CHARACTERIZATION AND FUNCTIONAL STUDY OF IONOTROPIC GABA RECEPTORS IN ALVEOLAR EPITHELIAL CELLS

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AD	Adult
AEC	Alveolar epithelial cells
AG	Attached collagen gel
AFC	Alveolar fluid clearance
AL	Air-liquid interface
ANOVA	Analysis of variance
ASF	Airway surface fluid
ASL	Alveolar surface liquid
AVSF	Alveolar subphase fluid
CaCC	Calcium activated chloride channel
CFTR	Cystic fibrosis transport regulator
CF	Cystic fibrosis
Ci	Curi
CMV	Cytomegalovirus
CNS	Central neuron system
Ct	Threshold cycle
DG	Detached collagen gel
DMEM	Dulbecco's Modified Eagle Medium
ENaC	Epithelial sodium channel
EHS	Engelbreth-Holm-Swarm
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GA	Glutaradehyde
GABA	γ-aminobutyric acid
GABRP	$GABA_A$ receptor π subunit
GAD	Glutamate decarboxylase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HRP	Horseradish peroxidase
IP	Immunoprecipitation
KCC	Potassium chloride co-transporter
KGF	Keratinocyto growth factor
LL	Liquid-liquid interface
LSD	Least significance difference
MEM	Minimal essential medium
mg	milligram
ml	milliliter

MOI	Multiplicity of infection
NB	New born
NE	Normalized expression
NKCC	Sodium potassium chloride co-transporter
NPPB	5-nitro-2-(3-phenylpropylamino) benzoic acid
SP-A, B, C, D	Surfactant protein A, B, C, D
PCR	Polymerase chain reaction
RNAi	RNA intereference
siRNA	Small intereference RNA
shRNA	Small hairpin RNA
TER	Transepithelial electrical resistance
TPMPA	tetrahydrofuran
μg	microgram
μl	microliter
ZO	Zonula occludens
AD	Adult
AEC	Alveolar epithelial cells
AG	Attached collagen gel
AFC	Alveolar fluid clearance
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RNAi	RNA intereference
siRNA	Small intereference RNA
shRNA	Small hairpin RNA
TER	Transepithelial electrical resistance
TPMPA	tetrahydrofuran
µg	microgram
µl	microliter
ZO	Zonula occludens

Chapter 1

INTRODUCTION

Ionotropic γ-aminobutyric acid (GABA) receptors were only recognized as the most important inhibitory mediators of neurotransmission in the brain. Their roles in peripheral organs and other functions have not been appreciated but began to draw more and more interests in recent years. Our laboratory has identified a $GABA_A$ receptor pi subunit (GABRP) in alveolar epithelial type II cells by DNA microarray analysis. This is the first report on the expression of GABA receptors in alveolar epithelial cells. This dissertation aimed to characterize the expression patterns of GABRP in cultured alveolar epithelial cells and all the subunits of GABA receptors in the lung and type II cells in physiological and pathological conditions. Due to the fact that chloride channels are essential for lung fluid homeostasis, the functional roles of ionotropic GABA receptors in adult rat lung fluid transport were further investigated. Our results show that functional ionotropic GABA receptors contribute to a novel fluid transport pathway in alveolar epithelial cells. This study would open a new aspect in the field of alveolar epithelial cell biology and may have a great potential for resolving cystic fibrosis or lung edema in the future.

