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MATURATION OF FIVE-LIPOXYGENASE METABOLISM IN CATTLE ALVEOLAR MACROPHAGES AND PERIPHERAL BLOOD MONOCYTES

By

Min-Chi Lu

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ABSTRACT

MATURATION OF FIVE-LIPOXYGENASE METABOLISM IN CATTLE ALVEOLAR MACROPHAGES AND PERIPHERAL BLOOD MONOCYTES

By

Min-Chi Lu

I examined changes in in 5-lipoxygenase (5-LO) metabolism in alveolar macrophages (AM) and peripheral blood monocytes (PBM) as cattle mature. AM and PBM from neonates as well as from adults were isolated and cultured. After a 17-hour culture period, AM and PBM have a purity, assessed by Giemsa and non-specific esterase stains, > 95% and >90%, respectively; and a viability, determined by trypan blue exclusion, > 90% in all animals. These cells were either [^{3}H]arachidonic acid (AA)-pre-labeled and studied for AA metabolism by RP-HPLC with radiodetection or homogenized and analyzed for the steady-state expression of 5-LO and 5-lipoxygenase-activating-protein (FLAP) by immunoblot analysis. In AM, two patterns of AA metabolism, i.e., juvenile (low 5-LO and high cyclooxygenase (CO) capacity) and adult patterns (high 5-LO and low CO capacity), were discovered. An upregulation of the 5-LO and FLAP protein expression parallels the age-dependent increase in 5-LO metabolic capacity. In contrast, neonatal and adult PBM displayed a similar AA metabolic pattern, for which 5-LO capacity was <10% that of respective AM. Whereas the FLAP expression in AM of neonates and adults, only differed a little, the 5-LO expression increased >100-fold from their respective PBM. This dramatic increase in 5-LO protein expression as cattle PBM differentiate into AM is likely important for the enhancement of 5-LO metabolic capacity. Furthermore, a prominent increase of 5-LO protein expression in neonatal and adult AM was induced by a combined treatment of 1,25-dihydroxy vitamin D_3 and transforming growth factor-$ß_1$.

This dissertation is dedicated to my parents and beloved wife

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LIST OF ABBREVIATIONS

aAM	adult alveolar macrophages
aPBM	adult peripheral blood monocytes
AM	alveolar macrophages
AA	arachidonic acid
BAL	bronchoalveolar lavage
FB	fibroblasts
Ca^{2+}	calcium ion
CaLB	calcium/phospholipid binding domain
Cd	crude cell mixture
CO	cyclooxygenase
Cs	cytosol fraction
$1,25(OH)_2$-D_3	1,25-dihydroxy vitamin D_3
EIA	enzyme immunoassay
FBS	fetal bovine serum
FB	fibroblasts
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
GC-MS	gas chromatography-mass spectrophotometry
5-HpETE	5-hydroperoxyeicosatetraenoate
5-HETE	5-hydroxyeicosatetraenoate
12-HETE	12-hydroxyeicosatetraenoate
15-HETE	15-hydroxyeicosatetraenoate
12-HHT	12-hydroxyheptadecatrienoic acid
IP_3	inositol 1,4,5-triphosphate
IL	interleukin

LT	leukotriene
LPS	lipopolysaccharide
5-LO	5-lipoxygenase
FLAP	5-lipoxygenase activating protein
M	Membrane fraction
MPS	mononuclear phagocyte system
nAM	neonatal alveolar macrophages
nPBM	neonatal peripheral blood monocytes
Nu	nuclear fraction
PBM	peripheral blood monocytes
PM	peritoneal macrophages
PAF	platelet activating factor
PLA_2	phospholipase A_2
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGI_2	prostaglandin I_2; prostacyclin
PGH_2	prostaglandin endoperoxide
PGHS-1	prostaglandin endoperoxide synthase isoform -1
PGHS-2	prostaglandin endoperoxide synthase isoform -2
PKC	protein kinase C
PMN	polymorphonuclear leukocytes
PIM	pulmonary interstitial macrophages
RIA	radioimmunoassay
ROS	reactive oxygen species
TxB_2	thromboxane B_2
TGF-$ß_1$	transforming growth factor-$ß_1$
TNF	tumor necrosis factor
VDR	vitamin D_3 receptor

INTRODUCTION

The lung is one of the vital organs and, distinctively, it is consistently exposed to the external air environment, in which it is confronted with potentially harmful foreign agents, such as infectious microorganisms and toxic substances. Several lines of defense mechanism, including mucociliary, phagocytic, and specific immune systems, have developed to protect the lung from infection and injury. Alveolar macrophages (AM), located in the surface of alveoli and in contact with the hyperoxic atmosphere and airborne particles, are resident phagocytes and one of the first-line defenders that constantly strive to maintain a relatively clean lower respiratory tract. The knowledge of alveolar macrophage biology began to grow rapidly in the early 1970s after the wide application of brochoalveolar lavage technique allowed the high-purity preparation of AM (Myrvik et al., 1962).

The alveolar macrophage is essential for lung defense and inflammation. The critical role of AM in lung defense against infection has been shown by the high frequency of pneumonia in severely neutropenic patients and in cases of lung macrophage dysfunction (Sibille and Reynolds, 1990). The antimicrobial function of AM requires identification of microorganisms. This recognition needs the surface receptors such as receptors for the Fc component of IgG and for the C3b component of complement (Johnson et al., 1986), as the first step for the phagocytosis and killing of invading microorganisms. The

destruction of microorganisms is achieved by reactive oxygen species such as superoxide and hydrogen peroxide, and by digestive enzymes in AM (Sibille and Reynolds, 1990).

It is now well recognized that AM also play important roles in the lung inflammatory and immune responses. During the initial phase of lung inflammation (Figure 1-1), recruitment of additional leukocytes is necessary when there are not enough local leukocytes for an adequate response. The AM achieve this by the release of chemotactic factors, such as LTB_4, platelet activating factor, complement C5a, and chemokines for leukocytes and additional macrophages. Leukotriene B_4 (LTB_4) is especially of interest because it is released significantly by native alveolar macrophages during inflammation and the immune response (Ford-Hutchinson et al., 1980; Martin et al., 1984).

Eicosanoids are important mediators of alveolar macrophage function. Eicosanoids, the family of molecules derived from arachidonic acid (AA), are composed primarily of prostaglandins, thromboxane, and leukotrienes. Biologically, eicosanoids are cellular mediators and exert their effect in an autocrine or a paracrine fashion by binding to the membrane G protein-linked receptors (Smith, 1989). Adult AM of human beings and rats release large quantities of 5-hydroxyeicosatetraenoic acid (5-HETE) and LTB_4, the 5-lipoxygenase (5-LO) products, and free AA, but much smaller amounts of prostanoids, including thromboxane A_2 (TxA_2), $PGF_{2\alpha}$, PGI_2, 12-hydroxyheptadeca-trienoic acid (12-HHT), and PGE_2 (MacDermont et al., 1984; Balter et al., 1989; Peters-Golden et al., 1990).

In the lung, eicosanoids evoke several functions. For example, TxA_2 is a constrictor of pulmonary airways and blood vessels (Samuelsson et al., 1978) and $PGF_{2\alpha}$ is a bronchoconstrictor and a vasoconstrictor (Widdicombe et al., 1989). On the contrary,

PGE_2 is a potent vasodilator, and has extensive anti-inflammatory actions, including suppression of PMN chemotaxis (Rivken et al., 1975). LTB_4 not only serves as one of the most potent chemoattractants for neutrophils, but increases pulmonary vascular permeability (Dahlèn et al., 1981), activates neutrophils, and modulates the functions of and B- and T-lymphocytes (Rola-Pleszczynski et al., 1983a and 1983b). Furthermore, the release of abundant 5-LO intermediate products by AM and neutrophils can be utilized transcellularly by the neighboring non-5-LO-containing cells for the production of LTC_4, D_4, and E_4, which are strong bronchoconstrictive substances (Lewis et al., 1990).

I have examined the 5-LO metabolism in neonatal and adult alveolar macrophage and its precursor cell, the peripheral blood monocyte. Neonates are susceptible to lung infections with severe manifestations of illness in most animal species, including cattle (Jensen and Mackey, 1979; Radostits and Acres; 1980; Andrews, 1992). As the first-line defender of the lung, the immaturity of AM from the neonates is one of the possible explanations. It has been shown that, in response to airway-administered lipopolysaccharide (LPS) or *Escherichia coli* as well as Pneumococci, the lungs of neonatal rats recruit significantly less PMN than those of the adults (Coonrod et al., 1987; Martin et al., 1995). Since LTB_4, a major product of AM, participates in the recruitment of additional PMN into the alveolar space (Martin et al., 1989), the alterations of arachidonate metabolism in neonatal AM may compromise the antimicrobial defense of the lung. Though several studies have been conducted in mononuclear phagocytes of adult animals, none have been performed in those of the neonates. Therefore, in my dissertation, I first determined the different patterns of AA metabolism in neonatal and adult AM of cattle and the mechanism for the age-related changes of 5-LO metabolism.

In adult animals, the AA metabolism has been shown to be enhanced via the 5-LO pathway in AM as compared to PBM. Marked increase in the expression of AM 5-LO and 5-lipoxygenase-activating protein (FLAP) parallels the change of 5-LO metabolic capacity. It is likely that the in vivo upregulation of both proteins requires lung-specific factor(s). Therefore, for the second part of my dissertation, I examined the 5-LO metabolic capacity and the 5-LO and FLAP protein expression in PBM and AM of the neonatal as well as adult cattle, from which I could attribute the upregulation of 5-LO metabolism to either age- or tissue (location, i.e., lung in my studies)-related regulation.

Since the immaturity of neonatal AM 5-LO metabolism might compromise the lung defense mechanism, it would be beneficial if the factor(s) that upregulate 5-LO metabolism can be discovered and, ultimately, used for clinical purposes. Studies performed in the adult rat PBM and AM found that 1,25-$(OH)_2$ vitamin D_3 ($1,25(OH)_2D_3$) is related to the upregulation of the 5-LO metabolism and protein expression. Furthermore, the effect of combined $1,25(OH)_2D_3$ and tumor transforming factor-ß (TGF-ß) treatment is even more dramatic in the 5-LO metabolism and 5-LO protein expression of leukemic cell lines HL-60 and Mono Mac 6 (Brungs et al., 1994; Brungs et al., 1995). Accordingly, for the last part of my dissertation, I determined the effect of TGF-ß and $1,25(OH)_2D_3$ on the upregulation of 5-LO protein expression in the neonatal and adult AM.

CHAPTER 1

LITERATURE REVIEW

ARACHIDONIC ACID METABOLISM AND ITS BIOLOGICAL REGULATION —*WITH AN EMPHASIS ON 5-LIPOXYGENASE METABOLIC PATHWAY*

Arachidonic acid (AA) and its metabolites, also known as eicosanoids, are 20-carbon polyunsaturated fatty acids, which include prostaglandins (PGs), thromboxane (Tx), leukotrienes (LTs), and hydroxyeicosatetraenoic acid (HETEs). Eicosanoids are widely distributed in the body and are important mediators both for the normal function and during the pathologic processes of cells. In the lungs, the major sites on which eicosanoids have effects are airway and vascular smooth muscle, airway epithelium, and pulmonary vascular endothelium; in addition, eicosanoids have been demonstrated to play crucial roles in the regulation of immune and inflammatory responses.

Arachidonic Acid Is Stored in and Released from Cell Membrane Phospholipids

In mammals, arachidonate is obtained either directly from diets or from the in vivo biosynthesis via the conversion of 18-carbon polyunsaturated fatty acid of the n-6 family (Chilton et al., 1996). This arachidonate is acylated and stored in the glycero-phospholipids of cell membranes, primarily at the sn-2 position. The incorporation of AA into several different glycerophospholipid molecular species, which are present in mammalian cells, including the inflammatory cells (Chilton and Murphy, 1986; Chilton

et al., 1987), is regulated by a number of CoA-dependent and -independent acyltransferase and transacylases (Chilton et al., 1996). The phospholipid pools and the enzymes for AA metabolism seem to be compartmentalized in subcellular localizations and, therefore, may be directly responsible for the production of particular lipid mediators (Chilton, 1996).

The scheme of arachidonic acid metabolism is shown in Figure 1-2, which indicates the enzymes participating in this process and the released metabolites. When cells are activated, arachidonate is liberated from membrane phospholipid by the action of phospolipases and the free arachidonate converted to various eicosanoids via lipoxygenase, cyclooxygenase, or epoxygenase pathway or reintegrated into glycerophospholipids.

PLA_2 is the primary enzyme that releases AA from membrane phospholipids. PLA_2 catalyzes the hydrolysis of the fatty acyl bond at the second (sn-2) position of glycerophospholipids. The observation that arachidonate is most abundantly found at the sn-2 position provides strong evidence that PLA_2 is likely the crucial enzyme for providing eicosanoid biosynthesis with AA substrate (Dennis, 1994). As a matter of fact, PLA_2 is not only the very first but also the rate-limiting enzyme for arachidonate metabolism (Glaser et al., 1993; Kudo et al., 1993). Other alternative enzymes that might participate in the release of AA include phospholipase C (PLC) or PLD followed by lipase(s) to mobilize AA.

The PLA_2 has several isoforms, including a secreted form (Groups I, II, and III) and a cytosolic form (Group IV and Ca^{2+}-independent type) (Dennis, 1994; Glaser et al., 1993). The group I, II, and III enzymes are mostly secreted and found in mammalian pancreas

and synovial fluid, and cobra and snake venoms. They are characterized by low molecular weight, high content of disulfide bond, and the requirement of Ca^{2+} for catalysis and a lipid-water interface, such as that of micelle or membrane, for complete activation (Kramer et al., 1989). Group II PLA_2 is associated with several mammalian cells and tissues or extracellularly when released in response to proinflammatory mediators such as interleukin-1 (IL-1) and IL-6 (Dennis, 1994).

Group IV enzyme, also known as $cPLA_2$, is present in a wide variety of cell types, including macrophages (Leslie et al., 1988), platelet (Kim et al., 1991), and kidney (Gronich, 1990), and U937 and J744 cell lines (Clark et al., 1990; Wijkander and Sundler, 1991). It has high molecular weight and is specific for the arachidonate moiety. The $cPLA_2$ is composed of a catalytic domain and a calcium/phospholipid binding (CaLB) domain and the CaLB domain is similar to that found in protein kinase C (PKC), GTP-activating protein, and phospholipase Cγ (PLCγ) (Dennis, 1994). When the cell is activated, this enzyme is translocated from its cytosolic location to the membrane fraction (Channon and Leslie, 1990) and the CaLB domain, in the presence of Ca^{2+}, is involved in the translocation and association of the enzyme to phospholipids in the cell membrane (Glaser et al., 1993).

Another group consisting of Ca^{2+}-independent PLA_2s was found in the myocardium of several species (Wolf and Gross, 1985) and in P388D1 macrophage-like cells (Dennis et al., 1985). These PLA_2s are activated by ATP rather than Ca^{2+}; however, their mechanism of action is not clear.

PLA_2s, through their participation in the releases of eicosanoids and other cellular mediators, presumably play a crucial role in a variety of cellular functions, including

inflammation and signal transduction. Since PLA_2s catalyze the same chemical reaction and several of them might be present in the same cell (Murakami et al., 1992), it is not feasible to assign the release of eicosanoids to certain PLA_2 based on our current understanding of PLA_2s.

Cellular Metabolism of Arachidonic Acid

The AA released from membrane phospholipids can be further metabolized to form various eicosanoids, including 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, LTB_4, LTC_4, LTD_4, 12-HHT, 15-HETE, 12-HETE, and 5-HETE (Figure 1-2). The important modulating enzymes for cyclooxygenase and 5-lipoxygenase pathways, rather than those for epoxygenase and 12- and 15-lipoxygenase pathways, are discussed in this section because these enzymes are expressed significantly in mononuclear phagocytes and their biochemical properties as well as physiological functions are better investigated.

Leukotrienes as AA Metabolites: 5-lipoxygenase and FLAP. 5-Lipoxygenase (5-LO) catalyzes the first two steps of leukotriene production, i.e., converting AA to 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and, then, 5HpETE to LTA_4 (Panossian et al., 1982; Maas et al., 1982). This 78-kDa protein is predicted to have no membrane-spanning domains (Dixon et al., 1988). ATP and calcium are found to stimulate 5-lipoxygenase activity, but the binding sites and the precise nature of the activation procedure are not yet determined. Distinctively, 5-LO is distributed with high concentration only in myeloid cells, including monocytes, macrophages, granular leukocytes, and B-lymphocytes (Shimizu, 1988). As a matter of fact, 5-LO products are the predominant eicosanoids released by AM.

The functional activation of 5-lipoxygenase is proposed to involve a membrane-binding process (Rouzer, C.A., and B. Samuelsson, 1987), in which a membrane protein, five-lipoxygenase-activating protein (FLAP), plays an essential role. Using an indole leukotriene inhibitor, MK-886 (Gillard, 1989), Miller and his colleagues isolated this 18-kDa protein, FLAP (Miller et al., 1990), and FLAP has since been found in a variety of leukocytes and cell lines. 5-LO has been shown to be located in the cytosol and, as determined by immunoblotting and immunohistochemistry, it moves to the nucleus following cell activation. (Peters-Golden and McNish, 1993; Brock et al., 1994). Using immunoelectron microscopy, Woods et al. (1993) localized FLAP to the nuclear envelope and endoplasmic reticulum in human peripheral blood leukocytes. Following activation, 5-LO could be also be found in the nuclear membrane (Brock, et al., 1994). Functional studies further revealed that the expression of both 5-LO and FLAP is required for leukotriene synthesis in intact osteosarcoma cells (Dixon, 1990).

Leukotrienes as AA Metabolites: LTA_4 Hydrolase. The enzyme LTA_4 hydrolase is a cytosolic protein that catalyzes the hydrolysis of LTA_4 to LTB_4. This 68-70-kDa monomeric protein is a suicidal enzyme that is inactivated by the substrate LTA_4 (Ohishi et al., 1987). Using a rabbit polyclonal antibody, the tissue distribution of LTA_4 was identified (Ohishi et al., 1990). In the lung, bronchial epithelium, smooth muscle, and arterial endothelial cells all contain LTA_4 hydrolase. LTA_4 hydrolase is present in mononuclear and PMN leukocytes as well. Based on the assumption that LTA_4, which is produced essentially by neutrophils and macrophages, can be transcellularly delivered for further processing, this wide distribution of LTA_4 hydrolase suggests that cells that do not contain 5-LO can amplify the signals of inflammation by converting LTA_4 received from

neighboring cells into chemoattractant LTB_4.

Leukotrienes as AA Metabolites: LTC_4 synthase. Under certain circumstances and primarily in mast cells, eosinophils, basophils, and macrophages, LTA_4 can alternatively be metabolized by LTC_4 synthase via a glutathione S-transferase reaction to form LTC_4 (Nicholson et al., 1992), which may subsequently transform into LTD_4 and, then, LTE_4 by γ-glutamyl transferase and dipeptidase (Anderson et al., 1982), respectively. For example, the IgE-dependent activation of mast cells and basophils releases LTC_4 (Levi-Schaffer et al., 1987; MacGlashan et al., 1986). As well, after seven days of coculture with fibroblasts, eosinophils develop the capability to respond to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) and produce a large amount of LTC_4 (Owen et al., 1990). Moreover, since LTC_4 is widely distributed among different cell types (Shimizu, 1988), such as capillary endothelial cells and platelets, the transcellularly provided LTA_4 during inflammation and immune responses is presumably utilized for the additional release of these cysteinyl leukotrienes.

Prostanoid Biosynthesis: Prostaglandin Endoperoxide Synthase Catalysis as the Rate Limiting Step. As a homodimer of two 72-kDa polypeptides, PGHS, also known as cyclooxygenase (COX), has bisdioxygenase and hydroperoxygenase activities to convert arachidonic acid to PGG_2, and thereafter to PGH_2. It is the first and rate-limiting enzyme for the biosynthesis of prostaglandins and thromboxane (Miyamoto et al., 1976; Ohki et al., 1979). PGH_2, an unstable intermediate metabolite, is rapidly metabolized to various novel prostanoids, which depend on the enzymes present in different type of cells and tissues.

Two related but unique isoforms of prostaglandin endoperoxide (PGH) synthase, isozymes-1 (PGHS-1 or COX-1) and -2 (PGHS-2 or COX-2), which carry 60% homologous protein sequence, have been identified. As integral membrane proteins, isoforms -1 and -2 are predominantly located at the cell endoplasmic reticulum and nuclear envelope, respectively (Rollins and Smith, 1980; DeWitt et al., 1981; Otto et al., 1994). PGHS-1 is present in most tissues and its constitutive high expression in various cell types, including vascular endothelial cells, platetelets, and renal collecting tubules, suggests its important role in the homeostasis of body organs (DeWitt et al., 1993). In contrast, the inducible PGHS-2 is quiescent in tissues, and the observation that its expression was increased during inflammation and accompanied that of other immediate early genes implies its participation in inflammation and cell cycle regulation (Smith, 1994).

Mononuclear phagocytes, as important immune and inflammatory cells, have been studied extensively for their prostanoid synthesis. In AM, peritoneal macrophages, monocytes and mouse RAW 264.7 cell line, both PGHS isoforms are present, with PGHS-1 constitutively expressed and PGHS-2 markedly induced by LPS or TPA, or various stimuli to cells previously primed with INF-γ. As compared to human monocytes, AM have more capacity for PGHS-2 induction with LPS stimulation (Goppelt-Struebe, 1995). Taking the above observations into consideration, it is feasible to assume that AM PGHS metabolism participates in lung inflammatory process and, possibly, the differentiation of monocytes into AM.

The Biochemical Coupling between Enzymes. Although there is no direct evidence showing that the supply of AA substrate for a particular lipoxygenase or cyclooxygenase

is coupled to a specific phospholipase, several studies have provided clues for separate pools of arachidonate. Based on the fact that a small amount of AA released by activated cells is further metabolized to eicosanoids, major subclasses of phospholipid with differential isotope-labeled AA/endogenous AA ratios have been employed to study the possible preferential pool for AA release as well as for specific eicosanoid production (Chilton et al., 1996). In mast cells, the free AA secreted and the AA for LTB_4 and LTC_4 generation appears to be from different engaged pools, the former is mostly from 1-alk-1-enyl-2-arachidonoyl-glycero-3-phosphoethanolamine and the latter from phosphatidyl-ethanolamine (Fonteh et al., 1994). Similar results have also been found in neutrophils and platelets.

