly regulated by substrate supply, at least in some cell types (23). The expression levels observed in the present work thus likely reflect the loading of the macrophages with cholesterol. Because of limitations in the sample material, it was not possible to evaluate the expression of CYP27A1 and ACAT-1 at the protein level.

However, as CYP27A1 can multiply hydroxylate cholesterol at the C-27 position, the total production of 27-OH and cholestenic acid, as well as the ratio between them, is dependent on the CYP27A1 expression level. It has previously been shown that as the level of CYP27A1 increases, so too does the amount of cholestenic acid produced relative to 27-OH (3). Thus, increased enzyme expression would be expected to result in a correlation between the total 27-oxygenated products and the cholestenic acid/27-OH ratio. There was no significant correlation between these factors in the serum of either the PAP patients or healthy controls or in the BAL of the healthy controls (results not shown). In the BAL of PAP patients, a significant correlation was observed, in contrast to the mRNA data. It

should be emphasized that the concentration of sterols present in BAL is determined by the contribution of the entire alveolar cell population, not just the alveolar macrophages. Based on the present data, it cannot be excluded that there may be some alteration in CYP27A1 protein levels, particularly in light of the large range in the mRNA levels.

One possible explanation for the variance in mRNA levels is that the BAL of PAP patients contains altered levels of several cytokines, including monocyte chemoattractant protein (MCP)-1, interleukin-8, interleukin-10, and M-CSF, the levels of which may vary with the severity of the disease. However, with the exception of M-CSF, the influence of these cytokines on the regulation of CYP27A1 mRNA expression has not been evaluated. According to experiments in this laboratory, supraphysiological levels (50 ng/ml) of M-CSF do not appear to affect the mRNA expression of CYP27A1, or the production of 27-oxygenated products by human monocyte-derived macrophages, compared with untreated cells (21). This is in contrast to a previous work (14) that demonstrated an induction of CYP27A1 after M-CSF treatment. An important difference in these experiments is that in those demonstrating induction, cells were differentiated in a serum-containing medium, suggesting that other competence factors may be required for the induction of CYP27A1 expression.

To exclude the possibility that abnormal hepatic metabolism of circulating 27-OH was involved in the increased levels, a pool of serum from seven of the PAP patients was analyzed for the presence of bile acids by gas chromatography-mass spectrometry. Normal levels of chenodeoxycholic acid, deoxycholic acid, and cholic acid were present, in addition to trace amounts of ursodeoxycholic acid. No unusual bile acids or bile acid precursors (e.g., di- and trihydroxycoprostanic acids) were observed in this material.


Based on the available data, the most likely explanation for the increase in the concentration of the 27-oxygenated metabolites observed in the present work is an increase in substrate availability, which is rate-limiting for the formation of 27-OH.

The entry of cholesterol into the mitochondrial lumen is generally accepted as the rate-limiting step in the production of 27-OH by CYP27A1 (24). In steroidogenic tissues, the steroidogenic acute regulatory (StAR) protein is a critical component of the mitochondrial cholesterol import mechanism (25). However, as StAR is not expressed outside of these tissues, it is unclear what mechanisms are responsible for the delivery of cholesterol to CYP27A1 in nonsteroidogenic tissues. The recent elucidation of a StAR protein family in which all members possess a START domain raises the possibility that a member of this protein family may be responsible for shuttling cholesterol to the mitochondrial lumen in nonsteroidogenic cells (26). Recently, a transgenic mouse model with a targeted deletion of the START domain of StARD3 was developed (27). However, no significant cholesterol accumulation was observed in homozygous animals, indicating that the presence of the StARD3 START domain is not essential for cholesterol delivery to mitochondria. To date, the role of other putative "cholesterol-delivery" factors has not been evaluated in alveolar macrophages.

The reason why the lung has a high capacity to produce cholestenic acid is unclear. As the lung derives most of its cholesterol from circulating lipoproteins, feedback regulation of lipoprotein receptors after the accumulation of cholesterol would be expected to downregulate the lipoprotein receptors and reduce the amount of cholesterol entering the lung. Moreover, it has been demonstrated that HDL can interact with both type II pneumocytes (28) and alveolar macrophages, indicating that both of these cell types have the capacity to participate in lipoprotein-mediated reverse cholesterol transport.

As alveolar surfactant is the first target for air-borne oxidants, the possibility may be considered that, as a result of its broad substrate specificity (29), CYP27A1 may be responsible for detoxifying oxygenated sterols that are unable to participate in the surfactant-recycling pathways. In this connection, it is notable that a recent study described the formation of several biologically active sterols after exposure to ozone (30), indicating that in addition to contributing to the elimination of nonrecyclable sterols, CYP27A1 may also be involved in the deactivation of cytotoxic sterols. A final hint of the possible role of CYP27A1 in the lung is the recent demonstration of an oxysterol-regulated ABCA1-mediated basolateral surfactant efflux pathway (31). As 27-OH has been shown, at least under some in vitro conditions (32), to be an activator of the liver X receptor family of nuclear receptors, CYP27A1 may be involved in the regulation of this proposed efflux pathway.

As surfactant represents a significant pool of cholesterol in the lung, there may be a coupling between the catabolism of surfactant lipids by alveolar macrophages and the production of cholestenic acid. In preliminary experiments, sterol 27-hydroxylase activity and CYP27A1 immunoreactivity were detected in purified lamellar bodies from human lung (S. Meaney, unpublished observations). This is in agreement with previous electron microscopy results that demonstrated immunopositive material in the lamellar bodies of tissue from healthy lung (33).

According to published data, an average pair of human lungs contains 2–3 g of cholesterol, of which 40–60 mg is believed to be associated with the surfactant. As the conversion of cholesterol into cholestenic acid results in the elimination of ~15 mg of cholesterol from the lung each day, we hypothesize that the elimination of cholesterol as cholestenic acid may be important in the processing of surfactant cholesterol by alveolar macrophages. Studies are in progress to test the relationship between surfactant cholesterol and serum cholestenic acid. 

This work was supported by Stiftelsen Lars Hiertas Minne, the Swedish Medical Research Council, the Swedish Heart-Lung Foundation, and National Institutes of Health Grant HL-67676 (M.J.T.).

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