1.1 Ionotropic GABA receptors

Ionotropic GABA receptors, together with nicotinic acetylcholine receptors, glycine receptors, and some other receptors such as 5-HT3 receptors and serotonin receptors, belong to the superfamily of ligand-gated ion channels (Jentsch et al. 2002b). Members of this superfamily have a common structural feature: the ion channel complex is formed by 5 subunits. Ionotropic GABA receptors can be classified into two subtypes: $GABA_A$ and $GABA_C$ receptors, based on the pharmacological characteristics. The third type of GABA receptor, $GABA_B$ receptor is a G protein-coupled receptor which modulates the conductance of Ca^{2+} and K^+ channels via G-protein. It has a very different molecular structure compared to $GABA_A$ and $GABA_C$ receptors. GABA is the common agonist of all three types of GABA receptors. $GABA_A$ receptors are competitively inhibited by bicuculline, whereas $GABA_C$ receptors are specificially blocked by (1,2,5,6-tetrahydropyridin-4-yl)methyl phosphonic acid (TPMPA). Both types of ionotropic GABA receptors can be non-competitively blocked by picrotoxin, a drug originally found in snake (Bormann 2000). Upon binding with a specific agonist, $GABA_A$ and $GABA_C$ receptors open the channels and increase the conductance to Cl^-, which results in a hyperpolarity of the plasma membranes in most of the circumstances; however, in some stages of development or in some organs, the activation of ionotropic GABA receptors generates excitatory effects (Ben Ari 2002). $GABA_B$ receptors, on the contrary, normally produce hyperpolarity by increasing the conductance of K^+ and inhibiting the elevation of cellular Ca^{2+} (Bowery et al. 2002), which results in a decrease of the membrane

excitability and inhibits the releasing of neurotransmitters.

Ionotropic GABA receptors may have thousands of different combination from the available subunits. Up to date, 7 subunits (α, β, γ, δ, π, ρ and ε) have been found, which can be further classified into 19 subfamilies. All the subunits are abundantly expressed in brain, except for π and ρ, which are most abundant in uterus and retina, respectively (Hedblom and Kirkness 1997, Enz et al. 1995). The most common type of $GABA_A$ receptors is αβγ, where α- and β-subunits are indispensable but γ-subunit can be replaced by δ-, π-, and ε-subunits (McKernan and Whiting 1996). $GABA_C$ receptors are mainly formed from ρ-subunits only. Some ρ-subunits can also assemble with other subunits to form "hybrid" receptors (Milligan et al. 2004). The assembly of different subunits results in functionally and pharmacologically distinct receptors as indicated by electrophysiological studies (Bowery et al. 2002, Korpi et al. 2002). The pharmacological diversity of the receptor subtypes is important clinically when selecting the appropriate drugs.

Ionotropic GABA receptors were thought to mediate Cl^- influx into the cells. Their ability to extrude Cl^-, depending on the transmembrane Cl^- electrochemical gradient, was realized only in the recent years (Ben Ari 2002). Those receptors can also transport HCO_3^- with a lower permeability than that of Cl^- (Kaila 1994). The rapid influx of Cl^- is normally observed in adult neurons, which hyperpolarizes the plasma membrane and inhibits the neuron activity (Jentsch et al. 2002b). The efflux of Cl^- is more often observed in immature neurons, which depolarizes the plasma membranes and results in an excitatory effect (Ben Ari

2002). The mechanisms behind this switch during development are not very clear; however, the ontogenic differential expression of Cl^- transporters and the modulation of intracellular Cl^- concentrations may play essential roles for the switch (Chen et al. 1996, Rivera et al. 1999, Clayton et al. 1998). In immature neurons, the Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) starts to express at the early embryonic stages. NKCC1 largely mediates an inward Cl^- transport. Consequently, cellular Cl^- is accumulated. The ionotropic GABA receptors are also expressed at the early stage of gestation. When the receptors are activated, the accumulated Cl^- is ready to be extruded through the opened channels (Clayton et al. 1998). After birth, the expression of the outward K^+-Cl^- co-transporter 2 (KCC2) increases. The KCC2 extrudes Cl^- which significantly lowers the cellular Cl^- concentration. In immature neurons, the equilibrium potentials for Cl^- are normally -20 to -30 mV, whereas in adult neurons, they are normally -60 to -70 mV. Therefore, in adult neurons, ionotropic GABA receptors are normally inhibitory, because the cellular equilibrium potentials for Cl^- are lower than the threshold to generate action potentials (Rivera et al. 1999, Clayton et al. 1998). Interestingly, although KCC1 and KCC2 are highly homogeneous in their amino acid sequences, genetic studies indicated that the shift of cellular Cl^- was due to the effect of KCC2 but not KCC1 (Ben Ari 2002, Rivera et al. 1999).