Further information about enzyme coupling was revealed by the use of leukotriene synthesis blocking agents. 5-LO protein in peritoneal macrophages and PMN at rest is found exclusively in the cytosol, and translocates to nuclear membrane upon A23187 stimulation, which is inhibited by FLAP inhibitor MK886, whereas in rat AM, its distribution is in both the cytosol and the nucleus, and MK886, even though it completely inhibits LT production, does not reverse the binding of 5-LO to the nuclear membrane (Coffey et al., 1992; Peters-Golden andMcNish, 1993). These findings indicate that the free AA released by the cell might not be utilized directly by 5-LO, and FLAP is not just a "docking" protein, but, most likely, presents AA substrate to 5-LO for LT generation, which strongly suggests a coupling mechanism between phospholipase and 5-LO.

Transcellular Metabolism of LTA_4 Can Amplify the Local Inflammatory Response. As mentioned previously, although LTA_4 hydrolase and LTC_4 synthase are widely distributed among different cell types, the enzyme 5-LO that provides the prerequisite

substrate LTA_4 is only present in limited cell types, mostly of hematopoietic origins. Presumably, the LTA_4 released by one cell can be transferred to neighboring cells and further metabolized. Many studies support this argument, for example, endothelial cells or platelets, which contain LTC_4 synthase but are incapable of LTA_4 production, have been shown to release LTC_4 when cocultured with neutrophils (Feinmark and Cannon, 1986; Maclouf and Murphy, 1988). During the lung inflammatory process, the local neutrophils and macrophages are markedly increased and the 5-LO metabolic intermediate produced by these cells is available for the adjacent epithelium, smooth muscle, and endothelial cells to liberate more LTs and augment the inflammatory response.

Biological Actions of Eicosanoids in the Lungs

The interaction between hormone (or ligand) and receptor is a prerequisite to trigger the release of eicosanoids for physiological function or in a disease state. For example, during the inflammatory process, bradykinin is present in plasma in large amounts; its effects appear to be mediated by its binding to the B_2 receptor, which causes the activation of phospholipase C. The resulting production of inositol 3-phosphate (IP_3) and diacyl glycerol (DAG) as well as the mobilization of calcium activates the cellular phospholipase especially the PLA_2, which subsequently releases free AA from membrane phospholipid (Henderson, 1987). The transducing signals that result in the release of arachidonic acid are not yet fully recognized, but G proteins, phospholipase C, and calcium seem to play central roles.

AA serves as a substrate for various oxidation pathways in the cell; it can be metabolized to form prostaglandins, thromboxane or leukotrienes depending on the activity of various enzymes in the cyclooxygenase and lipoxygenase pathways. The metabolites, being autacoids, primarily affect neighboring cells by binding to the plasma membrane receptors, which are presumably G-protein-linked (Smith, 1989). The transport of eicosanoids from intracellular site of production extracellularly to the receptors on the external surface of cytoplasmic membrane is necessary for functioning. LTB_4 and LTC_4 export in human neutrophils or eosinophils, for example, is a carrier-dependent saturable process that requires ATP hydrolysis (Lam et al., 1990; Keppler et al., 1992).

Leukotriene Functions. In vitro and in vivo experiments have shown that LTB_4 is a potent chemotactic substance for leukocytes with an effect comparable to that of C5a or other chemotatic factors (Martin et al., 1984; Martin et al., 1989). Upon recruitment of neutrophils, LTB_4 also induces their activation and degranulation and augments the release of lysosomal enzymes and oxygen radicals (Rase and Smith, 1981; Serhan et al., 1982). In addition, the effects of LTB_4 to increase microvascular permeability (Martin et al., 1989) and endothelial-neutrophil interaction/adhesion potentiate the local inflammatory response (Dahlén et al., 1981). It has been shown that LTB_4 indirectly stimulates T-suppressor cell function, which is probably mediated by the cytokines released from monocyte upon LTB_4 activation (Ford-Hutchinson, 1990). LTB_4 can also amplify the lymphokine activation of B-lymphocyte, which includes CD-23 marker expression, cell replication, and immunoglobulin synthesis (Dugas et al., 1990; Yamaoka et al., 1989). Two kinds of LTB_4 receptors with different affinities have been characterized in PMN (Goldman and Goetzl,

1982), their effect , similar to that of other chemotactic factors, is via the G-protein and IP_3-DAG-Ca^{2+} signaling pathway (Ford-Hutchinson, 1990).

Primarily in mast cells, eosinophils, and macrophages, LTA_4 can alternatively be metabolized to cysteinyl leukotrienes, LTC_4, LTD_4, and LTE_4, which are the major constituents of the mixture previously called "slow-reacting substance of anaphylaxis (SRS-A)." These LTC_4, LTD_4, and LTE_4 , by binding to LTD_4 receptors, act to cause bronchial smooth muscle contraction, increase pulmonary microvascular permeability, and induce airway epithelial secretion; these effects are important cellular components of immediate hypersensitivity (Dahlén et al., 1980). Although a specific receptor for LTC_4 has been found in several cell types such as guinea pig ileal smooth muscle cells, no evidence supports the presence of specific LTC_4 or LTE_4 receptor in human lungs; instead, LTC_4 and LTE_4 bind to a common LTD_4 receptor (Lewis et al.,1990; Ford-Hutchinson, 1994). The LTD_4 receptor is different from the LTB_4 receptor in binding specificity; however, its transduction mechanism is similar.

Prostaglandin and Thromboxane Function. Local production of PGE_2 and prostacyclin (PGI_2) by airway epithelium and vascular endothelium can modulate vascular and airway smooth muscle tone via EP_2 and IP receptors. These vasodilator prostanoids counteract the effects of circulating vasoconstrictor factors and maintain homeostasis of the lungs (Aiken and Vane, 1973). PGI_2 has strong pro-inflammatory effects and, in most systems, is more potent than PGE_2. $PGF_{2\alpha}$ functions as a bronchoconstrictor and a vasoconstrictor by binding to the FP receptor, and both $PGF_{2\alpha}$ and PGE_2 increase airway epithelial chloride secretion (Widdicombe et al., 1989). The unstable TxA_2, usually detected as its inactive derivative TxB_2, is a platelet and

neutrophil aggregator and vasoconstrictor. All the prostanoid effects seems to be mediated by various G-protein-activated signaling pathways (Smith, 1989).

Experimental Consideration: The Validity of [^{3}H]AA-Prelabeled Cells as a Tool for Measurement of Cellular AA Metabolism

Cell arachidonate metabolites can be measured using several different methods (Wescott et al., 1986) such as gas chromatography/mass spectrometry (GC/MS), radioimmunoassay (RIA), and enzyme immunoassay (EIA). Use of radiolabeled AA in cellular experiments has been a invaluable tool for studying cellular arachidonate metabolism. During the experiment, the actual phospholipid pools are labeled and utilized as sources for endogenous AA, and the eicosanoid released is analyzed by RP-HPLC separation with radiodetection. Previous studies have shown that these experiments which are based on the assumption that exogenously administered AA has been equilibrated among all phospholipid molecular species might not be valid. As a result, with a relatively short prelabeling period, the quantity of released radiolabeled eicosanoids may be different from that of endogenous eicosanoids measured by GC-MS or RIA (Chilton et al., 1996). This disagreement primarily results from the nonuniform distribution of radiolabeled AA in cellular phospholipid pools, which, presumably, is determined by the duration of cellular incubation with radiolabeled AA.

In the experiments using [^{3}H]AA-prelabeled cells, with an excess of radiolabel present in the culture medium, the uptake of [^{3}H]AA by the cells is determined primarily by the basal rate of phospholipid turnover, and the labeling efficiency can be enhanced by either reducing the amount of radiolabel in which the cells are incubated or plating more

cells in the culture dish. However, the spectrum of released [^{3}H]AA metabolites detected is related not to the labeling efficiency, but to the distribution of isotope in the membrane phospholipid pools. In my experiments, with 16-hr [^{3}H]AA labeling of AM membrane lipids, the resulting AA metabolic profile was similar to that measured by RIA (see Chapter 2). Studies of radiolabeled mast cells have also shown that cells prelabeled with < 0.1 μM [^{3}H]AA for 24-36 hr reach a state of radioactivity equilibrium among all phospholipid molecular species (Chilton, 1991). Therefore, the radiolabeling method, with equilibrium condition reached, results in an AA metabolic profiles reflecting the endogenous AA metabolism; and this method provides an easy and accurate approach to the examination of cellular phospholipid metabolism.

MATURATION OF ALVEOLAR MACROPHAGES FROM PERIPHERAL BLOOD MONOCYTES

The lung AM are part of the mononuclear-phagocyte system (MPS), which also includes the precursors, promonocytes in the bone marrow and monocytes in the circulation, and other macrophages in different organs, including Kupffer cells in the liver, Langerhans cells in the skin, osteoclast cells in bone, microglial cells in the central nervous system, and peritoneal and lung interstitial macrophages.

Alveolar Macrophages Originate from Peripheral Blood Monocytes

The origin of tissue macrophages has been debated for years. Although several studies suggest that local proliferation is a possible source of the heterogenous AM pools,

there is good evidence to indicate that the bone marrow is the source of these cells, including pulmonary AM.

Bone Marrow Origin of AM. Under steady state and during lung inflammation, the circulating peripheral blood monocytes (PBM) represent a direct source for replenishment of AM. Studies in animals have shown that the majority of AM are derived from PBM (Blussé van oud Alblas and van Furth, 1982). In patients with leukemia undergoing allogenic bone marrow transplantation, mononuclear cells from the donor repopulate the alveoli within three months. This sequence supports the concept that bone marrow cells and monocytes are the precursors of the pulmonary macrophages (Thomas et al., 1976). The experiments by Bowden and his colleagues showed that the direct precursor of the AM appears to be an interstitial cell derived from the circulating PBM, which in turn arises from a bone-marrow precursor cell (Bowden et al., 1969; Bowden et al., 1972).

Peripheral blood monocytes (PBM) have a circulating half-life in the range of days (van Furth et al., 1985; Whitelaw, 1972). Entering the tissue, cells undergo significant morphologic and functional transformations into macrophages and presumably never reenter the circulation (van Forth et al., 1979). The life span of an AM in the alveoli is estimated in the range of months (Thomas et al., 1976), and AM can be removed from the alveoli by a number of routes, e.g., by mucociliary movement or by lymphatics.

As mentioned above, under noninflammatory conditions, migration of PBM into different tissues appears to be a random phenomenon; the adhesion and sequestration of circulating monocytes are not promoted and the efflux of monocytes from the blood stream to the tissues ensures maintenance of the macrophage population in a given tissue compartment. In response to inflammation or injury, various mediators, cytokines, and

chemoattractants are released, which have been shown to initiate and regulate the margination and extravasation of monocytes to meet the increased demand for tissue macrophages (Beekhuizen and van Furth, 1993). Several adhesion molecules for PBM recruitment have been identified on monocytes, the best-characterized ones are L-selectin, CD14, and molecules that belong to the β_1- or β_2-integrin subfamily (Beekhuizen and van Furth, 1993). For example, L-selectin, a 90- to 100-kd glycoprotein, is constitutively expressed by most leukocytes, including monocytes, neutrophils, lymphocytes, and eosinophils, and it does not require external activating signals for function. L-selectin can interact directly with E-selectin and P-selectin on the surface of endothelial cells. Neutrophil L-selectin mediates a significant component of the integrin-independent adhesion to cytokine-stimulated, cultured endothelial cells. Thus it is likely that L-selectin mediates the initial leukocyte-endothelial cell interaction in vivo, which must occur under considerable shear forces due to blood flow (Beekhuizen and van Furth, 1993; Jutila, 1994). Once the cell has bound via L-selectin, other adhesive interactions take place and, eventually PBM migrate transendothelially into the lung (Jutila, 1994).

Local origin of AM. Whether AM can replicate within the lung has been controversial. Some evidence has suggested that a small portion of AM is locally reproduced. In normal persons, about 0.5% AM from healthy humans incorporate [^{3}H]thymidine, which is indicative of DNA synthesis, and a higher percentage is seen in inflammatory states and in smokers. These results suggest that a small fraction of the cell population replicates (Holian et al., 1990). Additionally, in patients with acute leukemia under intensive chemotherapy, the AM maintain their numbers through prolonged periods of monocytopenia and retain the ability to synthesize DNA. These findings suggest that in

man, the AM population can sustain itself by replication in the lung (Golde et al., 1974 and 1974b).

Transformation of Alveolar Macrophages from Peripheral Blood Monocytes. The tissue macrophages not only have a common origin, but also have a similar morphology and functions. The development of PBM into tissue macrophages is accompanied by the acquisition of endocytotic competence and surface expression of both complement and Fc receptors (Adams and Hamilton, 1984). Under light microscopy, the diameter of AM varies from 15 to 50 μM, and the cytoplasm is gray with numerous dark blue granules, which sometimes appear as foamy cytoplasm, when prepared with Giemsa stains. The ratio of nucleus/cytoplasm is commonly 1:3 and the nucleoli are frequently visible. Similar to other mononuclear phagocytes, AM contain a large amount of nonspecific esterase (α-naphthyl butyrase). AM express a wide variety of membrane receptors, including Fc receptors, complement receptors, and lectin receptors, and exhibit phagocytosis and antimicrobial activities (reviewed in Lohmann-Matthes et al., 1994). Several arrays of cell mediators are released by AM in response to different stimuli, including those involved in antimicrobial and antiviral activity (such as TNF-α, IFN-α, IFN-ß, and nitric oxide) and chemotaxis (IL-8, macrophage inflammatory proteins 1 and 2, and LTB_4) and other cytokines (IL-1α, IL-1ß, IL-6, and TGF-ß) (reviewed in Lohmann-Matthes et al., 1994).

Although all tissue macrophages share these common characteristics, each type isolated from a different anatomical location appears to have distinctive features that are specific for the tissue in which they live (Sibille and Reynolds, 1990). AM, as an example, express high MHC class II antigen and low CD14 levels, while the opposite is

observed on the surface of peritoneal macrophages (PM). Another group of lung macrophages, interstitial macrophages (PIM), which are considered as the intermediate precursors of AM, are smaller than AM and morphologically resemble more closely PBM (Lavnikova et al., 1993) Though PIM have a similar Fc-receptor-mediated phagocytosis ability as AM, they are much more competent in stimulating T-cell responses to antigens and replicate better (Holian et al., 1990), but function less effectively in Fc-receptor-independent phagocytosis, production of cytokines, such as TNF-α and IFN-α/ß, and release of oxygen radicals and eicosanoids. With these obvious differences, presumably, some tissue-specific stimuli take part in the process of the in vivo differentiation from PBM. In contrast to PIM and other macrophages, which interact with different cell types and extracellular matrix, AM uniquely inhabit the air-tissue interface of the alveoli, where they are not only directly exposed to high oxygen concentration, but also encounter inhaled microorganisms and environmental toxins. The AM, as a result, undergo special metabolic adaptations in the aerobic alveoli and become the first-line defender of the lung as they transform from their precursors, PBM.

Based on the density gradient separation, AM, as well as other tissue macrophages, have been separated into subpopulations, which differ in morphology and functional activities, such as cytotoxicity, migration, oxygen radical production, and tissue factor production (Holian et al., 1990). Differential distribution of AM subpopulations is observed in different diseases as well. For example, small monocyte-like AM tend to predominate in acute inflammation and larger macrophages in chronic inflammation. Thus, the heterogenous AM subpopulation may represent differing local conditions in the lung and/or be due to prior activation.

On the other hand, monocyte subtypes have been proposed based on the observation that PBM are heterogeneous regarding morphological features and functions, including the ability to synthesize eicosanoids (Goldyne and Stobo, 1979). Therefore, the clonal variation of myeloid progenitors with different functional characteristics is another possible mechanism to explain the programmed or predetermined maturation for AM heterogeneity.

Differentiation/Maturation of Mononuclear Phagocytes In Vitro

PBM and AM have been reliably isolated with a high yield and purity from several animal species (Peters-Golden and Shelly, 1988), including cattle (Czuprynski and Hamilton, 1985). In vitro culture has been convenient and useful for determining various cellular metabolic and biological activities of mononuclear phagocytes. Since PBM provide a common precursor pool that gives rise to the heterogeneous family of cells that constitute the human MPS (Kreutz and Andreesen, 1993), the terminal differentiation of PBM into mature macrophages has been studied in vitro as well (Johnson et al., 1977; Musson, 1983; Andreesen et al., 1990a).

The Growth and Differentiation of PBM and AM in Culture. The growth and differentiation of these cultured mononuclear phagocytes is related to the culture plate pretreatment, the concentration of fetal or adult serum, and the addition of other supplements. Human PBM, for example, maintained in culture for months using medium containing 10% fetal calf serum, 10% horse serum or a combination of both, increase in size and membrane activity, differentiate into fusiform or epithelioid shape, and retain phagocytic ability (Zuckerman et al., 1979). In addition to the development of the general

features of tissue macrophages, some of the monocytes kept in culture for 3 to 10 days are fused into large multinucleated cells (Schlesinger et al., 1984). Other phenotypic changes include increased fibronectin expression (Adams and Koerner, 1989), accumulation of tissue transglutaminase (Turpin and Lopez-Berestein, 1993), and enhanced TNF-α and neopterin production after activation (Andreesen et al., 1990b). The PBM-derived macrophage, showing a typical macrophage morphology and functional activity, have been a very useful model for investigating regulatory mechanisms and functional differentiation from precursor monocyte to mature macrophage (Musson, 1983).

Monocyte/Macrophage-Like Cell Lines as a Tool for Studying the Maturation of Macrophages. In addition to the information obtained from the studies of the primary monocyte/macrophage culture, the myelomonocytic cell lines, such as HL-60, Mono Mac 6, and M1 cells also provide invaluable contributions to the understanding of macrophage biology. These cell lines undergo monocyte/macrophage differentiation and acquire monocyte/macrophage characteristics following treatments with various inducing agents, and have been extensively used to study the in vitro differentiation of macrophages and the accompanying changes in cellular metabolism as well as the underlying biochemical mechanisms.

The HL-60 cell line, derived from a patient with acute promyelocytic leukemia, was developed in Dr. R. C. Gallo's laboratory at the National Institutes of Health in the United States in the late 1970s (Gallagher et al., 1978). Among monocyte/macrophage cell lines, HL-60 possesses distinct characteristics, e.g., it continuously multiplies in suspension culture with a doubling time of 36-48 hr and is capable of being induced to differentiate into several cell phenotypes by different inducers. For example, dimethyl

sulfoxide (DMSO; Collins et al., 1978) or retinoic acid (Breitman et al., 1980) markedly increases the differentiation of HL-60 toward mature granulocyte with morphological, surface antigen, and functional characteristics.

Several agents, including $1,25(OH)_2$-D_3 (McCarthy et al., 1983), γ-interferon (IFN-γ; Takei et al., 1984), and tumor necrosis factor (TNF; Trinchieri et al., 1986) differentiate HL-60 cells into monocyte-like cells exhibiting positive nonspecific esterase staining, monocyte surface antigens, and antibody-dependent cytotoxic ability. At concentrations as low as 0.1 nM, $1,25(OH)_2$-D_3, which could be used as a therapeutic agent, induces monocytoid differentiation (McCarthy et al., 1983). Furthermore, a synergistic effect has been shown on the induction of monocytoid differentiation when INF-γ was administered with tumor necrosis factor (Hemmi and Breitman, 1987) or retinoic acid (Trinchieri et al., 1986). The mechanism of synergism is not clear.

The primary inducer of macrophage-like HL-60 cells is phorbol 12-myristate 13-acetate (PMA or TPA), which, at > 0.5 nM concentration, causes cell differentiation presumably via activation of protein kinase C (PKC)-dependent signaling pathway (Nishizuka, 1984). Although these macrophage-like cells share a number of phenotypes with monocytoid HL-60 cells, they can be distinguished by the presence of specific surface antigens (Graziano etl al. 1983; Ferrero et al., 1983) and lack of chemotactic peptide receptors and NBT reduction activity (Newburger et al., 1981).

Aside from HL-60 cells, several other monocyte/macrophage lines have also been used to study cellular differentiation and metabolism. The maturation of mouse myelomonocytic cell line M1, human monoblastic cell lines U937 and THP-1, and more mature human monocytic cell line Mono Mac 6 toward monocyte/macrophage has been

induced by incubating cells with phorbol esters (Yen, et al., 1993; Ziegler-Heitbrock et al., 1994), $1,25(OH)_2$-D_3 (Yen, et al., 1993; Sellmayer et al. 1994; Abe et al., 1981), and retinoic acid (Sellmayer et al. 1994).

Induced Maturation of Mononuclear Phagocytes: The Effects of $1,25(OH)_2$-D_3 and TGF-ß

In the lung, the maturation of mononuclear phagocytes appears to be regulated by the local cellular and environmental factors. Several in situ cells, and the mediators they release have been studied for their roles in this process. For example, the binding and interaction of the recruited monocytes with the adhesion molecules on the vascular endothelium might be involved in the cell differentiation process (Beekhuizen and van Furth, 1993). Many cell mediators, including $1,25(OH)_2$-D_3, TGF-ß, granulocyte-monocyte colony stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF), have been shown to promote the differentiation of monocyte/macrophage.

$1,25(OH)_2$-D_3-Induced Monocyte/Macrophage Maturation. $1,25(OH)_2$-D_3, with its action discovered on bone and intestinal cells, has long been identified as one of the primary regulators for body calcium homeostasis (Adams, 1989). Its immunomodulatory effects have only been studied since the intracellular vitamin D_3 receptor (VDR) was discovered about fifteen years ago (Eisman et al., 1979) and observed in human PBM, macrophages, and lymphocytes (Provvedini et al., 1983; Bhalla et al., 1983). The effects of $1,25(OH)_2$-D_3 on monocytes include the reduced expression of differentiation-related surface receptors, such as HLA-DR and CD4+ (Rigby et al., 1990). On the macrophage,

$1,25(OH)_2$-D_3 enhances the ability to inhibit the proliferation of *Mycobacterium tuberculosis* (Barnes et al., 1989). In vitamin D_3-deficient mice, the activation of peritoneal macrophages has been shown to be defective, for example, in its inability to produce oxygen radicals (Gavison and Bar-Shavit, 1989).

Vitamin D, the prehormone, is produced in the skin and, through the action of enzymes in the liver and the kidney, becomes the active form, $1,25(OH)_2$-D_3. Local sources also contribute to the $1,25(OH)_2$-D_3 concentration; in the alveoli, for example, AM synthesize $1,25(OH)_2$-D_3 after stimulation with INF-γ or lipopolysaccharide (LPS) (Reichel et al., 1987a and b). In fact, Coffey et al. have shown that the concentration of $1,25(OH)_2$-D_3 in the alveolar lining fluid is 2.6-fold that in the serum (Coffey et al., 1994a).

Most of the biological actions of $1,25(OH)_2$-D_3 are mediated through a hormone-receptor complex in a manner analogous to the mechanism of action of classical steroid hormones. The sterol interacts stereospecifically with an intracellular receptor leading to gene regulation functions mediating various biological responses. PBM show a strong constitutive expression of VDR mRNA and VDR protein. The expression of VDR during the differentiation into PBM-derived macrophages in culture is accompanied by a down-regulation of VDR mRNA and protein expression (Kreutz et al., 1993). However, responsiveness to $1,25(OH)_2$-D_3 is, to some degree, preserved in matured macrophages, probably because VDR protein was upregulated after incubation with $1,25(OH)_2$-D_3 (Lee et al., 1989; Wiese et al., 1992).