Ionotropic GABA receptors mainly distribute in the CNS. In addition to mediating neurotransmission, they are also important for the development of the neuronal system (Owens and Kriegstein 2002, Behar et al. 2000, Maric et al. 2001). GABA acts as a signal molecule for promoting synaptogenesis, neuronal

differentiation and migration, probably via the depolarity effects generated by GABA receptors (Chen et al. 1996, Ganguly et al. 2001). The distribution and composition of ionotropic GABA receptors as well as their conductances are precisely regulated during neuron development (Lujan et al. 2005). As a result, knockout of some subunits leads to severe structural or functional abnormalities such as low neonate viability, fertile, growth retard and cleft palate in animal models (Jentsch et al. 2002a).

Ionotropic GABA receptors are also widely expressed in peripheral tissues and organs. However, their functions in those tissues are not well documented. GABA receptor subunits have been found in pancreas, kidney, ovary, fallopian tubes, pituitary, uterus and adrenal medullar (Akinci and Schofield 1999, Calver et al. 2000, Gamel-Didelon et al. 2002, Lux-Lantos et al. 2001, Moore et al. 2002). Interestingly, most of those organs have active secretory functions. In the pancreas, the activation of $GABA_A$ receptors increased both insulin release and fluid secretion (Brice et al. 2002, Park and Park 2000). In pituitary, $GABA_C$ receptors were only expressed in growth factor secreting cells but not the cells lacking secretory functions (Gamel-Didelon et al. 2003). The mechanism of GABA receptors to increase hormone secretion is unknown. In some cells, the activation of the GABA receptors results in elevation of cytosolic Ca^{2+}, which may act as a signal for hormone secretion (Predescu et al. 2005).

Because ionotropic GABA receptors mediate fast movement of ions, they may modulate cellular activities via changing the ion homeostasis. The activation of ionotropic GABA receptors regulates cell volume (Schomberg et al. 2003). In

addition, through cross-talk with other channels such as K^+-Cl^- co-transporters and purinergic receptors, ionotropic GABA receptors can change the conductance of several cations including Na^+, K^+ and Ca^{++} by different mechanisms (Hill et al. 1998, Panek et al. 2002, Limmroth et al. 1996). The reports on the role of ionotropic GABA receptors in fluid homeostasis are very limited. The blocking of $GABA_A$ receptors leads to brain edema (Iasnetsov and Novikov 1985). Some inflammatory neuron pains can be alleviated by the activation of $GABA_A$ receptors (Buritova et al. 1996, Dickenson et al. 1903). Some $GABA_A$ receptor agonists such as progesterone, allopregnanolone and muscimal, can be used to decrease plasma extravasation associated with meningitis (Limmroth et al. 1996, Lee et al. 1995).

The GABA receptor π subunit (GABRP) was cloned in 1997. Northern blot has shown that its expression is extremely abundant in the uterus and relatively enriched in the lung, but is very limited in the brain (Moats-Staats et al. 1995). GABRP constantly increases expression during the pregnancy in the uterus, but has a dramatic decrease at birth. The expression patterns suggest that GABRP may contribute to keep the uterus in a mostly quiescent status before parturition (Fujii and Mellon 2001, Neelands and Macdonald 1999). GABRP can co-assemble with other subunits, including α, β, and γ to form functional receptors (Neelands and Macdonald 1999). Recently, DNA microarray analysis indicated that GABRP was much more often expressed in breast tumors than normal mammary glands, suggesting that GABRP is a potential marker for breast tumors (Zehentner et al. 2002). Although only a few studies on GABRP have been

carried out, GABRP may play a role distinct from other subunits. When studying the genes involved in the differentiation pathways from alveolar type II cells to type I cells, our laboratory found that GABRP was only expressed in type II cells but not in type I cells (Chen et al. 2004). This is the first report of the expression of a GABA receptor subunit in alveolar epithelial cells. Because type II cells have essential roles to secrete surfactant, renew injured cells, and regulate fluid transport in the lung, GABRP or ionotropic GABA receptors may play a role to regulate those cellular activities in type II cells.

1.2 Lung and alveolar epithelial cells

Lung is a highly branched organ for gas-exchange. From proximal to distal region, the air conduction tubes of the lung include the cartilaginous trachea, bronchi, membranous bronchioles, respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. The proximal lung (cartilaginous airway) is covered with ciliated, secretory (mucus-goblet and serous cells), and basal cells. The distal (small noncartilaginous airway) is covered with ciliated and non-ciliated (Clara) bronchiolar cells. Alveoli are the blind ending, sac-like structures. The alveolar epithelium is mainly composed of two types of alveolar epithelial cells, type I and type II cell (Staub et al. 1988).