TGF-ß-Iinduced Monocyte/Macrophage Differentiation. TGF-ß is a multifunctional cytokine produced by several diverse cell types, including platelets, endothelial cells,

lymphocytes, monocytes, and macrophages (reviewed in Massagé, 1987; Robert and Sporn, 1990). TGF-β was originally found in transformed fibroblasts (Assoian et al., 1983) and is a 25-kd disulfide-linked homodimer. Three forms of TGF-β, named TGF-β_1, -β_2, and -β_3, have been isolated and they exert similar biological functions both in vitro (Cheifetz et al., 1990) and in vivo (Joyce et al., 1990). A high degree of conservation (99%) between the human and mouse TGF-β_1 sequences suggests that there is a critical biological role for the TGF-βs across species (McCartney-Francis and Wahl, 1994).

On different cell types, TGF-βs elicit a wide variety of biologic activities, which are regulated by complex factors including cell phenotype and the presence of other modulatory agents (Hooper, 1991). The effects include both the induction and inhibition of proliferation and differentiation, the regulation of gene expression, and the modulation of several metabolic pathways. For example, TGF-ß induces growth stimulation in fibroblasts and other mesencymal cells, whereas it inhibits the growth of several epithelial cell lines and keratinocytes.

TGF-ß is also implicated in the regulation of immune response and inflammation. It acts as a chemoattractant for human PBM (Wahl et al., 1987), and the recruited monocytes, as well as neutrophils, secrete TGF-ß. PBM in a resting state constitutively express type I and II receptors and are extremely sensitive to TGF-ß stimulation. Once PBM are activated, whether by LPS, INF-r, or TGF-ß itself, TGF-ß receptor expression decreases, resulting in a loss of sensitivity to TGF-ß in the activated population (McCartney-Francis et al., 1990). This might be meaningful because TGF-ß turns on PBM, which is in favor of the local inflammatory response, and then switches off the

activated cells in the alveoli in favor of the resolution of inflammation and the tissue repair.

TGF-ß moderates the growth and differentiation of several leukemic cell lines as well; for example, it substantially inhibits the proliferation of U-937 cells but has no effect on H-L60 cells. However, when administered with tumor necrosis factor-α (TNF-α), it induces differentiation and inhibits growth of both cells (De Benedetti et al., 1990). This observation parallels the findings that HL-60 cells have fewer TGF-ß receptors than U-937 cells, and TNF-α increases the expression of TGF-ß receptors dose-dependently (De Benedetti et al., 1990).

Several TGF-ß receptors expressed on cell surface have been identified: type I (~55 kDa) and type II (~75 kDa) of TGF-ß receptors are transmembrane serine/threonine kinases, participate in TGF-ß-induced signaling, and directly mediate biological activities of TGF-ß (Shum et al., 1995), whereas type III receptor (~280 kDa) is a proteoglycan that may participate in the facilitation, but not direct activation, of the type I and II receptor signaling process (Attisano et al., 1994).

The Effects of Combined Treatment of 1,25(OH)$_2$-D$_3$ and TGF-ß on Monocyte/Macrophage Differentiation. Synergistic effects on mononuclear phagocytes have been observed not only by the combined treatment of cytokines, but also by that of both 1,25(OH)$_2$-D$_3$ and TGF-ß. The phagocytic ability and the expression of surface antigen, such as CD14, of HL-60, U-937, and KG-1 leukemic cell lines, though increased with 1,25(OH)$_2$-D$_3$ treatment, are markedly enhanced with the combination of 1,25(OH)$_2$-D$_3$ and TGF-ß (Morikawa et al., 1990; Testa et al., 1993). Additional experiments showed that pretreatment of HL-60 cells with TGF-β followed by 1,25(OH)$_2$-D$_3$

incubation, but not vice versa, causes a similar effect on cell maturation as the combination treatment (Testa et al., 1993). Other studies have also shown the effect of combination treatment on cellular AA metabolism and will be discussed in a later section.

Experimental Consideration: Bronchoalveolar Lavage as a Useful and Valid Method to Obtain AM for the Study Adult as well as Neonatal AM Biology

The introduction of bronchoalveolar lavage (BAL) has been an important achievement not only for the examination of the inflammatory cells and cellular mediators present in the alveolar space but also for the in vitro study of AM biology (Myrvik et al., 1962). Although fiberoptic bronchoscopy is widely used to serve the above purposes, the application of plastic tubes of appropriate sizes in non-anesthetized experimental animals has been very useful. After a lavage tube is inserted and wedged in a subsegmental bronchus, sterile buffered saline is infused through the lumen of the tube in aliquots of 50 ml for cattle as well as human. Immediately, the lavage fluid is gently aspirated. A cumulative amount of several hundred ml of aspirated fluid is collected for cell experiments. In normal humans, the recovery of fluid averaged 60-70% (Harris et al., 1970).

The cells recovered from lavage of a normal humans (Sibille and Reynolds, 1990), rats (Peters-Golden et al., 1984), and cattle (Taylor et al., 1990), usually represent ~90% AM, as stained by Giemsa stain and verified by nonspecific esterase stain, and the rest include PMN and lymphocytes. After cell adherence to the culture dish and overnight incubation, the AM purity ordinarily reaches 95%. However, most of these data have been obtained from adult animals.

To study age-dependent maturation of 5-LO metabolism, in my experiments, similar procedures were performed in neonatal as well as in adult cattle and obtained AM for the examination of AA metabolism (Chapters 2 and 3). It has been reported that the adult AM (aAM) populations are heterogeneous, with subpopulations differing in morphology and function (Chandler and Fulmer, 1987). Since AM have a half-life in the order of months (Johnston, 1988) and are continuously replenished by PBM, the populations of aAM at equilibrium likely consists of AM at different maturation stages. However, for the neonates, AM rapidly enter the alveolar spaces during the first week of life (Bellanti et al., 1979) in response to undetermined stimuli, and by one week of age, there is a significant number of AM in the alveoli. Although neonatal AM (nAM) are largely composed of recently emigrated cells, in the experiments, I proved that nAM, similar to aAM, can be obtained from calves aged 8 day or older by BAL with high yield and purity for the study of AM biology (explained in Chapter 2).

5-LO METABOLISM IN AM AND PBM OF ADULT ANIMALS

During the past decade, arachidonate metabolism has been studied in several cell types, including alveolar epithelial cells, PBM, AM, and peritoneal macrophages. This has led to a better understanding not only of the AA metabolic profiles of individual cell types but also of the biomedical mechanisms underlying the differences in AA metabolism. The following is a brief summary of AA metabolism in mononuclear phagocytes from adult animals, with an emphasis on the 5-LO metabolism in AM and PBM.

In Adult animals, in Contrast to CO Pathway in PBM, 5-LO Pathway Is Predominant in AM

The 5-LO and CO metabolic capacities in AM and PBM. As PBM differentiate into tissue macrophages, they undergo a variety of functional changes. One dramatic difference is the enhanced AA metabolism via the 5-LO pathway in AM as compared to PBM. Although the two cell types release a similar quantity of endogenous AA in response to ionophore A23187 (Balter et al., 1989), marked alterations in the pattern of AA metabolism have been observed. In humans, the capacity of AM to metabolize endogenously released AA via the 5-LO pathway is more than 15-fold that of PBM as determined by [^{3}H]AA prelabeling technique as well as radioimmunoassay (RIA) (Balter et al., 1989, Pueringer et al., 1992; Coffey et al., 1994). The CO metabolic products are minor metabolites released by AM, however; together with 12-HETE, they are the major products of PBM AA metabolism. Similar results were obtained with zymosan stimulation as well (Balter et al., 1989). In fact, the 5-LO metabolic capacity of AM is greater than that of peritoneal macrophages or pulmonary interstitial macrophages (Peters-Golden et al., 1990).

The Biochemical Mechanisms for the Alternations of Arachidonate Metabolism during PBM to AM Differentiation. The two proteins responsible for 5-LO metabolism, 5-LO and FLAP, have been studied for their expression in AM and PBM from adult humans; at steady-state, the expression of 5-LO and FLAP in AM was about 7- and 40-fold that in PBM, respectively, as determined by Coffey et al. (1994). This result is comparable to the result of low 5-LO metabolic capacity in PBM. Therefore, the enhanced ability of

AM to synthesize 5-LO products possibly reflects cellular differentiation and induction of 5-LO and FLAP enzymes.

The CO metabolic capacity of AM, compared to that of PBM or peritoneal macrophages (PM), is low (Balter et al., 1989; Peters-Golden et al., 1990). A recent study has shown that the unstimulated rat AM contain only low levels of PGHS-1 and little PGHS-2 expression, as determined by immunoblot analysis (Wilborn et al., 1995). Although lipopolysaccharide (LPS) induces AM to increase the expression of PGHS-2 (O'Sullivan et al., 1992), the magnitude of the increment is much less that that of LPS-treated PM (Wilborn et al., 1995). Therefore, it is feasible that low CO capacity in AM can be explained, at least in part, by the poor expression of both PGHS-1 and PGHS-2.

Regulation of 5-LO Metabolism in Monocytic Phagocytes

Although AM and other tissue macrophages mature from the same precursor PBM, their arachidonate metabolic profiles are different, as described previously. For example, AM have more 5-LO activity than peritoneal macrophages (Balter et al., 1989). This shows that AA metabolism of tissue macrophages is specific for the tissue in which they live and also provides strong evidence that certain tissue-specific stimuli in the local microenvironment are responsible for the development of cell heterogeneity within the mononuclear phagocyte system.

Priming the Maturation of 5-LO Metabolism toward the Level of AM. In the lung, the recruited PBM transform into interstitial macrophages and then into AM, and they adapt to the unique lung milieu concurrently. During the transformation process, instead of in the interstitial space, the acquisition of 5-LO capacity occurs largely in the alveoli

(Peters-Golden et al., 1990). Since the alveoli are hyperoxic compared to the microenvironment in which other macrophages reside, Balter et al. (1989) examined the effect of normoxic culture on PBM AA metabolism, but learned that the aerobic environment alone is insufficient to explain the alterations of CO or 5-LO metabolism. Moreover, the prolonged culture of AM results in a time-dependent decline of A23187-induced LTB_4 and LTC_4 synthesis between 24 and 72 hr of culture. This might be due to alterations in cellular AA-containing phospholipid pools with consequent decreased coupling of AA release to 5-LO metabolism, depletion of a cofactor for the enzyme, or a decrease in the enzyme concentrations (Sporn et al., 1990).

Recently, Coffey et al. (1993) observed that AM from Vitamin D_3-deficient rats have low 5-LO capacity and FLAP protein expression. By incubating $1,25(OH)_2$-D_3-deficient AM in medium containing 50 nM $1,25(OH)_2$-D_3, the 5-LO capacity and the amount of FLAP protein expression were restored to a near normal level. Furthermore, rat PBM cultured with $1,25(OH)_2$-D_3 of the same concentration showed upregulation of both 5-LO capacity and FLAP protein as well (Coffey et al., 1994a). These results show that $1,25(OH)_2$-D_3 plays an important role in the maturation of 5-LO metabolism in AM and monocytes of rats.

Induction of 5-LO Metabolism in Monocyte/Macrophage-like Cell Lines. Several other investigations of 5-LO metabolism focused on the effects of the differentiation-inducing agents on 5-LO metabolism and the underlying biochemical mechanism in HL-60 cells have shown interesting results. After differentiation by DMSO or retinoic acid, granulocyte-like HL-60 increased the release of LTB_4 with a parallel enhancement of 5-LO and FLAP mRNA expression. This induction was time-dependent and reached a

plateau at approximately 48-72 hr (Bennett et al., 1993; Crooke et al., 1991). In addition, the effect of $1,25(OH)_2$-D_3 to differentiate HL-60 cells into monocyte/macrophage-like cells is similar to that of DMSO or retinoic acid, only the effect of the induction on 5LO mRNA is more prominent than that on FLAP mRNA (Bennett et al., 1993). Further examination, which showed an inhibition of mRNA induction by cycloheximide, suggested that the increase of 5-LO and FLAP mRNA was not likely due to transcriptional activation of their respective genes, but rather a postranscriptional event and likely requiring new protein synthesis. The mechanism probably involves the regulation of pre-mRNA processing or transport (Bennett et al., 1993; Crooke et al., 1991). However, the induction of HL-60 toward macrophage-like cell by PMA resulted in a smaller increase in FLAP mRNA expression but not 5-LO mRNA and the mechanism is not clear (Bennett et al., 1993).

A series of studies in the laboratories of Steinhilber and Samuelsson also demonstrated that 5-LO metabolic capacity, 5-LO protein, and 5-LO mRNA can be induced in DMSO-differentiated HL-60 and, less significantly, in mature monocyte-like Mono Mac 6 cells (Ziegler-Heitbrock et al., 1988) by DMSO, retinoic acid, $1,25(OH)_2$-D_3, or TGF-ß, and $1,25(OH)_2$-D_3 or TGF-ß, respectively (Brungs et al., 1994; Brungs et al., 1995). Moreover, a dramatic augmentation of the induction of 5-LO metabolic capacity, 5-LO protein, and 5-LO mRNA was observed in both cells when $1,25(OH)_2$-D_3 (24-50 nM) and TGF-ß (1 ng/ml) were administered simultaneously (Brungs et al., 1994; Brungs et al., 1995). This synergistic effect presumably requires biosynthesis of protein(s) that is essential for transcription or stabilization of mRNA (Brungs et al., 1995).

NEONATAL ALVEOLAR MACROPHAGES ARE IMMATURE

Neonatal lung is not well protected against invading microorganisms until some time after birth in most animal species (Solomon, 1971). In the cattle industry, calf lung infection, i.e., pneumonia, has been a major a major health problem (Jensen and Mackey, 1979; Radostits and Acres; 1980) and has caused great economic loss. AM, as the first-line cellular defenders of the respiratory system, have been shown to be essential in lung defense with their ability to phagocytize and kill infectious agents and to regulate local inflammatory and immune responses (reviewed by Fels and Cohn, 1986). Consequently, the morphological and functional differences between neonatal and adult AM have been studied in great detail.

The Rapid Increase in numbers of AM of Newborn Animal Is Due to Prenatal and Postnatal Influx into Alveolar Space

It has been shown that, in rabbit, calf, and human, very few AM can be obtained from the perinatal animal (Bellanti et al., 1979; Yeo et al., 1993; Ogden et al., 1984). In the rabbits, the AM number increases dramatically during the first postnatal week and continues to increase at a reduced rate throughout the first postnatal month (Bellanti et al., 1979). This phenomenon has also been observed in calves (Yeo et al., 1993). In fact, the start of AM influx actually precedes birth by at least several hours (Zeligs et al., 1977). As shown previously, the majority of AM originate from bone marrow-derived PBM, though some proliferation of macrophages in the alveoli has been observed in newborn rabbits (Evans et al., 1987).

The mechanisms for the influx of AM into alveolar space at birth have been under investigation. The timing of AM influx has been correlated well with the increased amount of intraalveolar surfactant (Bellanti et al., 1979). A low molecular weight component of surfactant is likely responsible for the AM migration during perinatal and postnatal periods (Jacobs et al., 1985), but this still requires further investigation.

Neonatal AM Have Distinctive Characteristics

Under light microscopy, the most prominent morphological feature in AM from neonatal rabbit is the presence of pale cytoplasmic inclusion bodies, particularly in those from the early postnatal period. This inclusion appears as a large phagolysosome containing mostly surfactant-related substances. Other remarkable findings include decreasing glycogen stores, an accumulation of lipid materials, and increased numbers of organelles such as Golgi apparatus, rough endoplasmic reticulum, lysosomes, and mitochondria (Bellanti et al., 1979). The increase in the number of mitochondria might be an adaptation of the neonatal AM to the high alveolar oxygen tension and an increased need to use oxidative phosphorylation as an energy source. The examination of biochemical activities showed that the activities of phagocytosis-related lysosomal enzymes, such as lysozyme, are increase during the postnatal period. This might be the response of neonatal AM to the stimuli of environmental particles, surfactants, and cellular debris (Bellanti et al., 1979).

The ability of AM to phagocytize particles, such as *Staphylococcus aureus* and *Escherichia coli* (Bellanti et al., 1979), *Candida albicans* (D'ambola et al., 1988), and zymosan (my observation) seems to be well developed in the early postnatal period.

However, the ability of phagocytizing opsonized group B streptococci via the alternative complement pathway has not matured (Hall and Sherman, 1990). The decreased expression of surface receptors, such as Fc, C3b, fibronectin and lectin receptors (Kradin et al., 1986), might explain the limited ability of specific phagocytosis. In addition, the chemotactic responses of newborn AM in response to endotoxin-activated serum and fMLP showed a dramatic increases age-dependently (Zeligs et al., 1984).

Furthermore, the microbicidal activity of AM does not develop until after the first postnatal week and not fully until the first postnatal month (Bellanti et al., 1979). The production of respiratory burst, which is an important mechanism of microbial killing, is significantly reduced in neonatal AM (Sherman and Lehrer, 1984). Additionally, a decreased concentration of digestive enzymes (Nerurkar et al., 1977) contributes to the deficient microbicidal ability of neonatal AM.

The Neonatal Animal Is Susceptible to Lung Infections: The Defective Alveolar Macrophage Defense Mechanism as a Possible Explanation

Lung infection has been a major cause of morbidity and mortality in the neonates of several animal species, including humans and cattle. For calf pneumonia, the etiologic agents consist of a wide range of microorganisms, including viruses, mycoplasma, and bacteria. In an epidemiological study, viral pneumonia accounted for ~70% of calf pneumonia at an early stage (Bryson et al., 1978). The viruses most frequently found have been bovine respiratory syncytial virus (BRSV), parainfluenza-3 virus (PI-3), and bovine viral diarrhea disease virus (BVD). In many cases, the primary viral infection precedes a secondary bacterial infection (Scott and Taylor, 1985; Trigo et al., 1984); it is possible

that viruses compromise the already immature lung defense of the neonate and act synergistically with bacteria to result in secondary pneumonia. The significant pathogenic bacteria of calf lung infections have been *Pasteurella haemolytica and mutocida*, *Haemophilus somnus*, and *Actinomyces pyogenes* (Bryson et al., 1978).

AM Immaturity Possibly Contributes to the High Incidence of Neonatal Lung Infection. Constituting 3-19% of human lung parenchymal cells (Crapo et al., 1982) and functioning as one of the first-line defense cells, the AM not only clear occasional aspirated particles, but also play a critical role in lung defense against infection by ingestion and killing of invading microorganisms, release of cell mediators, and recruitment of additional inflammatory cells into the lung. Since the quantity of AM in neonates older than one week is comparable to that in adults, as mentioned previously, the susceptibility of newborns to pneumonia is likely to be related to the functional deficiency of AM. One of the important roles of AM in lung infection is the recruitment of additional inflammatory cells, such as neutrophils, into the alveoli via the release of cellular mediators to augment the inflammatory response. It has been demonstrated that, in response to airway-administered lipopolysaccharide (LPS) or *Escherichia coli* as well as pneumococci, the lungs of neonatal rats recruit significantly less PMN than those of adult rats (Coonrod et al., 1987; Martin et al., 1995). This strongly indicates that the antimicrobial defense of neonatal lungs is compromised, which is a likely explanation for the prevalence of pulmonary infections in newborn animals including calves (Radostits and Acres, 1980).

A possible alteration in AA metabolism, especially 5-LO metabolism, of neonatal AM provides evidence for the defective defense of neonatal lung against invading microorganisms. Since eicosanoids are important AM mediators for the inflammation and

immue response of the lung, it is important to characterize the AA metabolism of AM from neonates. Although some studies have demonstrated AA metabolic profiles of several types of mononuclear phagocytes, including AM, PIM, PM, and PBM from adult animals (Balter et al., 1989; Coffey et al., 1994), the different pattern of AA metabolism (described in Chapter 2) in AM from neonates as compared to that from adults has never been published. In order to investigate the underlying mechanism responsible for the decrease in the release of 5-LO metabolites from neonatal AM, I next examined the expression of 5-LO and FLAP proteins (Chapter 2), which are required for 5-LO metabolism, in AM of different-aged cattle. Further study on the 5-LO metabolism of AM precursors, PBM, provides evidence for the relative contributions of age-dependent maturation and PBM-to-AM differentiation on the 5-LO metabolism of AM from neonatal and adult cattle (Chapter 3). Since the increase in 5-LO protein expression parallels and presumably plays an important role in the age-dependent enhancement of 5-LO metabolic capacity, I examined the effect of $1,25(OH)_2D_3$ and TGF-ß on the 5-LO protein expression in AM of neonates as compared to that of adults (Chapter 3). These studies lead to a better understanding of the maturation of 5-LO metabolism in cattle AM and PBM.

Figure 1-1. Initial phase of lung inflammatory response. Upon activation by attachment and phagocytosis of opsonized bacteria, AM produce reactive oxygen radicals (ROS) and proteases, which destroy the invading microorganisms. The activated AM also release cytokines and chemotactic molecules, such as LTB_4, C5a, and platelet activating factors (PAF), to enhance recruitment of additional inflammatory cells, including PMN and PBM, from the circulation.