Alveolar type I cells are large and squamous. Their main functions are gas exchange and fluid transport (Matthay et al. 2002, Johnson et al. 2002). Type II cells are small, cuboidal and normally located at the corner of alveoli. The main functions of alveolar type II cells is lung surfactant production, fluid transport, and

host defense (Bisetti 1989). Alveolar type II cells are also progenitors of type I cells. They proliferate to generate new type II cells and differentiate into type I cells. As a result, alveolar type II cells play a critical role in physiological renewing and pathological repairing of alveolar epithelium (Fehrenbach 2001). In contrast, alveolar type I cells are considered as terminal differentiated cells. Probably because type I cells are thin and large, they are more vulnerable to damages. For example, a prolonged hyperoxia exposure seriously damages type I cells, but most type II cells are intact (Pagano and Barazzone-Argiroffo 2003, Fehrenbach 2001).

Surfactant, a phospholipid-rich lipoprotein complex, lowers surface tension and prevents collapse of alveoli (Hutchison 1994). Surfactant is mainly synthesized and secreted by alveolar type II cells. However, Clara cells can also synthesize surfactant proteins. Four surfactant proteins (SP) A, B, C and D have been found. Among them, SP-C can only be synthesized and secreted by type II cells. Surfactant proteins are widely used as the markers of type II cells. Some of them, such as SP-D, function as host defense in the lung (Bisetti 1989, Whitsett 2005). The lamellar body membrane protein, LB-180, is another commonly used marker for type II cells (Mulugeta et al.). Type I cell markers include T1 alpha, a protein whose function is unknown yet, and aquaporin 5, a water channel (Rishi et al. 1995, Chen et al. 2004).

Type II cells undergo trans-differentiation when cultured *in vitro*. On plastic plates, type II cells lose most of the lamellar bodies after a 3-day culture and change completely to type I-like cells after a 7-day culture (Borok et al. 1998b,

Borok et al. 1998c, Shannon et al. 1992). The similar effects have been observed when those cells are cultured on attached collagen gel (AG), or when liquid is present on both the apical and basolateral surface of the cells (liquid-liquid interface, or LL). Some culture systems have been developed to maintain type II cell phenotypes (Shannon et al. 1987, Danto et al. 1995, Borok et al. 1998b, Dobbs et al. 1997, Xu et al. 1998, Mason et al. 2002). The culture models that partially maintain type II cell phenotype include: culturing cells on matrigel, adding keratinocyte growth factor (KGF) in the culture medium, culturing cells on the detached collagen gel (DG), and culturing cells on the air-liquid interface (AL). The mechanisms how those culture models work are not very clear. Interestingly, several culture models can modulate not only the cell phenotype, but also the expression of ion channels (Ridge et al. 1997, Liu and Mautone 1996, Jain et al. 2001, Wang et al. 1999, Yang et al. 2003). Culturing on the plastic plate decreases the expression of amiloride-sensitive Na^+ channels and cystic fibrosis transmembrane regulator (CFTR) in type II cells (Brochiero et al. 2004, Liu and Mautone 1996). On the contrary, matrigel, KGF, and AL increase the expression Na^+ or Cl^- transporters. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. The main components of matrigel include growth factors, laminin and collagen. Growth factors and laminin up-regulate the expression of CFTR and the epithelial Na^+ channel (ENaC), but collagen has no effects on cell differentiation and ion channel expressions (Yang et al. 2003, Wang and Chan 2000). The AL helps cells to maintain the differentiation status of type II cells. It also enhances

the expression of the α-subunit of the ENaC (Rossier et al. 2002, Matalon and O'Brodovich 1999). As a result, airway epithelial cells cultured on the AL have a significantly higher Na^+ absorption than those grown on the plastic plates. The expression of CFTR in type II cells also increases on the AL in comparison to that on plastic plates (Brochiero et al. 2004). In animals, KGF increases fluid clearance via the elevation of type II cell proliferation and thus the expression of ENaC (Wang et al. 1999). However, KGF increases fluid and electrolyte secretion in type II cells on the AL (Borok et al. 1998a, Prince et al. 2001). KGF modulates the expression of a number of Cl^- channels on the AL, increasing ClC1 and ClC7 but decreasing CFTR (Prince et al. 2001). The DG probably provides a continuous mechanical force to the cultured cells so that maintains the morphology of type II cells. Whether DG changes the expression of ion channels is not clear; however, most cells handle the mechanical force stimulation via ion channels (Kung 2005). Because GABRP is specifically expressed in type II cells, it may provide important information on the functions of GABRP in alveolar epithelial cells by examining its expression under those culture conditions.