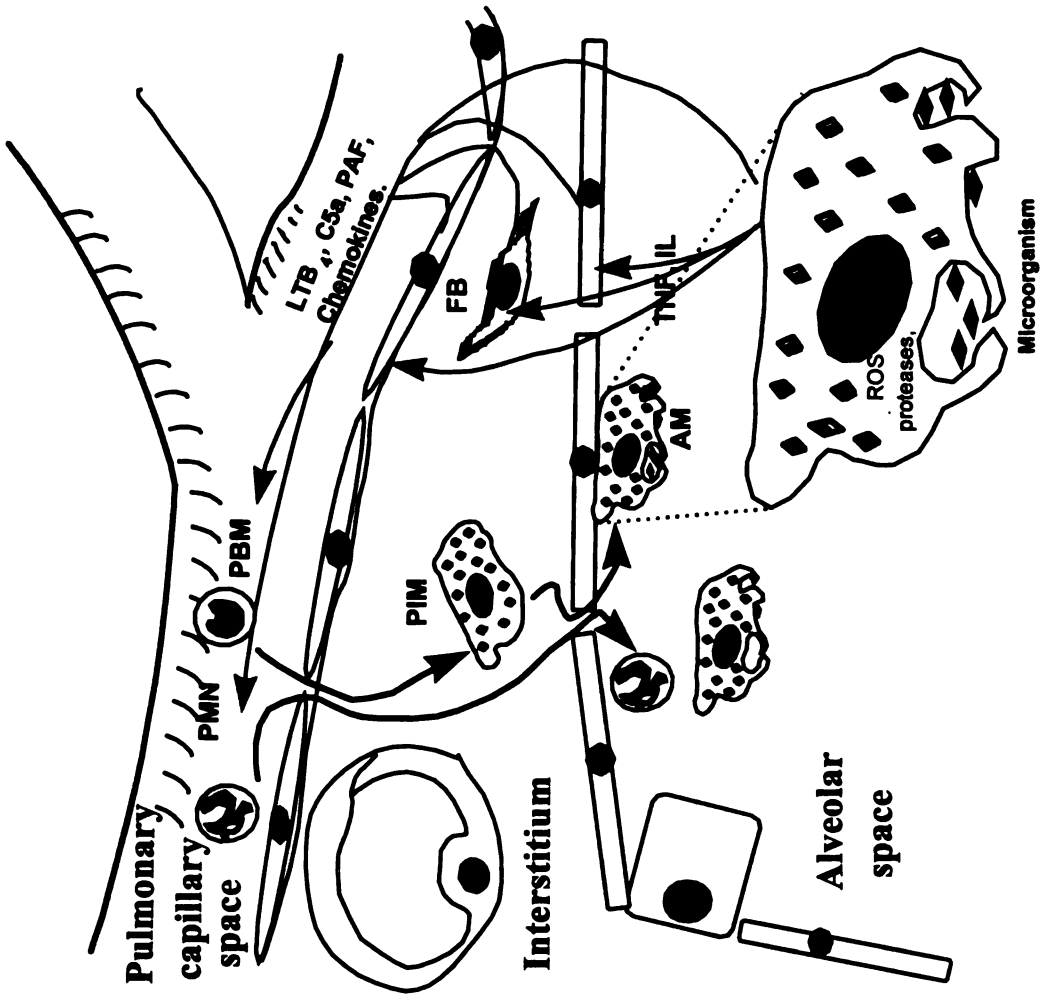

Pulmonary capillary space
PMN
PBM
LTB4, C5a, PAF, Chemokines
PIM
FB
TNF, IL
AM
ROS proteases
Microorganism
Interstitium
Alveolar space

Figure 1-1.

Figure 1-2. Arachidonic acid metabolism: A general scheme. Upon agonist stimulation of AM, AA is released from membrane phospholipid by phospholipases, among which PLA_2 is assumed to be the most important. AA is further metabolized into eicosanoids through various cyclooxygenase and lipoxygenase pathways (see text for details). In AM, AA is predominantly metabolized through 5-LO pathway.

Figure 1-2.

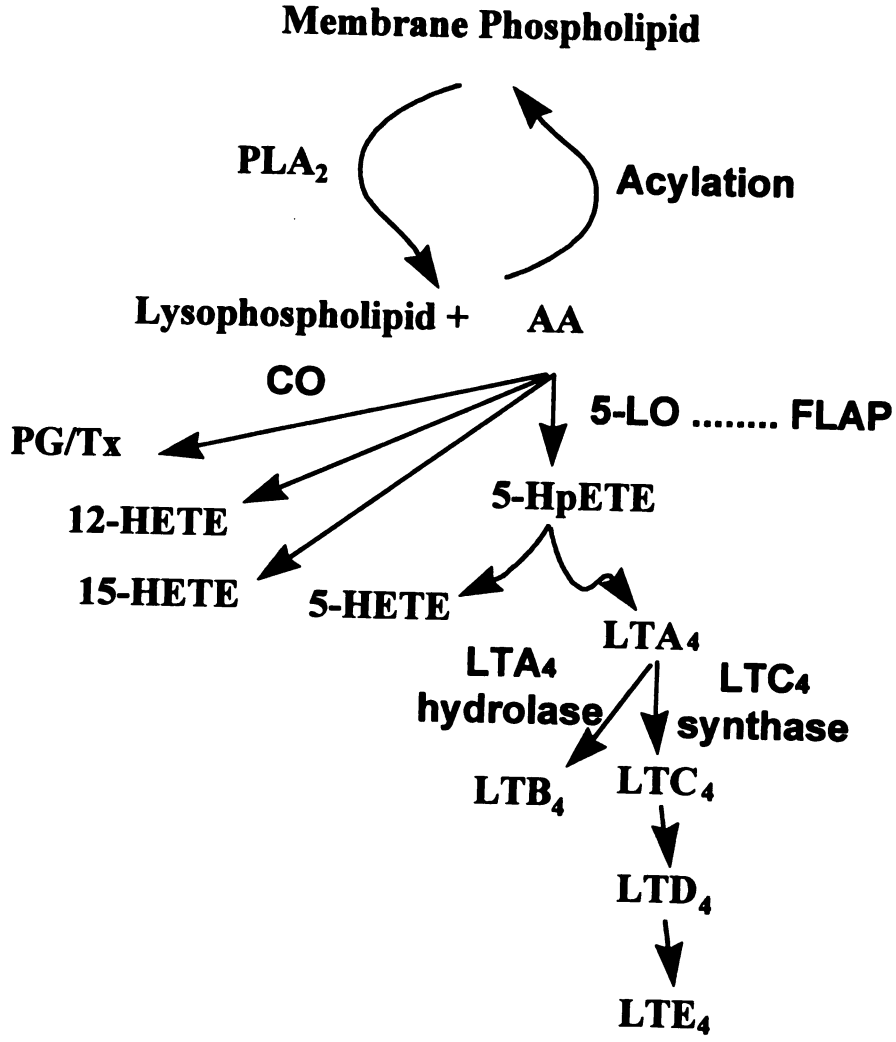
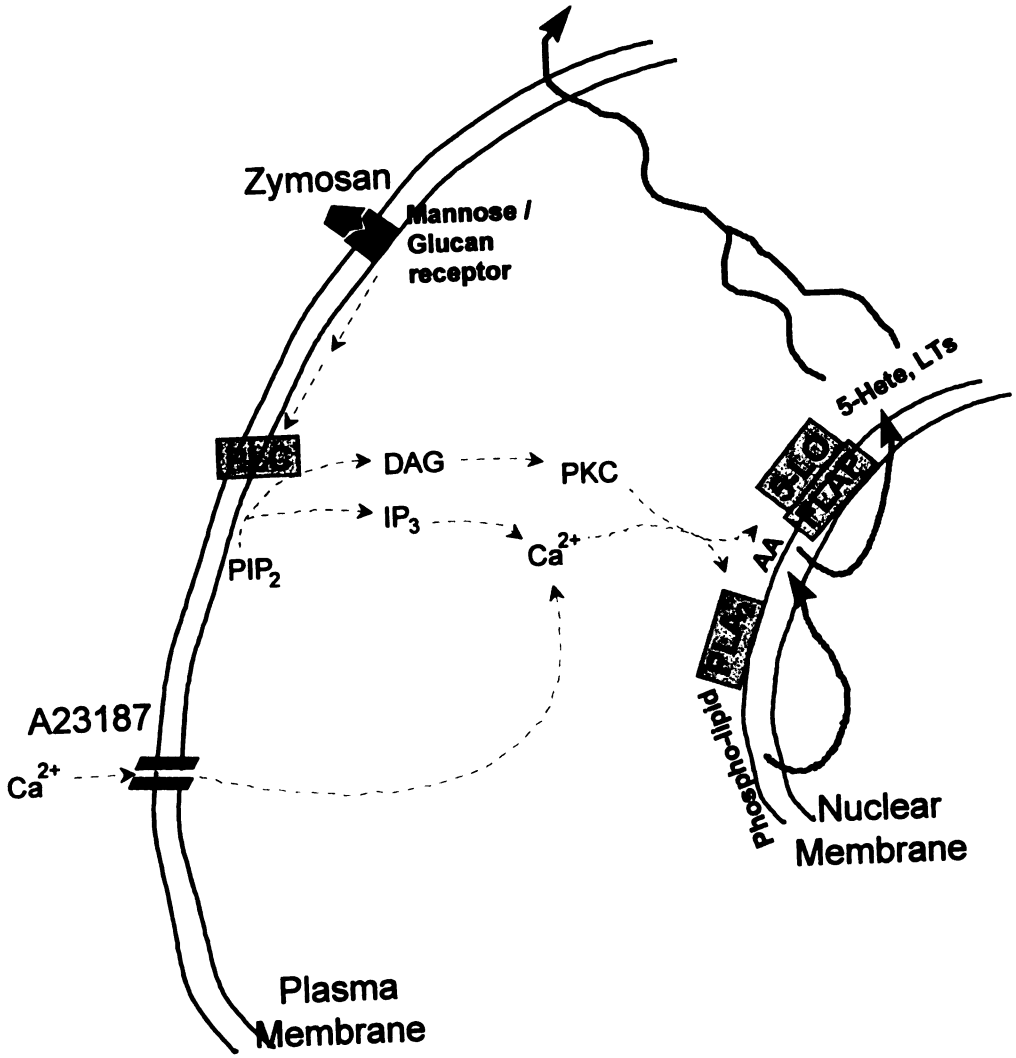

Membrane Phospholipid
PLA2
Acylation
Lysophospholipid +
AA
CO
PG/Tx
5-LO ..... FLAP
12-HETE
5-HpETE
15-HETE
5-HETE
LTA4
LTA4 hydrolase
LTC4 synthase
LTB4
LTC4
LTD4
LTE4

Figure 1-3. Release of 5-LO metabolites from AM by A23187 or Zymosan stimulation. Activation of AM by a soluble agonist (A23187) or particulate stimulus (zymosan) leads to the mobilization of intracellular calcium (Ca^{2+}) and activation of PLA_2. In contrast to the direct increase of intracellular Ca^{2+} by A23187, the activation by zymosan involves a complicated intracelluar signaling process via IP_3-DAG-Ca^{2+} cascade. Free AA, released by PLA_2, is metabolized to form leukotrienes by 5-LO. In the intact cell, the activation of 5-LO involves a Ca^{2+}-dependent mobilization of 5-LO from cytosol to nuclear membrane, where the integral membrane protein, FLAP, binds to 5-LO and is required for 5-LO metabolism.

Figure 1-3.


Zymosan
Mannose / Glucan receptor
PLC
DAG
PKC
IP3
PIP2
Ca2+
5-Hete, LTs
5-LO
FLAP
AA
PLA2
Phospholipid
A23187
Ca2+
Nuclear Membrane
Plasma Membrane

CHAPTER 2

ARACHIDONATE METABOLISM IN CATTLE ALVEOLAR MACROPHAGES: AGE-RELATED ENHANCEMENT OF 5-LIPOXYGENASE METABOLIC CAPACITY DUE TO INCREASES IN THE EXPRESSION OF 5-LIPO-XYGENASE AND 5-LIPOXYGENASE ACTIVATING PROTEIN

INTRODUCTION

Alveolar macrophages (AM) play a critical role in the defense of the lung by their intrinsic ability to phagocytize and kill invading microorganisms as well as by inducing and potentiating lung inflammation. The role of AM during the inflammatory response is achieved primarily via the release of cellular mediators, including arachidonic acid (AA) metabolites, i.e., eicosanoids. It is well established that the 5-lipoxygenase (5-LO) pathway is the predominant pathway of AM AA metabolism, generating leukotriene B_4 (LTB_4) and 5-hydroxyeicosatetraenoic acid (5-HETE) as the major products (MacDermot et al., 1984). These lipid mediators have important biological actions in the lung and other organs: LTB_4, for example, stimulates recruitment and degranulation of neutrophils and increases microvascular permeability (Martin et al., 1989). The first two steps in the 5-LO pathway leading to the production of leukotrienes are catalyzed by the enzyme 5-LO (Samuelsson, 1983). The activation of 5-LO involves both association with the nuclear membrane and interaction with a membrane protein, 5-lipoxygenase-activating protein (FLAP; Dixon et

al., 1990) In intact cells, the expression of both 5-LO and FLAP proteins is necessary for leukotriene synthesis (Dixon et al., 1990) from endogenous AA.

Newborn animals are very susceptible to the development of respiratory tract infections (Andrews, 1992) and they manifest more severe illness, which, presumably, is due to the insufficient lung defense of the neonate. Although few AM are present in the alveoli of animals at birth, the number and phagocytic function of AM approaches those of the adult by one week of age (Bellanti et al., 1979). These newly emigrated AM present in the neonate are defective in microbicidal activity (Bellanti et al., 1979). Although it is the source of important inflammatory mediators in AM from adults, the AM AA metabolic pathway has not been studied in AM from neonates. Therefore, in the present investigation, I examined the capacity of AM from newborn as well as from adult cattle to metabolize endogenous AA in response to either the soluble agonist calcium ionophore or the physiologically relevant particulate stimulus zymosan. Furthermore, I explored the biochemical mechanism for the age-related alteration of 5-LO metabolism by examining the expression of both 5-LO and FLAP in AM from different-aged cattle.

MATERIALS AND METHODS

Animals and bronchoalveolar lavage (BAL). Female Holstein cattle aged 9 days (range 8-10 days), 23 days (21-25 days), 2 years (22-26 months), and 6 years (70-74 months) were used in this study. In order to ensure that only healthy cattle were studied, a veterinarian excluded apparent infection on clinical grounds, and I also employed BAL cell counting to rule out leukocytosis which might be indicative of subclinical lung inflammation. The

calves were born at term. Each animal used in this study was lavaged once and the cells obtained were used for either metabolic study or protein analysis.

BAL was performed using different-sized tubes, 7-mm- and 1-cm-outer-diameter, respectively, for newborn (9-d and 23-d) and adult (2-y and 6-y) cattle. The tube was inserted through the nose without sedation of the animal and was wedged in a subsegmental bronchus. 400-600 ml of the phosphate-buffered saline solution, which included dextrose 10 mM, N-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES; Sigma, St. Louis, MO) 10 mM, and ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetatic acid (EGTA; Sigma) 0.2 mM, were utilized as lavage fluid and instilled in aliquots of 50 ml through the lumen of the tube. The lavage fluid was then gently aspirated with a syringe, collected into a sterile glass bottle, and transported on ice to the laboratory within one hour for cell isolation and culture.

Isolation and culture of AM. The lavage fluid from each animal was centrifuged at 250 × g for 10 min at 4°C. The pellet was resuspended in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) and subsequently filtered through a sterile 100-μm mesh filter (Tetko, Briarcliff Manor, NY). The cells were spun again at 250 × g for 8 min at 10°C and resuspended in serum-free Dulbecco's minimal essential medium (DMEM; Sigma). In all experiments, DMEM with or without heat- and chemically-inactivated (Bahnemann, 1976) fetal bovine serum (FBS; Hyclone, Logan, VT) contained 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Antibiotic-Antimycotic Solution; Sigma). Total recovered cell number was determined by counting cells by use of a hemacytometer and the yield was expressed as cells per ml of recovered fluid. Cell density was adjusted to 0.5×10^6 cells/ml and one ml of cell suspension was put into each well of a

24-well culture plate (Corning, Corning, NY) for AA metabolic experiments. For immunoblot experiments, 2×10^7 cells in 20 ml medium were plated onto a 100-mm culture dish (Corning). To allow AM adherence, cells were incubated for 1 hr at 37°C in humidified air with 5% CO_2. The non-adherent cells, mostly lymphocytes and polymorphonuclear leukocytes (PMN), were removed by gently washing twice with warm HBSS. After an additional 16-hr culture period in DMEM containing 10% FBS, purity and viability were assessed in adherent monolayers. Purity was determined by Giemsa and verified by non-specific esterase stains and viability was confirmed by trypan blue exclusion. Only the cultures with greater than 95% AM and exceeding 90% viable cells were utilized in the following experiments.

Prelabeling of membrane phospholipids with [^{3}H]AA. In the present study, I analyzed radiolabeled AA metabolites by HPLC, for which the results have been shown to correlate well with those from RIA technique (Peters-Golden and Thebert, 1987). In order to measure cell arachidonate metabolic profile by radiodetection, AM lipids were prelabeled by including 1 μCi of [^{3}H]AA (sp act 76 to 100 Ci/mmol, Dupont-New England Nuclear, Boston, MA) in the medium of each well of the 24-well culture plate during 16-hr culture as described (Peters-Golden et al., 1990). A 16-hr labeling interval was utilized both to allow isotopic equilibrium to be reached and to allow spontaneous release due to adherence to abate (Balter et al., 1989; Kouzan et al., 1988). Unincorporated label was removed by washing monolayers three times with HBSS and the cells were then incubated for an additional hr in DMEM containing 10% FBS. Cells were again washed three times with HBSS, and at this time, the cells in one well were scraped off into 1 ml of methanol and mixed with 10 ml of Safety-Solve scintillation fluid (Research Products International Corp.,

Mount Prospect, IL) and the radioactivity uptake per well was quantified by a liquid scintillation counter (LS 6000TA, Beckman Instruments, Fullerton, CA).

Agonist preparations and stimulation of AM. In the metabolic experiments, the soluble agonist calcium ionophore A23187 and the phagocytic stimulus zymosan were used to activate arachidonate metabolism of prelabeled AM. A23187 (Sigma) stock solution was prepared in dimethylsulfoxide (DMSO; Sigma) and stored at -20°C. Unopsonized zymosan A (Sigma) was boiled and washed, and the concentrated stock solution was stored at 4°C (Bonney et al., 1978). Immediately before the use of A23187 or zymosan, stock was diluted in medium to a final concentration of 10 μM (0.5% DMSO) or 1.0 mg/ml, respectively.

The optimal concentrations of A23187 and zymosan were determined in a preliminary experiment using AM from cattle of different ages by use of 5, 10, and 20 μM A23187 and 0.1, 0.5, 1.0, and 2.0 mg/ml zymosan (data not shown). In all ages, the eicosanoid release reached a plateau at 10 μM A23187 and 1.0 mg/ml zymosan. In an additional preliminary experiment using these agonist concentrations for varying periods of stimulation (15-, 30-, and 60-min), 30-min stimulation caused a maximal release of AA metabolites in cells from cattle of all ages. As a result, stimulation with either 10 μM A23187 or 1.0 mg/ml zymosan for a period of 30 min was used in all subsequent metabolic experiments.

Six different experimental treatments were designed to assess AM arachidonate metabolism in animals from each age group. In treatments 1, 2, and 3, the metabolic capacities of 5-LO and CO pathways were evaluated by the release of eicosanoids in DMEM in the absence of bovine serum albumin (BSA); while in treatments 4, 5, and 6, the liberation of free [^{3}H]AA was measured in medium containing 0.1% BSA, which prevents further reuptake or metabolism of AA by the cell so that all released radiolabel remains

trapped as free AA in medium. A23187 was added in treatments 1 and 4 and zymosan in treatments 2 and 5. Treatments 3 and 6 were controls.

Eicosanoid extraction. Radiolabeled AA and eicosanoids released into culture medium were extracted using the method of Wescott et al. (Wescott et al., 1986), with which recoveries of tritiated standards of thromboxane B_2 (TxB_2), LTC_4, and LTB_4 are similar and exceed 65% (Peters-Golden and Shelly, 1988), and that of free AA exceeds 90% (Balter et al., 1989). Briefly, at the end of stimulation, culture media (3 ml) from triplicate wells were collected and admixed with 6 ml of methanol, and the mixture was centrifuged at 480 × g for 5 min at 4°C. The resultant supernatant was decanted into 21 ml of 0.1 M sodium phosphate buffer (pH = 7.4) and the resulting concentration of methanol was 20%. This aqueous suspension was instilled into a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) prewashed with methanol followed by water. The cartridge was washed with 20% methanol in 0.1 M sodium phosphate buffer followed by water, and was then eluted with 3 ml of 80% of methanol in water. The eluted sample containing AA and eicosanoids was evaporated under nitrogen and the dry residue was stored at -70°C until analyzed.

RP-HPLC with on-line radiodetection analysis of AA metabolites. The separation and detection of radiolabeled AA metabolites follows the methods used previously by Peters-Golden and Thebert (1987) with some modification. Immediately before being injected into HPLC for analysis, the dry lipid extract was dissolved in 600 microliters of acetonitrile/water/trifluoroacetic acid (33:67:0.1, v/v/v) and filtered through a 0.45 μm CR Acrodisc 13 PTFE (Gelman Sciences, Ann Arbor, MI). Two hundred microliters were injected into the HPLC (Perkin Elmer, Norwalk, CT) and the eicosanoids were separated by a 5 μm μBondapak C_{18} column (30 × 0.4 cm; Waters Associates). The mobile phase was

pumped at a flow rate of 1 ml/min and was composed of varying ratios of acetonitrile, deionized water, and trifluoroacetic acid, all of which had been filtered through 0.2 μm nylon filter membranes (Gelman Sciences) prior to use. For a total 105-min run, CO metabolites were eluted during an initial 25-min isocratic phase (33:67:0.1, v/v/v), and 5-LO metabolites and free AA were eluted during the next 80 min with a stepwise gradient increase of acetonitrile to 100:0:0.1 (v/v/v). Eicosanoids were detected and quantified with an on-line radiodetector (FLO-ONE\Beta; Radiomatic, Meriden, CT) in which a scintillation fluid pump mixed Safety-Solve scintillation fluid with the eluate at a flow rate of 3 ml/min, and a liquid scintillation spectrophotometer continuously monitored the radioactive eicosanoids. In addition, standards of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, LTB_4, LTC_4, LTD_4, 12-hydroxyheptadecatrienoic acid (12-HHT), 15-HETE, 12-HETE, 5-HETE, and free AA (Cayman Chemical Company, Ann Arbor, MI) were utilized to determine the elution time of individual eicosanoids by a UV detector (Diode Array Detector 235, Perkin Elmer). The UV absorbance was 210 nm for CO metabolites and free AA, 280 nm for LTs, and 235 nm for monohydroxyeicosatetraenoic acids (mono-HETEs).

In metabolic experiments, 5×10^5 cells in a well were incubated in 1 ml medium containing 1 μCi [^{3}H]AA. A labeling efficiency of 21% was obtained after 16 hr incubation of AM, whereas in other studies with reported higher incorporation rates, cell prelabeling with a higher ratio of numbers of cells/amount of radiolabel was employed (Triggiani et al., 1994; Brown et al., 1988; Englen et al., 1989). However, the spectrum of released [^{3}H]AA metabolites detected is related not to the labeling efficiency, but to the distribution of isotope in the membrane phospholipid pools. In my experiments, a 16-hr

prelabeling time was chosen to allow the [^{3}H]AA to be equilibrated within the phospholipid pools.