1.3 Airway surface fluid (ASF) and alveolar surface liquid (ASL)

The inner surface of the lung is lined with a thin layer of fluid, namely airway surface fluid (ASF), which is a mixture of liquid and proteins. The ASF provides an ideal environment to trap foreign substances, such as bacteria, viruses, and particulate matter. And through efficient mucociliary movement, those unfavorable granules can be removed out of the lung through sputum. Some

inhaled unfavorable chemicals, such as hydrochloride gas, can be also dissolved and diluted by the ASF and finally be removed (Rubin 2002). The fluid covered on the surface of the alveoli is named alveolar surface liquid (ASL) or alveolar subphase fluid (AVSF). The ASL may be able to communicate with the ASF, however, it has very different component from the ASF. ASF contains a large amount of mucous that is secreted by the goblet cells whereas surfactant produced by type II cells is only in the ASL (Scarpelli 2003).

The ASF is formed by the lung epithelium. Similar to all the fluid secretions in the body, the active Cl^- secretion provides the main driving force for water to move to the airway lumens (Widdicombe et al. 1997). Through active re-absorption of Na^+, the ASF can be absorbed into circulation so that the volume of the ASF can be precisely controlled (Matthay et al. 2002, Tarran et al. 2001). Although there is disagreement about which types of cells are mainly responsible for the production of the ASF, Na^+ and Cl^- transporters have been found in many types of the airway epithelium and liquid secretion has been observed in all the segment of airways (Jeffery and Li 1997, Engelhardt et al. 1994, Rochelle et al. 2000b, Tarran et al. 2002, Brochiero et al. 2004). Because alveolar epithelium constitutes a large area of the airway, it is possible that alveolar epithelium is an important resource of the ASF productions (Matthay et al. 2002). Up to date, many kinds of Na^+ and Cl^- transporters such as ENaC, Na^+-K^+-ATPase, CFTR, purinergic receptors, and ClC, have been identified in type II or type I cells (Yue et al. 1995, Schneeberger and McCarthy 1986, Johnson et al. 2002, Borok et al. 2002, Brochiero et al. 2004, Qiao et al. 2003, Chen et al. 2004).

It is essential to control the volume and composition of ASF, which are the pre-requisite of proper pulmonary functions such as ciliary movement and lung defense. If the volume of the ASF is too much, the the ciliary movement may be prevented. However, if the volume of ASF is too low, the ability to trap foreign substance will be largely reduced and the lung will be more vulnerable to infections (Boucher 2004). The volume and composition of the ASL is equally important. If the volume is too much, the lung has low gas-exchange efficiency. However, if the volume is too low, alveoli lose the ability to circulate surfactant network and to exchange electrolyte with outside (Scarpelli 2003, Ng et al. 2004). To precisely regulate the volume and composition of the ASF, the expression and activity of epithelial ion transporters may vary under different physiological and pathological conditions (Rubin 2002, Widdicombe et al. 1997, Yue et al. 1995, Johnson et al. 1998, Boucher 2004).

The composition of the ASF is difficult to determine due to the extremely small volume. By using ultramicroanalytic techniques, scientists have found that the content of many ions of the ASF is close to the plasma, whereas the ion concentration of Na^+ and Cl^- may be only half of that of the plasma (Joris et al. 1993). Recently, X-ray microanalysis has begun to be used in determining the ionic composition of the ASF, which confirmed that in trachea, the ASF has a significantly lower concentration of Na^+ and Cl^- than the serum (Vanthanouvong et al. 2005, Kozlova et al. 2005). The composition of the ASL has been a mystery for many years until now. It is technically difficult to access the surface of the alveoli. However, it is generally agreed that the ASL is more acidic than serum,

because macrophages, which make up the defense layer of the alveoli, may generate a lot of metabolic acids (Scarpelli 2003).