Cell homogenization and preparation of subcellular fractions. In order to compare expression of 5-LO and FLAP proteins among AM of different-aged cattle, cell homogenates were prepared after 17 hr of incubation. HBSS-washed cell monolayers were scraped off the culture dish with a rubber policeman into a small volume of ice-cold hypotonic 40% TKM buffer (Tris-HCl 50 mM (pH = 7.4), KCl 25 mM and $MgCl_2$ 5 mM), to which diothiothreitol (DTT) 160 μg/ml and antiproteases (phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupeptin 1 μg/ml, and soybean trypsin inhibitor (SBTI) 60 μg/ml) had been freshly added. Cells were swelled by incubating on ice for 10 min, and then were disrupted with 60 to 70 strokes using a Dounce Tissue Grinder (Wheaton, Millville, NJ), achieving > 80% cell rupture with intact nuclei. The osmolarity of the homogenate was immediately restored by adding 1/4 volume of 3.4 × concentrated TKM with 1.25 M sucrose, and the final solute concentrations of crude cell lysate (Cd) were equal to those of TKM buffer with 0.25 M sucrose. One-fifth volume of Cd was aliquoted and saved for protein analysis and the rest was used in the following protocol (Brock et al., 1994) for preparing subcellular fractions.

To analyze protein distribution in subcellular compartments, Cd was centrifuged at 1,000 × g for 10 min at 4°C. The resulting pellet was washed with TKM, spun again at 1,000 × g for 10 min at 4°C, repelleted, and resuspended in TKM to generate the crude nuclear fraction (Nu). The supernatant was ultracentrifuged at 100,000 × g for 60 min. The resultant supernatant was designated as cytosol fraction (Cs) and the pellet, after gentle rinsing with TKM, was resuspended in TKM and designated as non-nuclear membrane

fraction (M). The Cd, Nu, and M preparations were sonicated for a total of 45 sec in Cell Disrupter (power level 7 and 50% duty cycle; model W-225R, Heat System-Ultrasonic, Inc., Plainview, NY) and, together with Cs were aliquoted and stored at -70°C. The protein concentrations in these samples were quantified by Bradford method using bovine serum albumin as standard.

SDS-PAGE and immunoblot analysis of 5-LO and FLAP contents. Equal quantities of proteins (~15 and ~25 μg per lane for 5-LO and FLAP, respectively) from Cd and subcellular fractions of AM from different-aged cattle were mixed with sample buffer and were loaded on 12.5% acrylamide gels by a method previously described (Coffey et al., 1992) for immunoblot analysis. Along with the samples, Rainbow® molecular weight markers (Amersham Corp., Arlington Heights, IL) were loaded to follow protein migration and transfer. Proteins were then transferred to nitrocellulose membranes overnight using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA). The membranes were then blocked with 5% non-fat dry milk in Tris buffered saline solution (TBS) for 1 hr and washed in TBS containing 0.1% Tween 20 (TBS-T). Steady-state quantities of 5-LO and FLAP proteins were determined by immunoblotting the membrane for 1 hr with rabbit polyclonal antisera (kindly provided by Dr. J. Evans, Merck Frosst Centre for Therapeutic Research, Claire-Dorval, Quebec, Canada) raised against either human leukocyte 5-LO (1:3000 diluted in TBS-T), or amino acid residues 41-52 of the human FLAP sequence (1:5000 dilution). Again, blots were washed in TBS-T and were incubated for another hr with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 diluted in TBS-T; Amersham). Finally the washed membrane was reacted with the ECL chemiluminescent Western blot system (Amersham), and exposed to hyperfilm (Amersham). Band densities of

the samples on ECL films, which reflect the relative amount of desired protein, were quantified by video densitometry using NIH Image software.

Data and statistical analysis. The data from metabolic experiments and immunoblot analysis are expressed, where applicable, as mean ± standard error (SEM). For the metabolic study, the data means of different-aged animals were compared by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (hsd) post-hoc test; means of different treatments within the same age group were compared by the unpaired Student's *t*-test. The evaluation of densitometric bands between different age groups used the paired *t*-test. In all comparisons, a p value < 0.05 was considered significant.

RESULTS

Cells recovered from BAL fluids. To determine the ages of calves to be used, in a preliminary experiment, cells obtained by BAL from cattle of various ages (1-d, 2-d, 4-d, 6-d, 8-d, 14-d, and 28-d) were counted, cultured, and stained using methods described above. The results from repeated experiments on cattle 6-d or younger showed that cells per ml of BAL fluid tended to increase with age and the purity of AM after 16 hr of culture was almost always less than 90%. Similar to previous reports, the contaminating cells were largely polymorphonuclear leukocytes (PMN) (Bellanti et al., 1979). By contrast, the overnight-cultured cells from 8-d or older cattle ordinarily consisted of greater than 95% AM. Therefore, 8-day-old (8-d) cattle were the youngest in which I studied AM AA metabolism. Due to the availability of animals, 9 ± 1-d and 23 ± 2-d cattle were assigned to the newborn group and 2-y ± 2-m and 6-y ± 2-m to the adult group. In all these ages, the

recovered fluid volume was 60-70% of the input volume. The numbers of cells recovered from 9-d, 23-d, 2-y, and 6-y cattle did not vary significantly (n= 4 or 6 in each age group; Table 2-1).

Metabolism of endogenous AA; 5-LO and CO metabolic capacities. The uptake of [^{3}H]AA by 9-d, 23-d, 2-y, or 6-y cattle AM after 16-hr culture, expressed as mean ± SEM, was 276,260 ± 25,336, 229,933 ± 39,842, 245,194 ± 33,732, or 265,007 ± 43,481 cpm/well, respectively (p = NS, n = 4 or 5 in each age group). Without any agonist stimulation, AM from all ages released very little free [^{3}H]AA, and virtually no eicosanoids were detected (data not shown). The representative AA metabolic profiles of adult and neonatal AM stimulated with A23187 are displayed in Figure 2-1. Adult cattle AM (aAM; Figure 2-1B) released large quantities of 5-HETE and LTB_4 (5-LO products) as well as free AA, and those from newborns (nAM; Figure 2-1A) released predominantly free AA and CO products including 12-HHT, TxB_2, PGE_2, and $PGF_{2\alpha}$. Figure 2-2 demonstrates the mean 5-LO and CO metabolic capacities assessed by the release of all 5-LO and CO products, respectively, and expressed as a percentage of total radioactivity released. The 5-LO metabolic capacity increased with age with both A23187 and zymosan stimulation

Table 2-1. The number of cells recovered from BAL fluid obtained from different-aged cattle

	9-d	23-d	2-y	6-y
Cell number per ml of BAL fluid ($\times 10^{-5}$)	1.65 ± 0.21	1.11 ± 0.30	1.17 ± 0.08	1.31 ± 0.20

(Figure 2-2A). For 23-d nAM and 2-y and 6-y aAM, the ionophore-stimulated 5-LO metabolic capacity was 2.6-, 3.7-, and 4.4-fold that of 9-d nAM, respectively. The corresponding increases for zymosan-stimulation were 4.1-, 10.6-, and 10.2-fold, respectively. The CO metabolic capacity tended to decrease with age (Figure 2-2B). With A23187 and zymosan stimulation, the CO capacity of nAM was 1.6- to 1.8-fold and 1.8- to 3.6-fold that of aAM, respectively. Release of individual eicosanoids is summarized in Table 2-2. In the progression from neonate to adult, changes in synthesis of the preponderant eicosanoids paralleled those observed for the total 5-LO or CO metabolic capacity. In addition, the releases of 12-HETE and 15-HETE from AM stimulated either with A23187 or zymosan decreased with age (Table 2-2)

As shown in Figure 2-1, free AA and its major metabolites, including 6-k-$PGF_{1\alpha}$, TxB_2, $PGF_{2\alpha}$, PGE_2, PGD_2, 12-HHT, LTB_4, LTC_4, LTD_4, and 5-,12-, and 15-HETE, was detected with this method. Comparing AA metabolic profiles of AM to those obtained by radiodetection or RIA (which does not depend on the incorporation of isotopic AA), my data from adult AM match those obtained from adult AM of several species including cattle (Peters-Golden et al., 1990; Taylor et al., 1990; O'Sullivan et al., 1990). This strongly supports the contention that the radiolabeling method is valid and my data are indeed representative of the entire spectrum of metabolites produced.

Release of free [^{3}H]AA from prelabeled AM. HPLC data demonstrated shifts in distribution of metabolites from CO to 5-LO with age. To determine if changes in total AA release contributed to changes in eicosanoid synthesis, prelabeled cells were stimulated in the presence of 0.1% BSA. Under this condition, released AA is trapped in the medium bound to BSA and, in a preliminary experiment, more than 90% of released [^{3}H]AA

remained unmetabolized (data not shown). In Figure 2-3, the total [^{3}H]AA release is expressed as a percentage of the total radioactivity incorporated into the phospholipid pool of AM. Without agonist stimulation, both nAM and aAM demonstrated a similar low level of release. When stimulated with A23187 or zymosan, there was a marked increase in AA release, but the quantity was similar among cells from animals of different ages and between different agonists. This suggested that the availability of AA for eicosanoid production was similar in nAM and aAM and between A23187 and zymosan. Thus, differences in metabolic profiles reflect post-phospholipase mechanisms.

Immunoblot analysis of 5-LO and FLAP. To determine if the cellular expression of 5-LO or FLAP proteins is responsible for the age-related increase in 5-LO metabolic capacity of cattle AM, immunoblot analysis was used. The polyclonal antisera specific for 5-LO and for FLAP, proven useful in rat and human species (Brock et al., 1994, Coffey et al., 1994), worked well in cattle (Figures 2-4A and B). As can be seen in the left-hand lanes of Figures 2-4A and B, the amount of 5-LO and FLAP in crude lysate of AM increased with age. Densitometric analysis of those bands from several experiments showed that there were age-related increases of the total steady-state expression of both 5-LO and FLAP. For 23-d nAM and 2-y and 6-y aAM, the expression of 5-LO was 1.60 ± 0.12-, 1.90 ± 0.04-, and 1.70 ± 0.08-fold that of 9-d nAM, respectively (Figure 2-4C). The corresponding increases for the expression of FLAP were 1.56 ± 0.20-, 2.33 ± 0.85-, and 1.86 ± 0.56-fold, respectively (Figure 2-4D). Changes in 5-LO metabolic capacity could also reflect changes in the subcellular localization of 5-LO or FLAP. The distributions of 5-LO (Figure 2-4A) and FLAP (Figure 2-4B) among subcellular fractions, including Nu, M, and Cs, of unstimulated AM were compared among different-aged cattle. Blots showed that, in aAM,

5-LO was present in all three fractions with predominance in Nu and M, and FLAP was present only in Nu and M. These results are similar to those previously reported for aAM from other species (Brock et al., 1994; Coffey et al., 1994). In nAM, the distribution of 5-LO was similar to that in aAM. However, FLAP in nAM was present in Nu, but there was little in M.

DISCUSSION

In the present study, nAM and aAM from cattle exhibited different patterns of arachidonate metabolism in response to either A23187 or zymosan stimulation. In aAM, endogenously released AA was converted predominantly to LTB_4 and 5-HETE, with only a minimal degree of conversion to CO products. This "adult pattern" of AM AA metabolism is consistent with reports from cattle as well as other species (Balter et al.,1989; MacDermot et al., 1984; Peters-Golden et al., 1990; Taylor et al., 1990). In nAM, by contrast, the major AA metabolites released were prostanoids rather than 5-LO metabolites (the "juvenile pattern"). The age-related enhancement of AM 5-LO metabolic capacity was paralleled by increases in both 5-LO and FLAP protein expression of a similar magnitude. It is plausible that these changes in protein expression explain most of the changes in 5-LO metabolic capacity.

Several lines of evidence in the present investigation led us to the above conclusion. The age-dependent increase in the release of LTB_4 was in parallel with that of 5-HETE, which suggested that the upregulation occurred at a step proximal to the LTA_4 hydrolase. The AM ability to hydrolyze AA from membrane phospholipids did not differ significantly among cattle of different ages, indicating similar phospholipase capacity. This pointed to

the 5-LO enzyme as the regulated step probably responsible for the age-related increase in 5-LO metabolic capacity. In intact cells, activation of the enzyme 5-LO to synthesize leukotrienes requires interaction with the membrane-associated protein, FLAP (Dixon et al., 1990). Quantitative analysis of blots indicated that total levels of both 5-LO and FLAP in AM increased with age, in parallel with increases in 5-LO metabolic capacity. Between 9-d neonates and adults, the expression of both 5-LO and FLAP in AM approximately doubled and the release of 5-LO metabolites in response to A23187 increased about 4-fold. Intermediate degrees of enhancement were seen at 23-d, in which the expression of both 5-LO and FLAP increased 1.6-fold and the release of 5-LO metabolites increased 2.6-fold, as compared to 9-d cells. Independent of receptor function, A23187 increases the intracellular concentration of Ca^{2+}, which directly activates the Ca^{2+}-dependent processes of 5-LO metabolism. It would be plausible to infer from my data that increases in expression of both 5-LO and FLAP were responsible for the age-dependent increase of AM 5-LO capacity and that the contribution of each protein was similar. The 5-LO metabolic capacity of AM from 6-y cattle increased ~30% as compared to that from the 2-y. This increment was not accompanied by further elevation of 5-LO or FLAP expression; therefore, other factor(s), such as the cellular mechanisms for the supply of substrate AA and cofactor ATP for 5-LO metabolism, might account for this augmentation of 5-LO metabolic activity. Identification of this mechanism is beyond the scope of the present study.

Unlike the non-specific agonist A23187, zymosan is phagocytized and stimulates cellular AA metabolism via the activation of the intracellular messenger system(s), presumably the inositol 1,4,5 triphosphate (IP_3)-diacyl glyceride (DAG)-Ca^{2+} cascade (Hoffman et al., 1991). The result that similar age-dependent changes in AA metabolism

were observed following stimulation by either A23187 or zymosan signified that this phenomenon is not agonist-specific. In view of the importance of phagocytosis by AM in host defense, extending the data derived from A23187 to the more relevant agonist zymosan suggests that the relative inability of nAM to generate 5-LO products has physiological importance. The zymosan-stimulated 5-LO metabolic activity of aAM and of 23-d nAM was ~10- and ~4-fold that of 9-d nAM, respectively. This more prominent difference between aAM and nAM, as compared with A23187 stimulation, suggests that the intracellular signaling process(es) involved in responses to zymosan in newborn AM may be immature as well. Similar comparisons of adult and neonatal PMN revealed that neonatal PMN released less LTB_4 with zymosan but not with A23187 stimulation (Viggiano et al., 1994), which suggests that neonatal PMN also have impaired response to receptor-mediated stimuli in spite of their normal 5-LO metabolic capacity. Although the capacities for AA release and CO metabolism were similar in response to optimal concentrations of A23187 and zymosan, the 5-LO metabolic capacity was always higher with A23187 stimulation. Considering that calcium ionophore results in a higher and more long-lived Ca^{2+} transient than receptor-mediated agonist (Wong et al., 1991), this result is consistent with the finding that the activation of 5-LO requires higher concentration of intracellular Ca^{2+} than that of CO (Aderem and Cohn, 1988).

Though no study comparing the 5-LO metabolism of neonatal and adult mononuclear phagocytes has been published, Ibe and Raj investigated the leukotrienes generated by ovine lungs stimulated with A23187 and showed that their release increased with age (Ibe and Raj, 1995). Since AM are one of the major cell types and an important source of leukotriene release in the lung, the low 5-LO metabolic capacity of neonatal lung likely reflects the

contribution of nAM. As for the age-dependent decrease in CO product release by AM, my result is consistent with the finding that PGE_2 release by AM of newborn rabbits is higher than that of adults (Tomai et al., 1992). The agreement of AA metabolic profile of cattle nAM with that of other species implies that this age-dependent phenomenon is not specific to cattle.

Most of the AM originate from peripheral blood monocytes (PBM), which have a circulating half-life of about three days and leave the circulation for the alveoli where they differentiate into AM (Johnston, 1988). Significant morphologic and functional changes accompany this process (Johnston, 1988), including an increase in the 5-LO and a decrease in the CO metabolic capacity (Balter et al., 1989). However, in the neonates, AM metabolize the endogenous AA predominantly to CO products, which is similar to PBM from the adults. Studies comparing AA metabolism in AM and PBM from adult human showed that the A23187-stimulated capacity of AM to produce 5-LO metabolites from endogenous AA is more than 15-fold (Balter et al., 1989; Coffey et al., 1994), and the expression of 5-LO and FLAP is about 7- and 40-fold that of adult PBM, respectively (Coffey et al., 1994). In the present study with cattle, the A23187-stimulated 5-LO metabolic capacity of aAM was 1.6- to 4-fold and the steady-state expression of 5-LO and FLAP proteins was twice that of nAM. In spite of differences in animal species and methods to isolate PBM and AM among these studies, nAM seem to be more competent in 5-LO metabolism and to express more 5-LO and FLAP proteins than adult PBM.

These substantial differences between nAM and adult PBM can be explained as follows. In cattle and other species, there are only a few AM at birth. During the first week of life, AM rapidly enter the alveolar spaces (Bellanti et al., 1979; my observations) in

response to undetermined stimuli, and by one week of age, there is a significant number of AM in the alveoli. As for adult animals, AM have a half-life in the order of months (Johnston, 1988) and are continuously replenished by PBM. As well, the AM populations are heterogeneous, with subpopulations differing in morphology and function, including the release of CO products (Chandler and Fulmer, 1987). Thus, it is reasonable to assume that nAM were largely composed of recently emigrated cells with low 5-LO metabolic capacity, whereas the population of aAM at equilibrium consisted mainly of "older" AM with high capacity. Moreover, the increase in the expression of 5-LO and FLAP proteins from 9-d to 23-d cattle nAM is likely to reflect the maturation of AM AA metabolism in the alveoli. I speculate that the processes of maturation towards the adult pattern of AA metabolism require lung factor(s), although only by studying the AA metabolism of macrophages in other organs can I determine if this age-dependent change is unique to the lung.

In summary, the age-related increases of 5-LO metabolic capacity observed in AM parallel increases in the expression of both 5-LO and FLAP proteins. It has been demonstrated that, in response to airway-administered lipopolysaccharide (LPS) or *Escherichia coli* as well as pneumococci, the lungs of neonatal rats recruit significantly less PMN than those of the adults (Coonrod et al., 1987; Martin et al., 1995). Since leukotrienes are important mediators of inflammation and immune responses (Chen et al., 1989 and 1994) and LTB_4, a major product of AM, is a potent chemoattractant that participates in the recruitment of additional PMN into the alveolar space (Martin et al., 1989), the immature pattern of AA metabolism in nAM may compromise the antimicrobial defense of the lung and explain, at least in part, the prevalence of pulmonary infections in newborn animals including calves (Radostits and Acres; 1980).

Table 2-2. Distribution of radiolabeled AA metabolites released by different-aged cattle AM stimulated with A23187 (2-2A) or zymosan (2-2B)[a]

2-2A.

	Percent of eluted radioactivity			
AA Metabolites	9-d	23-d	2-y	6-y
6-k-$PGF_{1\alpha}$[b]	1.2 ± 0.3%	0.9 ± 0.2%	1.0 ± 0.5%	0.8 ± 0.3%
TxB_2[b]	5.7 ± 0.9%	4.8 ± 1.6%	2.4 ± 0.4%	2.8 ± 1.0%
$PGF_{2\alpha}$[b]	2.7 ± 0.8%	4.2 ± 0.7%	1.8 ± 0.7%	2.8 ± 0.6%
PGE_2[b]	3.0 ± 1.6%	1.6 ± 0.4%	0.8 ± 0.4%	1.0 ± 0.5%
PGD_2[b]	0.9 ± 0.5%	0.8 ± 0.4%	0.4 ± 0.2%	0.3 ± 0.2%
12-HHT[b]	6.5 ± 1.5%	6.1 ± 0.4%	3.7 ± 0.6%	3.9 ± 1.1%
LTC_4[b]	0.3 ± 0.2%	0.2 ± 0.1%	0.6 ± 0.3%	0.4 ± 0.2%
LTD_4[b]	0.6 ± 0.1%	0.8 ± 0.2%	0.6 ± 0.3%	0.6 ± 0.4%
LTB_4[b]	2.5 ± 0.2%	5.8 ± 0.9%	10.2 ± 1.1%	12.8 ± 1.8%
5-HETE[b]	4.4 ± 0.3%	13.1 ± 2.1%	14.7 ± 2.0%	20.6 ± 0.7%
12-HETE[c]	3.0 ± 0.7%	2.6 ± 0.2%	0.6 ± 0.2%[*,#]	1.4 ± 0.5%
15-HETE[c]	1.8 ± 0.9%	2.2 ± 0.1%	0.7 ± 0.3%	0.9 ± 0.1%
free AA	67.5 ± 7.0%	56.9 ± 2.5%	62.7 ± 2.4%	51.7 ± 4.1%

2-2B.

	Percent of eluted radioactivity			
AA Metabolites	9-d	23-d	2-y	6-y
6-k-$PGF_{1\alpha}$[b]	1.1 ± 0.3%	1.1 ± 0.1%	0.6 ± 0.3%	0.6 ± 0.1%
TxB_2[b]	7.1 ± 1.8%	4.8 ± 0.7%	1.8 ± 0.5%	2.5 ± 0.6%
$PGF_{2\alpha}$[b]	4.3 ± 1.2%	4.3 ± 0.6%	1.5 ± 0.4%	2.6 ± 0.4%
PGE_2[b]	3.7 ± 1.2%	2.0 ± 0.3%	0.7 ± 0.4%	1.1 ± 0.4%
PGD_2[b]	0.9 ± 0.5%	0.7 ± 0.1%	0.1 ± 0.1%	0.4 ± 0.2%
12-HHT[b]	7.9 ± 2.1%	6.0 ± 0.7%	2.3 ± 1.0%	3.0 ± 0.4%
LTC_4[b]	0.5 ± 0.3%	0.1 ± 0.1%	0.3 ± 0.1%	0.1 ± 0.1%
LTD_4[b]	0.2 ± 0.1%	0.8 ± 0.2%	0.3 ± 0.1%	0.3 ± 0.1%
LTB_4[b]	0.2 ± 0.1%	1.8 ± 0.1%	4.8 ± 1.5%	4.6 ± 1.0%
5-HETE[b]	0.5 ± 0.2%	2.9 ± 0.8%	9.0 ± 2.2%	8.8 ± 2.3%
12-HETE[c]	3.4 ± 0.8%	2.7 ± 0.3%	0.6 ± 0.2%[*,#]	1.7 ± 0.3%
15-HETE[c]	2.7 ± 0.5%	2.3 ± 0.2%	0.6 ± 0.3%[*,#]	0.7 ± 0.1%[*,#]
free AA	67.5 ± 7.7%	70.7 ± 2.0%	77.5 ± 1.8%	73.6 ± 3.1%

[a] [^{3}H]AA-prelabeled AM were stimulated with either A23187 (in table A) or zymosan (in table B) and the released eicosanoids were extracted from medium and separated by HPLC. For each eicosanoid, results are expressed as the percentage of total radioactivity eluted by HPLC. The data are mean ± SEM from 4 or 5 cattle in each age group.

[b] The total metabolic capacities of CO (metabolites include 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, and 12-HHT) and 5-LO (metabolites include LTB_4, LTC_4, LTD_4, and 5-HETE) are illustrated in Figure 2-3B and 2-3A, respectively.

[c] Data means for 12-HETE and 15-HETE were compared among different-aged cattle by ANOVA with Tukey's hsd post-hoc test (* = significantly different ($p < 0.05$) from 9-d; $^\#$ = significantly different ($p < 0.05$) from 23-d).

Figure 2-1. Representative HPLC profiles of eluted radioactivity from prelabeled 9-d (in panel A) and 2-y (in panel B) cattle AM. Prelabeled AM were incubated with 10 μM A23187 and the released eicosanoids were extracted from medium and separated by HPLC. Radioactivity was detected by an on-line radiodetector and is expressed as counts per 30 sec. The retention times of authentic standandards are shown in the chromatogram.

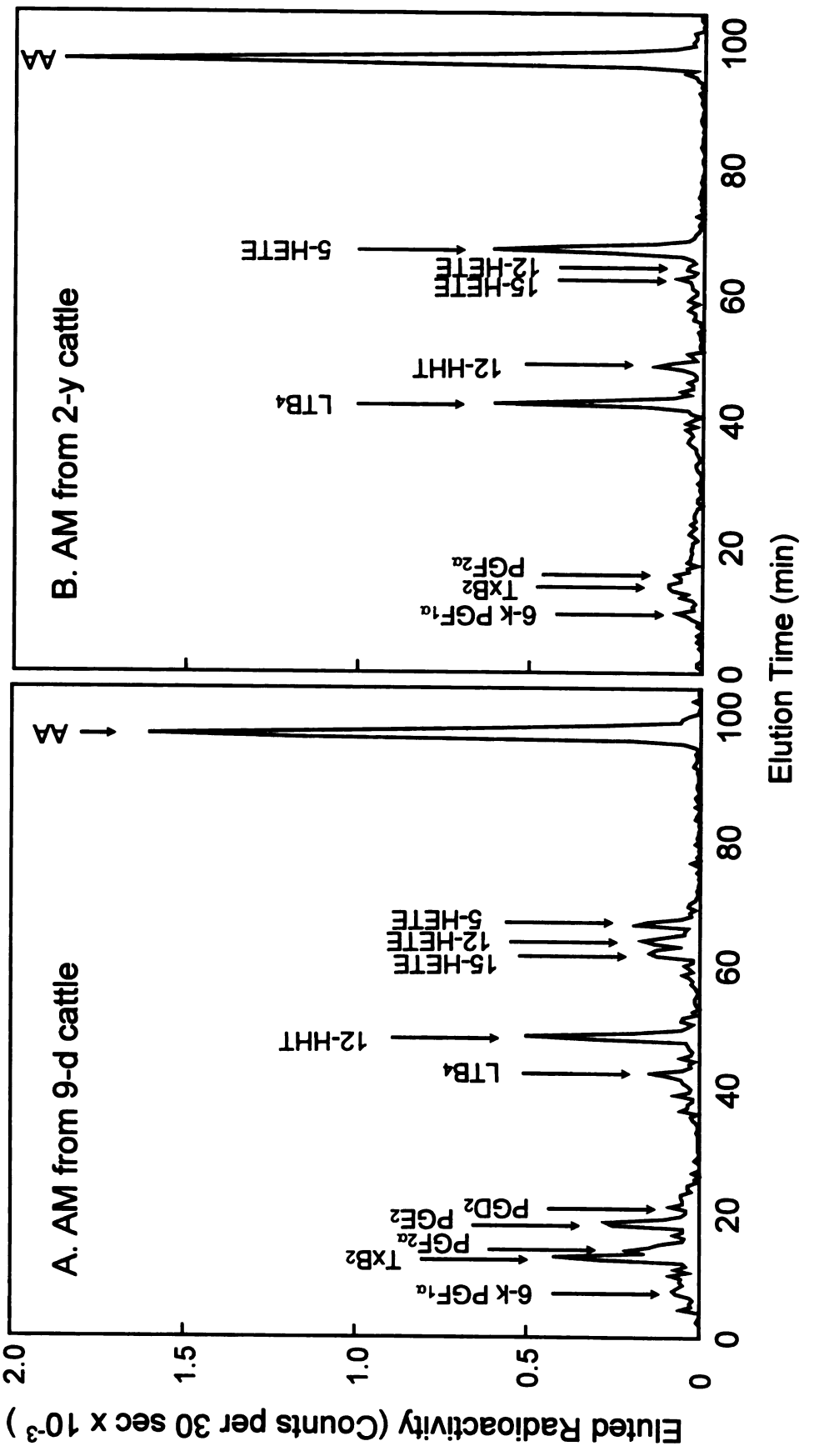

A. AM from 9-d cattle
B. AM from 2-y cattle
AA
6-k PGF1a
TxB2
PGF2a
PGD2
PGE2
LTB4
12-HHT
15-HETE
12-HETE
5-HETE
Eluted Radioactivity (Counts per 30 sec x 10-3)
Elution Time (min)
0
0.5
1.0
1.5
2.0
0
20
40
60
80
100

Figure 2-1.

Figure 2-2. Release of 5-LO (in panel A) and CO (in panel B) metabolic products by different-aged cattle AM stimulated with either A23187 or zymosan. Prelabeled AM were incubated with 10 μM A23187 or 1 mg/ml zymosan for 30 min, and eicosanoids were extracted from medium and analyzed by HPLC with on-line radiodetection. Data are expressed as a percentage of the total released radioactivity, and the results represent the mean ± SEM (n = 4 or 5 in each age group). The data means of different-aged animals were compared by ANOVA with Tukey's hsd post-hoc test (* = significantly different ($p < 0.05$) from 9-d; # = significantly different ($p < 0.05$) from 23-d).

Figure 2-2.

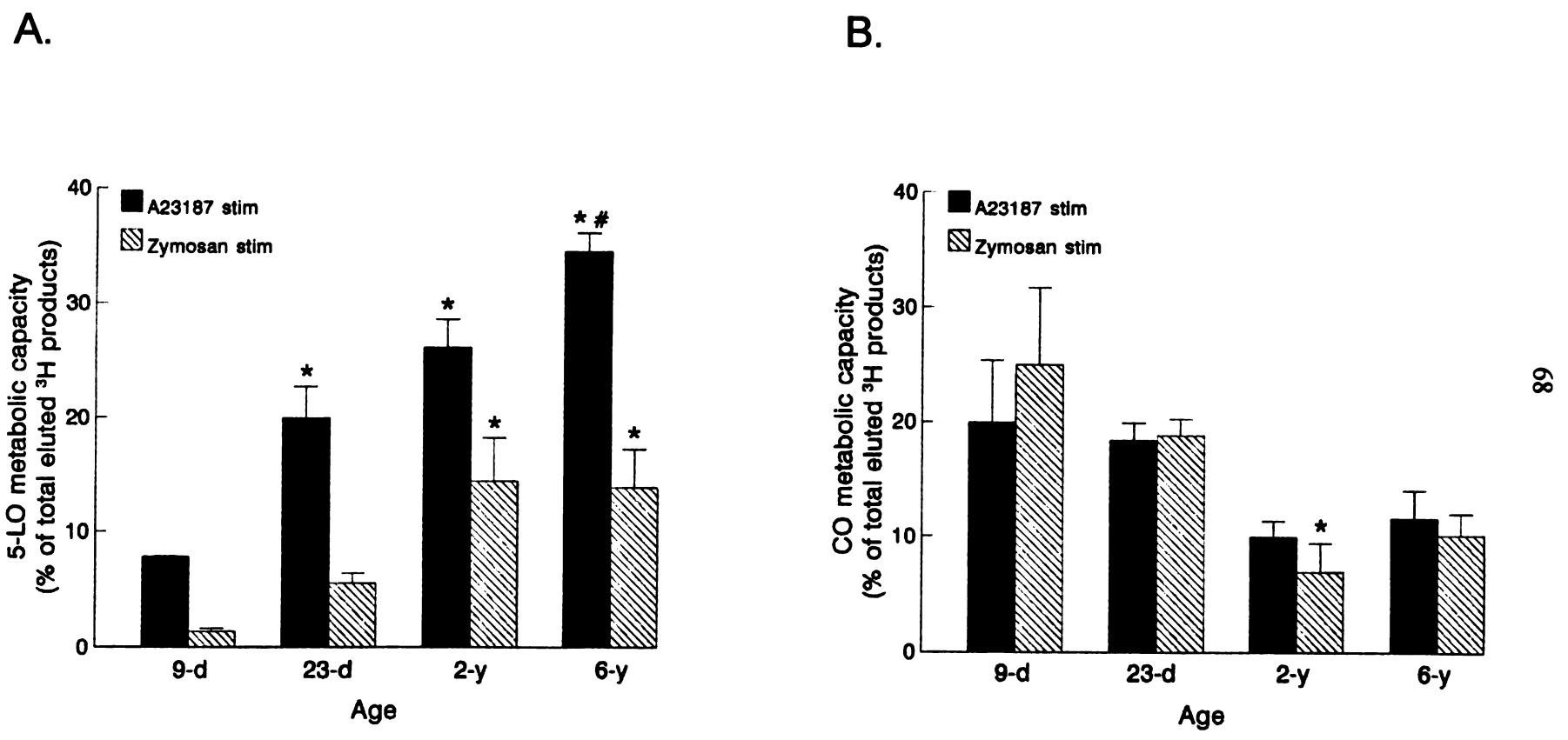

A.
B.
A23187 stim
Zymosan stim
5-LO metabolic capacity
(% of total eluted ³H products)
CO metabolic capacity
(% of total eluted ³H products)
0
10
20
30
40
9-d
23-d
2-y
6-y
Age
*
*#

Figure 2-3. Release of radiolabeled arachidonate by prelabeled AM from different-aged cattle. Prelabeled AM were incubated for 30 min in medium containing 0.1% BSA in the absence or presence of 10 μM A23187 or 1 mg/ml zymosan. Arachidonate was then extracted from medium and analyzed by HPLC with on-line radiodetection. The released arachidonate radioactivity was not different among cattle of different ages (n = 4 or 5 in each age group). Data are expressed as a percentage of the incorporated radioactivity and the results represent the mean ± SEM.

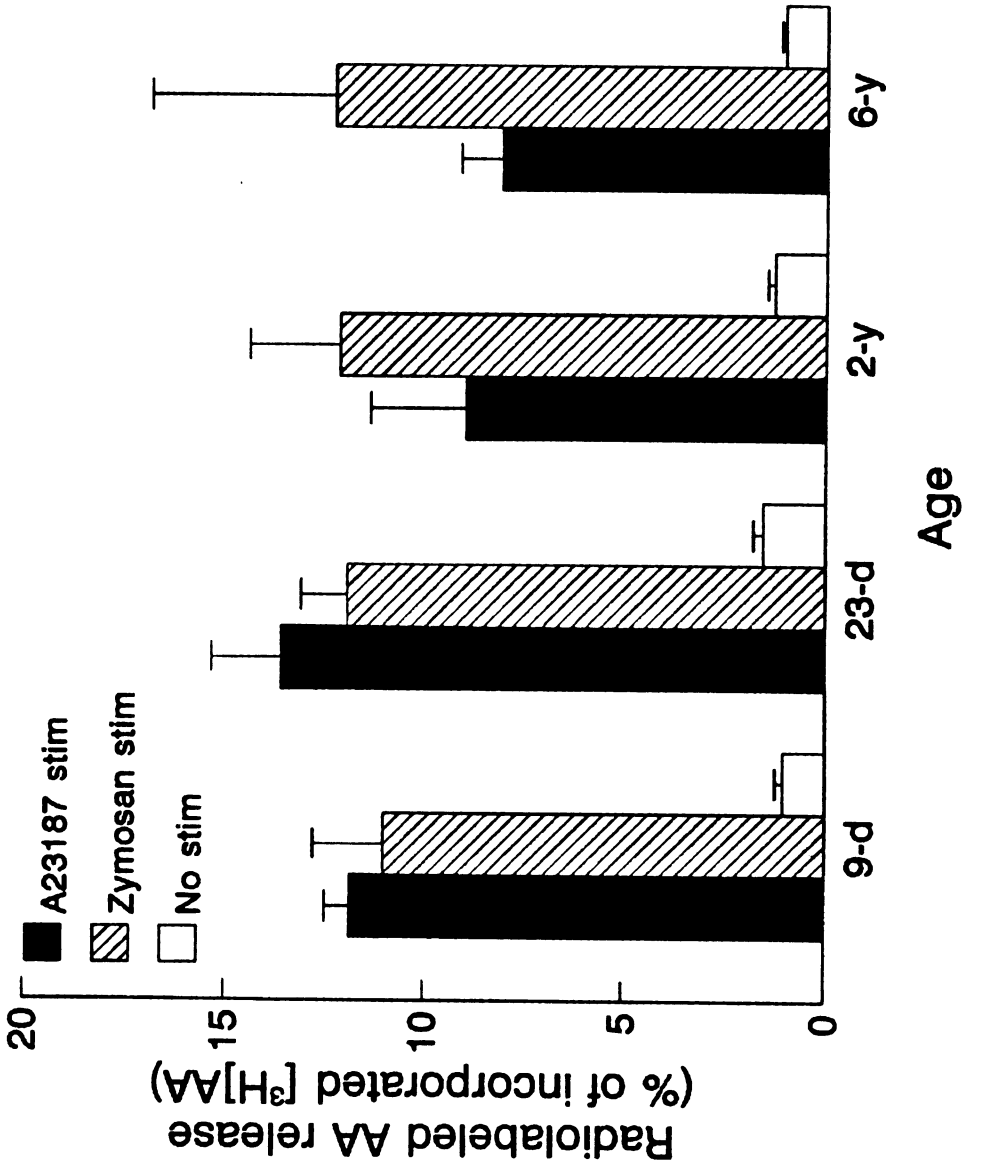
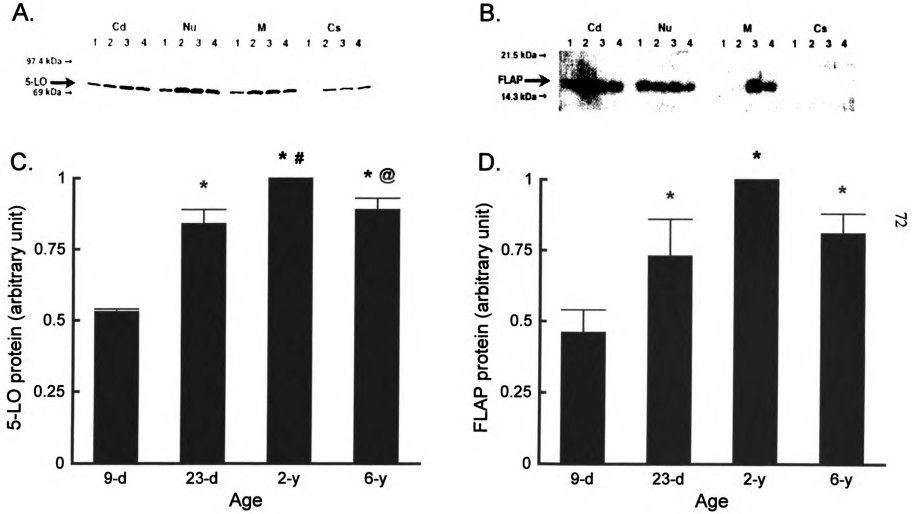

A23187 stim
Zymosan stim
No stim
Radiolabeled AA release
(% of incorporated [3H]AA)
0
5
10
15
20
9-d
23-d
2-y
6-y
Age

Figure 2-3.

Figure 2-4. Immunoblot analysis of 5-LO (~78 kD; in panels A and C) and FLAP (~18 kD; in panels B and D) in samples of crude cell lysates (Cd) and nuclear (Nu), membrane (M), and cytosol (Cs) fractions from different-aged cattle AM. Samples were prepared from overnight-cultured AM of 9-d, 23-d, 2-y, and 6-y cattle; equal amounts (~15 μg for 5-LO or ~25 μg for FLAP determination) were loaded on acrylamide gels and transferred to nitrocellulose membranes. Quantities of 5-LO and FLAP were determined by immunoblot analysis. A and B: Autoradiograph of representative experiments. Migration of molecular mass markers is indicated. Lanes 1, 2, 3, and 4 are identified as samples (Cd, Nu, M, or Cs) prepared from AM of 9-d, 23-d, 2-y, and 6-y cattle, respectively. C and D: Results of densitometric analysis of protein bands of Cd from 4 (for 5-LO) and 3 (for FLAP) independent experiments. The unit of densitometric analysis is arbitrarily defined: For either 5-LO or FLAP band density, "1" represents 2-y Cd band density, and 9-d, 23-d, and 6-y Cd densities are expressed as a percentage of 2-y Cd density. The data are expressed as mean ± SEM. * = significantly different ($p < 0.05$) from 9-d, # = significantly different ($p < 0.05$) from 23-d, and @ = significantly different ($p < 0.05$) from 2-y by paired t-test.

Figure 2-4.


A.
Cd
Nu
M
Cs
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
97.4 kDa
5-LO
69 kDa
B.
Cd
Nu
M
Cs
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
21.5 kDa
FLAP
14.3 kDa
C.
5-LO protein (arbitrary unit)
1
0.75
0.5
0.25
0
*
* #
* @
9-d
23-d
2-y
6-y
Age
D.
FLAP protein (arbitrary unit)
1
0.75
0.5
0.25
0
*
*
*
9-d
23-d
2-y
6-y
Age

CHAPTER 3

5-LIPOXYGENASE METABOLISM IN NEONATAL AND ADULT MONONUCLEAR PHAGOCYTES AND INDUCED EXPRESSION OF ALVEOLAR MACROPHAGE 5-LIPOXYGENASE BY 1,25-DIHYDROXYVITAMIN D_3 AND TRANSFORMING GROWTH FACTOR-ß

INTRODUCTION

Five-lipoxygenase (5-LO) metabolic products have important biological functions, especially in the inflammatory and immune responses: leukotriene B_4 (LTB_4), for example, not only serves as one of the most potent chemoattractants for neutrophils, but increases pulmonary vascular permeability (Dahlen et al., 1981), activates neutrophils, and modulates the functions of B- and T-lymphocytes (Rola-Pleszczynski et al., 1983a and 1983b). The enzyme 5-LO catalyzes the first two steps in the 5-LO pathway (Samuelsson, 1983) and converts arachidonic acid (AA) into LTA_4, which is soon metabolized to form various leukotrienes by LTA_4 hydrolase and LTC_4 synthase (Lewis et al., 1990). It has been shown that the production of leukotrienes from endogenous AA by 5-LO in intact cells requires a nuclear membrane protein, 5-lipoxygenase-activating protein (FLAP; Dixon et al., 1990)

Alveolar macrophages (AM), as the resident phagocytes in the alveoli and one of the first-line defenders, participate in the induction and potentiation of lung inflammation

against infection primarily via the release of cellular mediators (Sibille and Reynolds, 1990), such as the AA metabolite LTB_4. Furthermore, the abundant 5-LO intermediate LTA_4 generated by AM and neutrophils can possibly be utilized by the neighboring non-5-LO-containing cells, such as capillary endothelial cells, for the production of LTC_4, D_4, and E_4 (Feinmark and Cannon, 1986; Maclouf and Murphy, 1988), which are strong bronchoconstrictive substances and result in an augmentation of local inflammatory and immune responses (Dahlén et al., 1980). Accordingly, inadequate AM 5-LO metabolism could possibly result in an ineffective lung defense.

The induced maturation of mononuclear phagocytes with an upregulation of 5-LO metabolism has been demonstrated in a few studies using primary cultured peripheral blood monocytes (PBM) and leukemic cell lines. 1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2$-D_3) is abundant in the lung (Coffey et al., 1994a) and, with its receptor discovered on human PBM and macrophages (Provvedini et al., 1983; Bhalla etal., 1983), regulates the expression of mononuclear phagocyte maturation-related surface receptor, for example, it reduces HLA-DR and CD4 expression by monocytes (Rigby et al., 1990). $1,25(OH)_2$-D_3 has been shown to modulate the 5-LO metabolic capacity and the expression of 5-LO protein in rat AM (Coffey et al., 1994a) as well as in leukemic cell lines, such as HL-60 and Mono Mac 6 (Brungs et al., 1994 and 1995). In the presence of the multifunctional cytokine transforming growth factor-ß (TGF-ß), which is also a chemoattractant for human PBM (Wahl et al., 1987), $1,25(OH)_2$-D_3 further enhanced phagocytic ability and expression of surface antigen, such as CD14, in HL-60, U-937, and KG-1 leukemic cell lines (Morikawa et al., 1990; Testa et al., 1993). Additionally, the combined treatment dramatically augments the induction of 5-LO metabolic capacity, 5-LO protein, and 5-LO

mRNA in DMSO-differentiated HL-60 and mature monocyte-like Mono Mac 6 cells (Brungs et al., 1994 and 1995).

Previously, I have demonstrated that the AM of neonatal cattle (nAM), as compared to the adult AM (aAM), are defective in the release of 5-LO metabolites, which is parallel with the low level of 5-LO and FLAP expression. There are at least two possible explanations for the variation between nAM and aAM 5-LO and FLAP protein expression. First, the precursor cells, neonatal PBM (nPBM) and adult PBM (aPBM), may express different levels of 5-LO and FLAP proteins, i.e., the inherited differences from precursor cells and/or other age-related factors might be responsible for the subsequent difference between nAM and aAM. Second, certain lung (location-dependent) factor(s) or mononuclear phagocyte receptors, which are essential for the upregulation of the 5-LO and/or FLAP expression in PBM and/or nAM toward that in aAM, might be deficient in neonatal animals. Therefore, the primary goals of this study are to determine if a similar age-dependent change is present in the 5-LO metabolic capacity and the 5-LO and FLAP protein expression of cattle PBM, and, furthermore, by comparing PBM and AM of different ages, to specify if translocation of PBM into the lung with concomitant maturation plays an essential role. Furthermore, since the immature 5-LO metabolism of neonatal AM presumably compromises the lung defense mechanism, it would be helpful if the factor(s) that upregulate 5-LO metabolism can be discovered and, ultimately, investigated for possible clinical benefits. The next goal, accordingly, was to examine if $1,25(OH)_2D_3$ and TGF-ß induce the expression of 5-LO protein in AM of neonatal and adult cattle.

MATERIALS AND METHODS

Animals. Newborn (9 days, range 8-10 days) and adult (2 years, range 22-26 months) Holstein cattle obtained from MSU and a local dairy farm were used in this study. In order to ensure that only healthy cattle were studied, animals with apparent illness were excluded by a veterinarian on the basis of clinical examination and the neonates used were born at term. The AM or PBM obtained from these cattle were utilized for either metabolic study or protein analysis.