Probably because the adult lung is a relatively “dry” organ in comparison with other organs, and the volume of ASF is tiny compared with other body fluids, the absorption functions of the airway epithelium have been substantively studied (Matthay et al. 2002). The fluid-secretion of the fetal lung is also well studied (Folkesson et al. 1998, Olver et al. 2004a, Bland and Nielson 1992), however, the importance of fluid secretion in the adult lung is realized for only ~ 20 years. Fluid secretion in the adult lung is now considered to be at least equally important as absorption. How the ASF is dynamically regulated all the time is not clear. The leading hypothesis assumes that the airway epithelium is absorptive most of the time; however, when there are breaks of fluid absorption, there is significant fluid secretion so that the volume and composition of the ASF are maintained (Boucher 2004). Before the wide-acceptance of the concept of active absorption or secretion, there was a theory that Na^+ is absorbed through epithelium but Cl^- is transported passively through the paracellular tight junctions (Kim et al. 1991). That theory is largely rejected now. Paracellular ion transport rate is only 1/3 of the transcellular transport (Kim et al. 2005). As a result, paracellular transport may not meet the requirement of electronic equilibrium.

1.4 Fluid transport during lung development

The lung is not filled with air during the fetal stage. The fetal lung is a fluid-secretion organ at the time that lung bud forms (Olver et al. 2004b). The lung

fluid secretion increases during early gestation, peaks at mid-gestation, and gradually declines before birth (Harding and Hooper 1996). The liquid secretion, accompanied with the increasing volume of the lung, generates a distensing pressure against the lung/chest wall, which provides the crucial force for the lung growth (Olver et al. 2004b). If the lung fluid secretion is impaired during gestation, it results in the lung hypoplasia, which is morphologically characterized with insufficient lung growth, reduced number of airway generation, reduced pulmonary vascular bed, and reduced acinar size (Moessinger et al. 1990).

The lung fluid is largely secreted by the fetal lung epithelium. The epithelial cells, including type II cells, type I cells, clara cells, and ciliated cells, may all contribute to the formation of the fetal lung fluid (Olver et al. 2004b, Cotton et al. 1988, Rao and Cott 1991). Alveolar epithelial cells may be the main source of lung fluid, because they constitute large part of the total lung surfaces. Cl^- secretion is the main driving force for the fluid secretion (Olver and Strang 1974). Composition analysis of the fetal lung fluid indicated that the concentration of Cl^- in the fetal sheep lung lumen is much higher than the plasma (157 mM v.s. 107 mM). How this fluid is produced is not fully understood. A widely accepted theory describes the mechanism as follows: NKCC and Na^+-K^+-ATPase are expressed at the early stage. Na^+-K^+-ATPase pumps Na^+ out of the cells and results in a relative insufficient Na^+. NKCC is sensitive to the lack of cytosolic Na^+ and co-transports the Na^+, K^+, and Cl^- into the cells from the mesenchyme. Extra Na^+ can be still be pumped out through the Na^+-K^+-ATPase, whereas K^+ and Cl^- are accumulated in the cells. The Cl^- is then ready to be extruded from the apical

surface of the epithelium because of the accumulated gradient. K^+ is secreted together with Cl^- to keep the electronic balances. This statement also explains why fetal lung epithelium can continuously secrete Cl^- against its gradient (Olver and Strang 1974). A big “gap” of this theory is that the Cl^- channels responsible for fetal lung fluid secretion are still unknown. CFTR was assumed to be important for lung fluid secretion. However, knock-out study has indicated that the lung has normal development even without the expression of CFTR (O'Neal et al. 1993). ClC mutations result in many serious problems, such as Dent’s disease, osteopetrosis, sterile, and deafness (Jentsch et al. 2002b). However, lung development appears to not be a problem for those ClC knockout mice. The expression of GABRP in type II cells provides a potential novel candidate to fill the gap of the current theory. If the ionotropic GABA receptors are expressed in fetal lungs, they may have a role in the production of fetal lung fluid.