Preparation of PBM. Neonatal and adult PBM (nPBM and aPBM, respectively) were isolated following the procedures of Czuprynski et al. (1985) with some modifications. Four to six hundred ml of blood were obtained by jugular venipuncture and sodium citrate were used as anticoagulant with a final concentration of 0.38%. The blood was centrifuged at 300 × g for 20 min at 22°C and then the upper platelet-rich plasma layer was removed. The remnant was spun again at 850 × g for 20 min at 22°C. The white blood cell-rich buffy coat was collected in order to eliminate most of the contaminating erythrocytes, a hypotonic lysis procedure was performed on the buffy coat cells by adding 5 × volume of distilled water for 8~10 sec and, subsequently, the same volume of 2 × concentrated phosphate-buffered saline (2 × PBS) solution to restore the osmolarity. The unconcentrated PBS included dextrose 10 mM, N-[hydroxyethyl]-piperazine-N'-[2-ethanesulfonic acid] (HEPES; Sigma, St. Louis, MO) 10 mM, and ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetatic acid (EGTA; Sigma) 0.2 mM. The mixture was centrifuged at 250 × g for 10 min at 22°C. The pellet was resuspended with Hanks balanced salt solution (HBSS), the resulting fluid was underlaid with Ficoll-Hypaque (specific density = 1.077; Pharmacia Laboratory Separation, Piscataway, NJ) , and spun at

450 × g for 30 min at 22°C. The mononuclear cell layer at the gradient interface was carefully collected and washed three times with cold HBSS and centrifuged at 250 × g for 8 min at 4°C. The final pellet was resuspended in RPMI 1640 (Sigma) containing 0.5% fetal bovine serum (FBS; Hyclone, Logan, VT) at the density of 4×10^6 cells/ml and cells were plated onto 35-mm and 100-mm polystyrene culture dishes at the desired density (8×10^6 cells/2 ml and 6×10^7 cells/15 ml, respectively) and incubated at 37°C in humidified air with 5% CO_2. In all experiments, the RPMI 1640 contained 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Antibiotic-Antimycotic Solution; Sigma) and the FBS had been chemically inactivated before it was used (Bahnemann, 1976). After ~1.5 hr, non-adherent cells were removed by washing twice with warm Hank's balanced salt solution (HBSS; Gibco, Giand Island, NY), and the attached cells were incubated in RPMI 1640 with 10% FBS. The PBM purity was determined by Giemsa and verified by non-specific esterase stains and viability was confirmed by trypan blue exclusion after 16 hr of culture. In all experiments described below, the PBM monolayer purity was greater than 90% and the viability was above 90%.

Preparation of AM. Neonatal and adult AM (nAM and aAM, respectively) were obtained by bronchoalveolar lavage (BAL) as described previously (Chapter 2) . Briefly, the tube, 7-mm- or 1-cm-outer-diameter for 9-d or 2-y cattle, respectively, was inserted through the nose without sedation of the animal and was wedged in a subsegmental bronchus. About 500 ml PBS were utilized as lavage fluid and instilled in aliquots of 50 ml through the lumen of the tube. The lavage fluid was then gently aspirated with a syringe, collected into a sterile glass bottle, and transported on ice to the laboratory within one hour for cell isolation and culture.

The lavage fluid was centrifuged at 250 × g for 10 min at 4°C. The pellet was resuspended in HBSS and subsequently filtered through a sterile 100-μm mesh filter (Tetko, Briarcliff Manor, NY). The cells were spun again at 250 × g for 8 min at 10°C and resuspended in serum-free RPMI 1640. Cell density was adjusted to 0.75×10^6 cells/ml, and 1.5×10^6 cells/2 ml were put into a 35-mm culture dish for AA metabolic experiments. For immunoblot experiments, 1.5×10^7 cells in 20 ml medium were plated onto a 100-mm culture dish (Corning). To allow AM adherence, cells were incubated for 1 hr at 37°C in humidified air with 5% CO_2. The non-adherent cells were removed by gently washing twice with warm HBSS. The attached monolayer cells were next incubated in RPMI 1640 with 10% FBS. After overnight culture, the AM purity was greater than 95% as determined by Giemsa and verified by non-specific esterase stains and the viability was above 90% as confirmed by trypan blue exclusion.

AM and PBM culture and AM incubation with TGF-ß$_1$ and 1,25(OH)$_2$-D$_3$. For all experiments, AM and PBM were cultured in RPMI 1640 containing 10% FBS at 37°C in humidified air with 5% CO_2. Both types of Day 1 (D1) cells had been incubated in medium without any additives for 17 hr before AA metabolic capacity and the expression of 5-LO an FLAP proteins were determined.

The Day 5 (D5) AM had been cultured in FBS-containing medium with or without other additives for 120 hr before cells were collected for the determination of 5-LO protein expression. Four D5 treatment groups were used, which included cells cultured 1) in plain FBS-containing medium as a control, 2) in medium with TGF-ß$_1$ and 1,25(OH)$_2$-D$_3$, 3) in medium with TGF-ß$_1$ alone, and 4) in medium with 1,25(OH)$_2$-D$_3$ alone. TGF-ß$_1$ was obtained from Genezyme (Lot B50044, activity > 1,000 unit/μg; Cambridge, MA)

and stocked as 1 μg/ml in RPMI containing 10% FBS in -20°C. 1,25$(OH)_2$-D_3 was from ICN Biochemicals Inc. (Lot 71447; Aurora, OH) and was reconstituted in ethanol and stored as 100 μM stock solution in -20°C. Before adding to the medium, the stock TGF-$ß_1$ and 1,25$(OH)_2$-D_3 solutions were diluted to a final concentration of 1 ng/ml and 50 nM (a final ethanol concentration of 0.05%), respectively. These inducer concentrations were selected because of their documented capability to cause differentiation and to upregulate 5-LO protein expression in monocyte/macrophage cell lines, including HL-60 and Mono Mac 6 (Brungs et al., 1994 and 1995).

Analysis of cellular AA metabolic capacity. In order to measure AA metabolic capacity in both D1 AM and D1 PBM, cell membrane phospholipids were prelabeled with [^{3}H]AA for 16 hr. The labeling procedures were described previously (Chapter 2) with some modification. Briefly, 2 μCi of [^{3}H]AA (sp act 76 to 100 Ci/mmol, Dupont-New England Nuclear, Boston, MA) was added to each 35-mm culture dish containing 2.5-ml medium, and after overnight culture, unincorporated label was removed by washing monolayers three times with HBSS. The cells were then incubated for an additional hour in RPMI 1640 containing 10% FBS. Immediately before A23187 stimulation, cells were again washed three times with HBSS. At this moment, the cells in one dish were scraped off into 2 ml of methanol and mixed with 10 ml of Safety-Solve scintillation fluid (Research Products International Corp., Mount Prospect, IL) and the radioactivity uptake per dish was quantified by a liquid scintillation counter (LS 6000TA, Beckman Instruments, Fullerton, CA).

Stimulation of PBM or AM arachidonate metabolite release was induced by incubating cells of a 35-mm dish in 3 ml of Dulbecco's minimal essential medium

(DMEM; Sigma) containing 10 μM A23187 for 30 min. A23187 (Sigma) stock solution was prepared in dimethylsulfoxide (DMSO; Sigma) and stored at -20°C. Immediately before the use of A23187, stock was diluted in medium to a final concentration of 10 μM (0.5% DMSO). Because the uptake of [^{3}H]AA per dish of AM was almost twice that of PBM (data shown in Result section), for the metabolic experiments, in contrast to the medium from a dish of AM used, that from two dishes of PBM stimulated with ionophore was collected for eicosanoid analysis. In a preliminary experiment, the optimal concentrations of A23187 stimulation were determined using 0.1, 1, 5, or 10 μM A23187 for nPBM and aPBM, and 1, 5, 10, or 20 μM for nAM and aAM. With 30 min of stimulation, the release of [^{3}H]AA reached a plateau at 1 μM for nPBM and aPBM and at 10 μM for nAM and aAM. Therefore, in all experiments, a concentration of 10 μM A23187 was used to activate PBM and AM AA metabolism.

Eicosanoid extraction and RP-HPLC analysis of AA metabolites. Radiolabeled AA and eicosanoids released into culture medium upon agonist stimulation were extracted and analyzed using the methods described previously (Chapter 2) with some modification. Briefly, at the end of stimulation, culture media from one or two dishes of AM or PBM, respectively, were collected and admixed with 2 × volume of methanol, and the mixture was centrifuged at 480 × g for 5 min at 4°C. The resultant supernatant was decanted into 0.1 M sodium phosphate buffer (pH = 7.4) to reach a 20% final methanol concentration. This aqueous suspension was instilled into a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) prewashed with methanol followed by water. The eicosanoid-bound cartridge was washed with 20% methanol in 0.1 M sodium phosphate buffer followed by water, and was then eluted with 3 ml of 80% of methanol in water.

The eluted sample was evaporated under nitrogen and the dry residue was stored at -70°C until analyzed.

Immediately before being injected into HPLC for analysis, the dry lipid extract was dissolved in 400 microliters of acetonitrile/water/trifluoroacetic acid (33:67:0.1, v/v/v) and filtered through a 0.45 μm CR Acrodisc 13 PTFE (Gelman Sciences, Ann Arbor, MI). Two hundred microliters were injected into the HPLC (Perkin Elmer, Norwalk, CT) and the eicosanoids were separated by a 5 μm μBondapak C_{18} column (30×0.4 cm; Waters Associates). The mobile phase was pumped at a flow rate of 1 ml/min and was composed of varying ratios of acetonitrile, deionized water, and trifluoroacetic acid, all of which had been filtered through 0.2 μm nylon filter membranes (Gelman Sciences) prior to use. During a 105-min run, CO metabolites were first eluted during an initial isocratic phase (33:67:0.1, v/v/v), and 5-LO metabolites and free AA were eluted during a subsequent stepwise gradient increase of acetonitrile to 100:0:0.1 (v/v/v). Eicosanoids were detected and quantified with an on-line radiodetector (FLO-ONE/Beta; Radiomatic, Meiden, Conn.) in which a scintillation fluid pump mixed Safety-Solve scintillation fluid with the elute at a flow rate of 3 ml/min, and a liquid scintillation spectrophotometer continuously monitored the radioactive eicosanoids. In addition, standards of 6-keto-$PGE_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, LTB_4, LTC_4, LTD_4, 12-hydroxyheptadecatrienoic acid (12-HHT), 15-HETE, 12-HETE, 5-HETE, and free AA (Cayman Chemical Company, Ann Arbor, MI) were utilized to determine the elution time of individual eicosanoids by a UV detector (Perkin Elmer). The UV absorbance was 210 nm for CO metabolites and free AA, 280 nm for LTs, and 235 nm for monohydroxyeicosatetraenoic acids (mono-HETEs).

SDS-PAGE and immunoblot analysis of total cellular 5-LO and FLAP contents. In order to compare 1) 5-LO and FLAP contents among D1 PBM and D1 AM of neonatal and adult cattle, after 17 hr of incubation and 2) 5-LO amount among four treatment groups of D5 AM after 120 hr of culture, cell monolayers were washed with HBSS and were scraped off the culture dish with a rubber policeman into a small volume of ice-cold TKM buffer, to which antiproteases, including diothiothreitol (DTT) 160 μg/ml, phenylmethylsulfonyl fluoride (PMSF) 1 mM, leupeptin 1 μg/ml, and soybean trypsin inhibitor (SBTI) 60 μg/ml had been freshly added. The TKM buffer included Tris-HCl 50 mM (pH = 7.4), KCl 25 mM and $MgCl_2$ 5 mM. The cell suspension was sonicated for a total of 45 sec in Cell Disruptor (power level 7 and 50% duty cycle; model W-225R, Heat System-Ultrasonic, Inc., Plainview, NY), aliquoted, and stored at -70°C. The protein concentrations in the sample quantified by Bradford method using bovine serum albumin as standard.

Equal quantities of proteins (~25 μg per lane) from crude cell lysate of D1 or D5 cells from different-aged cattle were mixed with sample buffer and were loaded on 12.5% acrylamide gels by a method previously described (Coffey et al., 1992 and Chapter 2) for immunoblot analysis. Along with the samples, Rainbow molecular weight markers (Amersham Corp., Arlington Heights, IL) were loaded to follow protein migration and transfer. Proteins were then transferred to nitrocellulose membranes overnight using a Bio-Rad Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA). The membranes were then blocked with 5% non-fat dry milk in Tris buffered saline solution (TBS) for 1 hr and washed in TBS containing 0.1% Tween 20 (TBS-T). Steady-state quantities of 5-LO and FLAP proteins were determined by immunoblotting the membrane for 1 hr with rabbit

polyclonal antisera (kindly provided by Dr. J. Evans, Merck Frosst Centre for Therapeutic Research, Claire-Dorval, Quebec, Canada) raised against either human leukocyte 5-LO (1:3000 diluted in TBS-T), or amino acid residues 41-52 of the human FLAP sequence (1:5000 dilution). Again, blots were washed in TBS-T and were incubated for another hr with horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 diluted in TBS-T; Amersham). Finally the washed membrane was reacted with the ECL chemiluminescent Western blot system (Amersham), and exposed to hyperfilm (Amersham). Band densities of the samples on ECL films, which reflect the relative amount of desired protein, were quantified by Osiris imaging software (University Hospital of Geneva, Geneva, Switzerland) analysis of prints developed from ECL films.

Data and statistical analysis. The data from metabolic experiments and immunoblot analysis are expressed, where applicable, as mean ± standard error (SEM). For the metabolic study, the data means of D1 PBM and D1 AM from different-aged animals were compared by one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (hsd) post-hoc test. The evaluation of densitometric bands among D1 PBM and D1 AM or D1 and differentially treated D5 AM of different age groups used the paired *t*-test. In all comparisons, a p value < 0.05 was considered significant.

RESULTS

5-LO and CO metabolic capacities of PBM and AM from neonate and adult cattle. To investigate if a similar age-dependent alteration in AA metabolism is present in AM and their precursors, PBM, AA metabolites were assayed in the 9-d neonatal and the 2-y adult PBM as well as AM. After overnight culture, D1 cells were stimulated with A23187, and

the 5-LO and CO metabolic capacities were assessed. Just before agonist stimulation, another dish of cells from 9-d and 2-y cattle was assessed for the uptake of [^{3}H]AA and the data was expressed as mean ± SEM; the result for PBM was 463,482 ± 71,478 and 475,089 ± 53,028 cpm/dish, respectively (p = NS, n = 4 or 5), and that for AM was 964,731 ± 57,974 and 810,435 ± 150,951 cpm/dish (p = NS, n = 4 in each age group), respectively, for 9-d and 2-y cattle. The representative AA metabolic profiles are displayed in Figure 3-1. 5-HETE and LTB_4 were the principal AA products released by aAM (Figure 3-1D) and nAM produced a much smaller amount of 5-LO metabolites and a relatively larger quantity of prostanoids (Figure 3-C) as compared to aAM. On the other hand, 12-HETE was the main metabolite released by nPBM and aPBM (Figure 3-1A and B). The result of quantitative comparison is shown in Figure 3-2: the 5-LO metabolic capacity of aAM was ~3.5-fold that of nAM and ~18- and ~36-fold that of aPBM and nPBM, respectively (Figure 3-2A). As for the CO metabolites, nPBM, aPBM, and aAM released similar amounts, which are ~50-60% that of nAM (Figure 3-2B). Table 3-1 further demonstrates the cellular ability to release 12-HETE, in which nPBM and aPBM were alike and were ~15-fold and ~40-fold higher than nAM and aAM, respectively. The predominant release of 12-HETE products by aPBM of cattle is also observed in other species, including humans and rats (Balter et al., 1989; Peters-Golden et al., 1990).

Immunoblot analysis of 5-LO and FLAP protein expression in PBM and AM of the neonate and the adult. The steady-state expression of two proteins, 5-LO and FLAP, that are crucial for cellular 5-LO metabolism was next examined on crude lysates of nPBM, aPBM, nAM, and aAM. The polyclonal antisera specific for 5-LO and for FLAP have been proven useful in cattle (Chapter 2). As can be seen in Figure 3-3A, the amounts of 5-LO and

FLAP expressed were the highest in aAM, and moderate expression of both proteins was seen in nAM. While very little 5-LO protein was present in nPBM and aPBM, the FLAP expression of both cells was higher than that of nAM. Densitometric analysis of the protein bands from several experiments showed that the expression of 5-LO in aAM was ~2.5-fold that in nAM and more than 100-fold that of either nPBM or aPBM (Figure 3-4C). For the FLAP expression, aAM, aPBM, and nPBM were ~2.8-, ~2.4-, and ~1.8-fold higher than nAM, respectively (Figure 3-4C).

5-LO and FLAP protein expression in 1,25(OH)$_2$-D$_3$- and TGF-ß$_1$-treated AM. The induction of 5-LO protein expression in nAM and aAM was studied using 1,25(OH)$_2$-D$_3$ and TGF-ß$_1$ with a concentration (50 μM and 1 ng/ml, respectively) that was reported to upregulate 5-LO protein expression in HL-60 and Mono Mac 6 leukemic cell lines (Brungs, et al., 1994 and 1995). As demonstrated in Figure 3-4A, the expression of 5-LO protein in both nAM and aAM decreased with time in culture medium, and the magnitude of decrement was greater in nAM than in aAM. After 5 days of culture, nAM and aAM expressed 5-LO protein approximately 0.29 ± 0.18 and 0.73 ± 0.17, respectively, the amount found in D1 cells (Figure 3-4B and C). After incubation with 1,25(OH)$_2$-D$_3$, TGF-ß$_1$, or 1,25(OH)$_2$-D$_3$ and TGF-ß$_1$ for 5 days, the 5-LO protein expression increased. Combined 1,25(OH)$_2$-D$_3$ and TGF-ß$_1$ treatment for 5 days resulted in a 2.0- and 1.5-fold increase in 5-LO expression for nAM and aAM, respectively, as compared to the controlled cells; this effect was more prominent than that of an individual inducing agent (Figure 3-4D and E), and the expression of 5-LO protein in D5 aAM reached the D1 level, but that in D5 nAM only achieved about half of their D1 value.

DISCUSSION

Previously, I determined the juvenile and adult patterns of AM AA metabolism and demonstrated that neonatal cattle AM possess a lower 5-LO metabolic capacity than do adult AM, which is accompanied by a comparable difference in the expression of 5-LO and FLAP protein (Chapter 2). Since these age-related changes between nAM and aAM might derive from the intrinsic difference between nPBM and aPBM, in the present study I have compared the neonatal and adult cattle PBM as well as AM for their endogenous AA metabolic capacity and steady-state expression of 5-LO and FLAP protein expression. Several distinctive findings were discovered. First, the 5-LO metabolic capacity of mononuclear phagocytes was dependent not only on the age of the animals, but also, for both neonates and adults, on the location of the cells, i.e., PBM in the circulation vs. AM in the alveoli. Second, although, for the 5-LO protein expression of cattle PBM as well as AM, age-dependent increases (~1.9- and ~3.5-fold, respectively) between 9-d and 2-y animals were observed, the most striking difference was that, in both neonatal and adult cattle, the 5-LO protein expression in PBM was less than 1% that in AM. This low 5-LO protein expression presumably contributes to the very low 5-LO metabolic capacity in PBM. Third, the increase of FLAP expression of PBM and AM was age-dependent, about 1.5- and 2.8-fold, respectively, higher in adult cattle than in neonates. The aAM also demonstrated a small (1.2-fold) increase of FLAP expression over aPBM. On the other hand, nAM expressed only about 0.6-fold amount of FLAP protein of nPBM, and are the least effective cells among all these four cells in terms of FLAP protein expression. Last of all, combined treatment with TGF-$ß_1$ and $1,25(OH)_2$-D_3 for 5 days resulted in a prominent increase in the expression of 5-LO protein. Seeing that

the 5-LO protein expression decreased with time in culture, it is possible that TGF-$ß_1$ and $1,25(OH)_2$-D_3 are important lung cellular mediators to maintain the constitutive expression of 5-LO in neonatal as well as adult AM.

The higher A23187-stimulated 5-LO metabolic capacity (~4-fold) and 5-LO (~2 fold) and FLAP (~2-fold) protein expression have been consistently demonstrated in cattle aAM (Chapter 2 and this study) as compared to those in 9-d nAM. In this study, the examination of AA metabolism in the precursor PBM of the neonate and adult showed that the ability to release arachidonate metabolites, mainly 12-HETE and prostanoids, was fairly similar among different ages, although ~1.9-fold greater 5-LO capacity was observed in aPBM ($p > 0.05$). This shows that the age-related 5-LO phenomenon is more obvious in the phagocytes of the alveoli than in the circulating PBM, suggesting a crucial role of the lung milieu for the regulation of 5-LO metabolism. Furthermore, in adult cattle, AM were shown to have ~18-fold greater 5-LO metabolic capacity than their precursor cells, PBM, this value is quantitatively similar to those reported in humans and rats (Balter et al., 1989; Peters-Golden et al., 1990; Pueringer et al., 1992; Coffey et al., 1994). Similar comparison in the neonate demonstrated that ~10-fold more 5-LO capacity was present in nAM than in nPBM. This dramatic acquisition of 5-LO metabolic capacity presumably occurs as PBM are recruited into the lung and interact with lung cells and cell mediators, and the more significant increase in the adult cells implies that the adult lung microenvironment and/or the phagocytes are more effective in the induction of AM 5-LO metabolism.

In intact cells, both 5-LO and FLAP proteins are essential for converting endogenously released AA into 5-HpETE and LTA_4 for further production of various

biologically active leukotrienes (Dixon et al., 1990). Despite the fact that the ratio of AM/PBM 5-LO metabolic capacity in adult animals is similar in several species, the protein that is presumably critical for the difference between PBM and AM 5-LO capacity is likely different: This study showed that 5-LO (>100-fold upregulation in this study) protein is important in cattle AM, whereas Coffey et al. demonstrated that FLAP seems to be the key protein in human AM (~40-fold; 1994). The reason for the difference between these two species is not clear. Furthermore, in neonatal cattle, the 5-LO protein expression in nPBM was less than 1% that in nAM as well, which is possibly a major contributor to the very low 5-LO metabolic capacity of cattle nPBM. However, the 5-LO protein expression in cattle PBM, unlike that in AM, did not differ significantly between 9-d and 2-y animals.

The expression of FLAP protein, similarly, increased age-dependently in AM (2.8-fold greater in aAM than in nAM) as well as in PBM (1.5-fold greater in aPBM than in nPBM), and the magnitude of increase is comparable to the concomitant increase in 5-LO protein (~3.5- and ~1.9-fold, respectively) of AM and PBM. However, in contrast to the relatively higher level of FLAP content per unit of total cellular protein (1.2-fold; $p > 0.05$) in aAM than in aPBM, the amount in nAM was only 60% that of nPBM ($p > 0.05$); in fact, the level of FLAP expression in nAM was the lowest among the four mononuclear phagocytes I examined. This failure of the FLAP protein upregulation of nAM in neonatal lung presumably contributes to the low 5-LO metabolic capacity of nAM as well.