At birth, fluid has to be removed from the lung and allow the lung to be filled with air. The fluid absorption is driven mainly by ENaC and Na^+-K^+-ATPase. Through active Na^+ transport, the fluid is forced to transport from the lumen and accumulate in the interstitial space of the lung (O'Brodovich et al. 1990, Chapman et al. 1990). The fluid in the interstitium is finally removed through the vascular and lymphatic systems (Bland et al. 1982) in several hours. The role of Na^+ transporters in perineonatal lung fluid clearance is well studied. Consistent with the developmental change of the lung fluid, the expression of ENaC in the lung is low in the early embryotic stages. It begins to increase from the mid-gestation, peaks at birth and remains at a high level after birth (Chapman et al.

1990, Matalon and O'Brodovich 1999, Watanabe et al. 1998).

The roles of Cl^- channels in fetal lung development are well studied; however, their functions at birth are not clear. The ontogenic expression of some Cl^- channels including CFTR, ClC and NKCC has been reported (Tizzano et al. 1994, McGrath et al. 1993, Harris et al. 1991, Thiemann et al. 1992, Lamb et al. 2001, Edmonds et al. 2002, Rochelle et al. 2000a). CFTR and most ClC are expressed at a much higher level in fetal lungs compared to adult lungs (McGrath et al. 1993, Tizzano et al. 1994, Harris et al. 1991, Thiemann et al. 1992, Lamb et al. 2001, Edmonds et al. 2002). ClC2 has a dramatic decrease at birth (Thiemann et al. 1992). There is no report about the increase of Cl^- channels at birth yet. This phenomenon is quite confusing, because as we know, to keep the electronic equilibrium, when the Na^+ is absorbed, a Cl^- or other anion should be equally absorbed. Further, recent studies indicated that the absorption of Na^+ is dependent on the absorption of Cl^- (O'Grady et al. 2000). A strong evident is that in cystic fibrosis (CF) mouse, because the mutation of the CFTR, a major Cl^- channel, β-agonist failed to increase the fluid clearance even there is damage to the Na^+ channels (Fang et al. 2002a). In addition, the fetal lung fluid has very high concentration of Cl^-, which has to be absorbed at birth. Probably because the role of Cl^- channels is realized for only the recent 20 year, the main Cl^- channels that help to remove lung fluid at birth have not been figured out yet. Therefore, in addition to the current known Cl^- channels, other Cl^- channels exist in the developing lungs and play a role in the Cl^- absorption at birth. Ionotropic GABA receptors are Cl^- channels. Whether they play a role in fluid clearance at

birth is not clear.

1.5 Fluid homeostasis in hyperoxic lung injury

Lung fluid homeostasis is important in both normal and pathological conditions. Supplying a high concentration of O_2 is commonly used clinical practice for patients with acute lung injury or severe hypoxemia, and most often for pre-maturely delivered babies (Weaver 1992). However, prolonged exposure to a high concentration of O_2 may lead to hyperoxic lung injury. A high concentration of O_2 produces reactive oxidative species (ROS), which damage the endothelium and alveolar type I cells, increase the permeability of the alveolar epithelium to water and protein, and finally result in lung edema (Freeman et al. 1993). Alveolar epithelial cells play an important role to resolve the lung edema. In subacute hyperoxic lung injury models, the transcripts and activity of ENaC and Na^+-K^+-ATPase in the lung are increased. As a result, the fluid clearance ability of the lungs increases (Fehrenbach 2001, Matthay et al. 2002, Yue et al. 1995, Matalon and O'Brodovich 1999). In severe hyperoxic lung injuries, excess ROS damage the ion channels and hampers the lung to clear edema fluid (Yue et al. 1995, Matalon and O'Brodovich 1999). The functions of Cl^- channels are not clear in hyperoxia-injured lungs. Because aborption of Na^+ requires Cl^-, Cl^- channels may also play a role in the resolution of hyperoxic lung edema.