The 5-LO protein is constitutively expressed in mononuclear phagocytes, and it is clear from this study that the increase in age as well as the differentiation from nPBM to

nAM resulted in an upregulation of 5-LO protein expression and 5-LO metabolic capacity. This upregulation occurs primarily in the lung (Peters-Golden et al., 1990; this study). On the other hand, in vitro prolonged culture results in a time-dependent decline of AM leukotriene synthesis (Sporn et al., 1990), for which the decreased expression of 5-LO protein demonstrated in this study is likely an important reason.

It has been shown that $1,25(OH)_2$-D_3 and TGF-ß induce differentiation and growth of mononuclear phagocytes (Kreutz and Andreesen, 1990) and leukemic cell lines, such as HL-60 and Mono-Mac 6 (Provvedini et al., 1983; Bhalla et al., 1983). Individually, $1,25(OH)_2$-D_3 and TGF-ß have been shown to upregulate the expression of 5-LO protein in HL-60 and Mono Mac 6 cells, and a synergistic effect, which plateaus at day 2 to 3 of culture, has been observed when both agents were given simultaneously (Brungs et al., 1994 and 1994). In cattle nAM as well as aAM, a combined treatment with $1,25(OH)_2$-D_3 and TGF-$ß_1$ for 5 days resulted in a consistent increase in the expression of 5-LO protein as compared to the control group, and this effect was also more prominent than that induced by either agent alone. In this study, the amount of 5-LO protein expressed in aAM after 5 days of induction reached that in D1 aAM, however, the quantity of 5-LO expression in D5 nAM only approached ~50% that in D1 nAM. This difference might be due to an inadequate response of immature nAM to $1,25(OH)_2$-D_3 and/or TGF-$ß_1$ or an accelerated degradation of 5-LO in nAM. On the other hand, the effect of $1,25(OH)_2$-D_3 and TGF-$ß_1$ on the primary cultured AM is not as dramatic as that on the leukemic cell lines, and the combined treatment of AM with both inducers did not enhance the AM 5-LO metabolic capacity after 5 days of incubation (preliminary data not shown). Since the transformed cells might behave differently from normal cells and the developmental

stages of the cell lines are probably not comparable with those of the in vivo cells (Rutherford et al., 1993), this result is not inconceivable. Furthermore, taking into consideration that both $1,25(OH)_2$-D_3 and TGF-ß are released as autacoids by several types of in situ lung cells, including AM (Nathan, 1987; McCartney-Francis and Wahl, 1994), and the concentration of $1,25(OH)_2$-D_3 was higher in the alveoli than in the plasma (Coffey et al., 1994), these two inducers are possibly important lung cellular mediators for the maintenance of the constitutive expression of 5-LO protein in neonatal as well as adult AM, though other factors might be required for 5-LO to be fully expressed.

In summary, the age-dependent increases of 5-LO metabolic capacity and 5-LO protein expression in AM of neonatal as well as adult cattle are mostly related to the maturation of mononuclear phagocytes in the lung. $1,25(OH)_2$-D_3 and TGF-ß, when administered simultaneously, resulted in a prominent upregulation of nAM and aAM 5-LO protein expression, and both are likely important lung factors for 5-LO metabolism.

Table 3-1. Distribution of radiolabeled AA metabolites released by different-aged cattle PBM and AM stimulated with A23187[a]

	Percent of eluted radioactivity			
AA Metabolites	nPBM	aPBM	nAM	aAM
total CO products[b]	10.44 ± 1.33%	8.87 ± 1.71%	17.81 ± 2.37%	9.59 ± 0.36%
total 5-LO products[b]	1.12 ± 0.16%	2.17 ± 0.83%	11.71 ± 4.88%	40.43 ± 2.78%
12-HETE[c]	19.47 ± 2.69%	25.78 ± 7.88%	1.55 ± 0.20%	0.56 ± 0.11%
15-HETE[c]	0.05 ± 0.01%	0.09 ± 0.02%	0.15 ± 0.05%	0.08 ± 0.02%
free AA	68.45 ± 2.83% [*,#]	62.28 ± 5.55% [*,#]	67.48 ± 7.56%	48.59 ± 2.56%

[a] [^{3}H]AA-prelabeled AM were stimulated with A23187 and the released eicosanoids were extracted from medium and separated by HPLC. The products of various cyclooxygenase and lipoxygenase pathways have been calculated and the results are expressed as the percentage of total radioactivity eluted by HPLC. The data are mean ± SEM from 3 or 5 cattle in each age group.

[b] The total metabolic capacities of CO (metabolites include 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TxB_2, PGE_2, PGD_2, and 12-HHT) and 5-LO (metabolites include LTB_4, LTC_4, LTD_4, and 5-HETE) are illustrated in Figure 3-2B and 3-2A, respectively.

[c] Data means for 12-HETE and 15-HETE were compared among PBM and AM of different-aged cattle by ANOVA with Tukey's hsd post-hoc test ([*] = significantly different ($p < 0.05$) from nAM; [#] = significantly different ($p < 0.05$) from aAM).

Figure 3-1. Representative HPLC profiles of eluted radioactivity from prelabeled 9-d PBM (in panel A) and AM (in panel C) and 2-y PBM (in panel B) and AM (in panel D). Prelabeled cells were incubated with 10 μM A23187 and the released eicosanoids were extracted from medium and separated by HPLC. Radioactivity was detected by an on-line radiodetector and is expressed as counts per 30 sec. The retention times of authentic standandards are shown in the chromatogram.

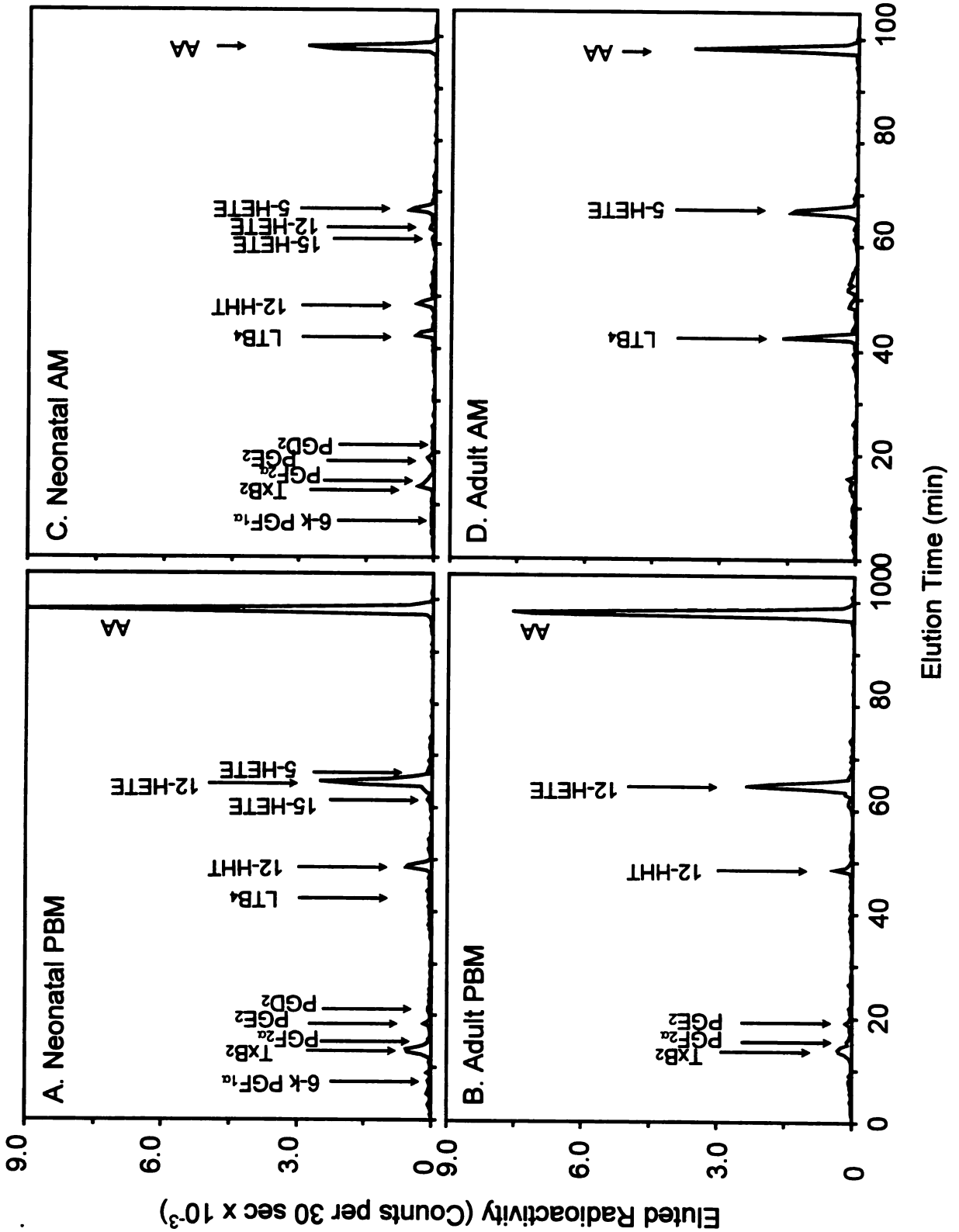

A. Neonatal PBM
B. Adult PBM
C. Neonatal AM
D. Adult AM
AA
6-k PGF1a
TxB2
PGF2a
PGE2
PGD2
LTB4
12-HHT
15-HETE
12-HETE
5-HETE
Eluted Radioactivity (Counts per 30 sec x 10-3)
Elution Time (min)
0
3.0
6.0
9.0
20
40
60
80
100

Figure 3-1.

Figure 3-2. Release of 5-LO (in panel A) and CO (in panel B) metabolic products by different-aged cattle PBM and AM stimulated with A23187. Prelabeled AM were incubated with 10 μM A23187 for 30 min, and eicosanoids were extracted from medium and analyzed by HPLC with on-line radiodetection. Data are expressed as a percentage of the total released radioactivity. The results represent the mean ± SEM (n = 3 or 5 in each age group). The data means of different-aged animals were compared by ANOVA with Tukey's hsd post-hoc test (* = significantly different ($p < 0.05$) from nPBM; # = significantly different ($p < 0.05$) from aPBM); @ = significantly different ($p < 0.05$) from nAM); $ = significantly different ($p < 0.05$) from aAM).

Figure 3-2.

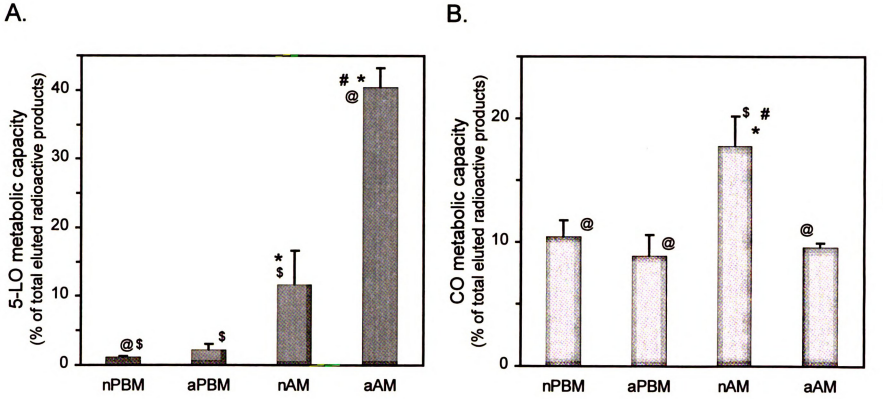

A.
5-LO metabolic capacity
(% of total eluted radioactive products)
0
10
20
30
40
@ $
$
* $
# * @
nPBM
aPBM
nAM
aAM
B.
CO metabolic capacity
(% of total eluted radioactive products)
0
10
20
@
@
$ # *
@
nPBM
aPBM
nAM
aAM

Figure 3-3. Immunoblot analysis of 5-LO (~78 kD; in panels A and C) and FLAP (~18 kD; in panels B and D) in samples of crude cell lysates (Cd) from neonatal and adult cattle PBM (nPBM and aPBM, respectively) and AM (nAM and aAM, respectively). Samples were prepared from overnight-cultured cells of 9-d and 2-y cattle; equal amounts (~25 μg for 5-LO and FLAP determination) were loaded on acrylamide gels and transferred to nitrocellulose membranes. Quantities of 5-LO and FLAP were determined by immunoblot analysis. A and B: Autoradiograph of representative experiments. Migration of molecular mass markers is indicated. Results of densitometric analysis of protein bands of Cd from 5 (for 5-LO) and 4 (for FLAP) independent experiments. The unit of densitometric analysis is arbitrarily defined: For either 5-LO or FLAP band density, "1" represents 2-y Cd band density, and 9-d, 23-d, and 6-y Cd densities are expressed as a percentage of aAM Cd density. The data are expressed as mean ± SEM. * = significantly different ($p < 0.05$) from nPBM, # = significantly different ($p < 0.05$) from aPBM; @ = significantly different ($p < 0.05$) from nAM; $ = significantly different ($p < 0.05$) from aAM.

Figure 3-3.

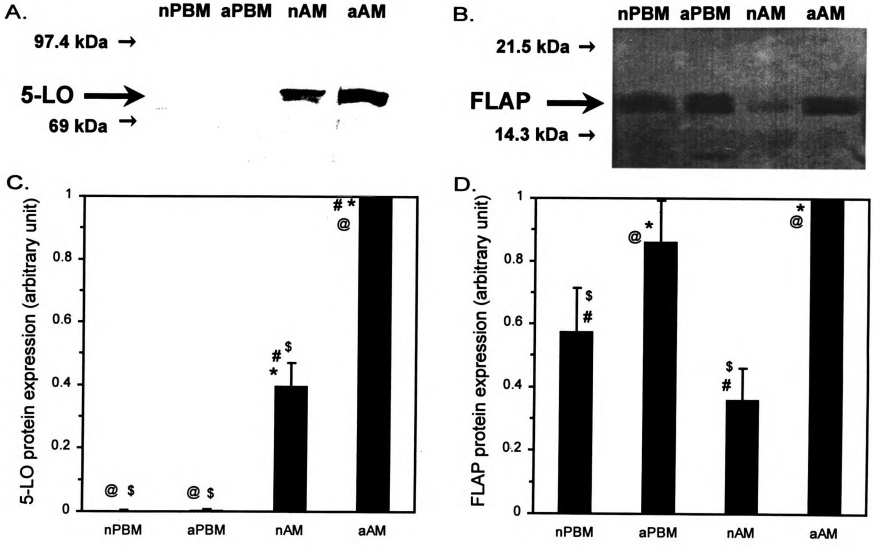
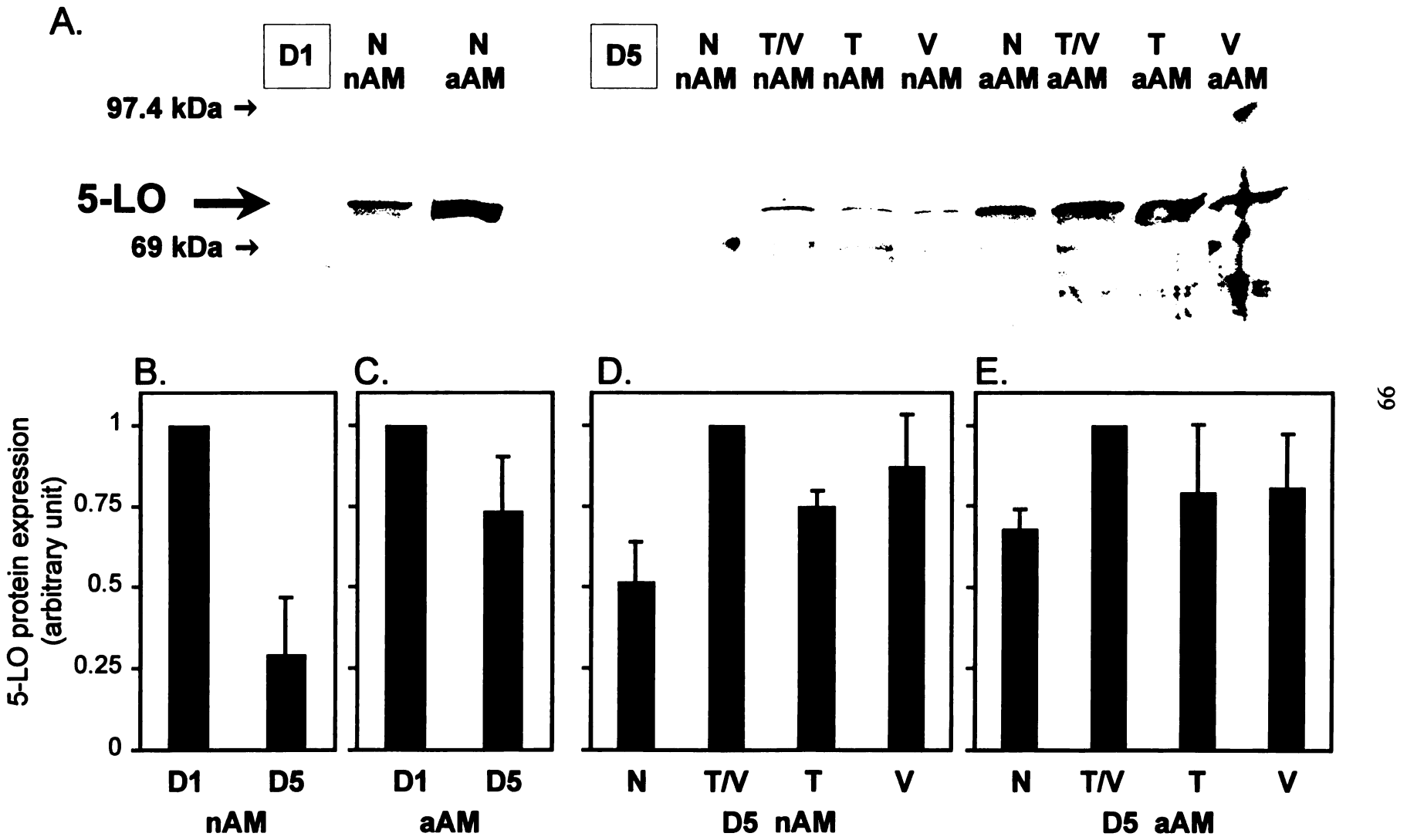

A.
nPBM aPBM nAM aAM
97.4 kDa →
5-LO →
69 kDa →
B.
nPBM aPBM nAM aAM
21.5 kDa →
FLAP →
14.3 kDa →
C.
5-LO protein expression (arbitrary unit)
1
0.8
0.6
0.4
0.2
0
@ $
@ $
#*
*
#$
*
@
nPBM
aPBM
nAM
aAM
D.
FLAP protein expression (arbitrary unit)
1
0.8
0.6
0.4
0.2
0
$
#
@*
*
$
#
*
@
nPBM
aPBM
nAM
aAM

Figure 3-4. Immunoblot analysis of 5-LO in crude cell lysates (Cd) of cultured Day 1 (D1) and Day 5 (D5) nAM (in panels A, B, and C) and aAM (in panels A, D, and E). Samples were prepared from D1 and D5 AM of 9-d and 2-y cattle; equal amounts (~25 μg) were loaded on acrylamide gels for 5-LO immunoblot analysis. A: Autoradiograph of representative experiments. Migration of molecular mass markers is indicated. Left two lanes are D1 nAM and aAM samples and right eight lanes are D5 nAM and aAM control (N), combined $1,25(OH)_2D_3$ and TGF-ß-treated (T/V), $1,25(OH)_2D_3$ (V)-treated, and TGF-ß (T)-treated groups. B, C, D and E: Results of densitometric analysis of protein bands of Cd from 2 independent experiments. The unit of densitometric analysis is arbitrarily defined: For 5-LO band density, "1" represents band density of D1 nAM (panel B), D1 aAM (panel C), D5 T/V-treated nAM (panel D), or D5 T/V-treated aAM (panel E). Other densities in each panel are expressed as a percentage of their respective standard density (i.e., "1").

Figure 3-4.


A.
D1
N
nAM
N
aAM
D5
N
nAM
T/V
nAM
T
nAM
V
nAM
N
aAM
T/V
aAM
T
aAM
V
aAM
97.4 kDa →
5-LO →
69 kDa →
B.
C.
D.
E.
5-LO protein expression (arbitrary unit)
1
0.75
0.5
0.25
0
D1
D5
nAM
D1
D5
aAM
N
T/V
T
V
D5 nAM
N
T/V
T
V
D5 aAM

CHAPTER 4

SUMMARY AND CONCLUSIONS

On the basis of my studies, several conclusions concerning the maturation of 5-LO metabolism in AM and PBM of neonatal and adult cattle have been reached. First, two patterns of AM arachidonate metabolism have been discovered, that is: 1) "Juvenile pattern," which is characterized by low 5-LO and high CO metabolic capacity, and 2) "adult pattern," which is characterized by high 5-LO and low CO metabolic capacity. Failure of AM from neonatal cattle to release significant quantities of leukotrienes may result in insufficient lung defense and may explain, at least in part, the high incidence of neonatal pneumonia.

Second, the age-related enhancement of AM 5-LO metabolism by receptor-independent ionophore stimulation is paralleled by, and presumably due to, the increase in the expression of 5-LO and FLAP protein. The even more prominent reduction in receptor-mediated 5-LO capacity of neonatal AM suggests that these cells have additional defect(s) in their intracellular signaling pathway(s).

Third, unlike AM, neonatal and adult PBM had a similar AA metabolic pattern and only minor differences in 5-LO capacity and 5-LO and FLAP protein expression were observed.

Fourth, the maturation of AM from PBM, in both neonates and adults, is accompanied by a remarkable upregulation of 5-LO metabolic capacity and a striking increase of 5-LO protein expression. However, for the FLAP protein expression, only a small increase in adult cattle and a decrease in the neonates were recognized. Furthermore, the magnitude of the aforementioned increase in 5-LO metabolic capacity and 5-LO protein expression was always more significant in the adult than in the neonate. These imply that 5-LO protein is probably the determinant for the upregulation of cattle PBM to AM 5-LO metabolism.

Last of all, a combined treatment with $1,25(OH)_2$-D_3 and TGF-$ß_1$ resulted in an increase in the expression of neonatal and adult AM 5-LO protein, which is more prominent than the effect by individual inducer alone. Although the induction by a combined treatment of both inducers upregulates the amount of D5 5-LO protein expression to the D1 level in adult AM but not in neonatal AM, it is likely that $1,25(OH)_2$-D_3 and TGF-$ß_1$ are important lung cellular mediators that maintain the constitutive expression of AM 5-LO protein.

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