1.6 Airway and alveolar epithelial Cl^- channels

Since the role of Cl^- channels in lung fluid transport is only realized in recent years, only a few Cl^- channels have been studied on airway and even less on alveolar epithelial cells, which include CFTR, Ca^{2+}-activated Cl^- channel (CaCC), ClC, NKCC, KCC, and purinergic receptor P2X (Harris et al. 1991, Thiemann et al. 1992, Lamb et al. 2001, Edmonds et al. 2002, Rochelle et al. 2000a, Brochiero et al. 2004, Fang et al. 2002b, Blaisdell et al. 2000, Murray et al. 1996, Rochelle et al. 2000b, Murray et al. 1995, Lee et al. 2003, Brochiero et al. 2004, Chen et al. 2004). All of those channels are also widely expressed in other organs (Jentsch et al. 2002b). Those channels may not be all the members of Cl^- channels in the lung, because none of those channels were proved to be indispensable for lung development.

The most widely studied Cl^- channel is CFTR, which is encoded by a gene of ~250 kb (Riordan et al. 1989). It is a voltage-independent anion channel that requires phosphorylation for efficient activity (Riordan et al. 1989). The single-channel conductance of CFTR is between 6~10 pS (Berger et al. 1991). In the airway and alveolar epithelium, CFTR is located on the apical plasma membrane. Earlier studies have showed that CFTR is mainly distributed in submucus glands and distal airways (McGrath et al. 1993, Tizzano et al. 1994). Recent reports have demonstrated that CFTR is also expressed in type II cells (Brochiero et al. 2004). CFTR is constitutively expressed in the lung but its distribution changes during the development (McGrath et al. 1993, Tizzano et al. 1994). In adult lungs, CFTR plays a crucial role for lung fluid secretion in the airway (Boucher 2004,

Sheppard and Welsh 1999, Donaldson and Boucher 2003, Quinton 1983). Patients with a mutated CFTR have a significantly lower ASF volume than that of normal people (Donaldson and Boucher 2003, Quinton 1983). CFTR is important for alveolar fluid clearance as well. In the CFTR(-/-) mouse, isoproterenol could not stimulate the alveolar fluid clearance (Fang et al. 2002a). However, CFTR is not required for lung development. Up-to-date, more than 1000 kinds of mutations for CFTR have been found (Boucher 2004). CF mice are mostly born with a structurally normal lung (O'Neal et al. 1993). The early death of CF patients is generally due to the fact that they have significantly reduced ASF, which lowers their ability to resist the recurrent bacterial infections (Boucher 2004).

CaCC is another widely studied Cl^- channel located on the apical membrane of airway epithelial cells (Jentsch et al. 2002b, Anderson and Welsh 1991, Tarran et al. 2002). CaCC is activated by the elevation of intracellular Ca^{2+} (specifically from the apical part of the cytoplasma) and mediates an outward Cl^- current (Paradiso et al. 2001). cAMP, which activates CFTR, inhibits the activity of CaCC (Anderson and Welsh 1991). The molecular identity of CaCC is not clear yet, although several molecular candidates have been proposed (Hartzell et al. 2005, Jentsch et al. 2002a). Probably due to the ambiguous molecular identity, the single channel conductance of CaCC has been recorded from 1-70pS (Jentsch et al. 2002a). Because it secretes Cl^-, CaCC is an attractive candidate to replace CFTR in case of CF diseases (Wagner et al. 1991). In cultured airway epithelial cells, CaCC has been observed to increase the volume of ASF (Tarran et al.

2002). However, lack of specific activators and inhibitors make it difficult for clinic use (Boucher 2004). CaCC may be not expressed in alveolar epithelial cells.

ClC belongs to the voltage-gated Cl^- channels. Their mRNA and proteins have been identified in the lung (Thiemann et al. 1992, Lamb et al. 2001, Edmonds et al. 2002, Blaisdell et al. 2000). ClC is probably located on the apical membrane of epithelium. Their high expression in early gestation and low expression in adult lungs suggest that they may play a role in fetal lung fluid secretion (Thiemann et al. 1992, Lamb et al. 2001, Edmonds et al. 2002, Blaisdell et al. 2000). On cultured fetal lung, down-regulation of ClC2 hampers the growth of the cysts (Blaisdell et al. 2004). In other organs, ClC plays an important role in fluid homeostasis. Mutation of ClC5 results in Dent's disease (proteinuria and kidney stones). There are no reports about their roles in adult lungs (Jentsch et al. 2002a).

NKCC and KCC are both located on the basolateral membranes. NKCC is proposed to accumulate Cl^- gradient in fetal lung epithelium (Olver and Strang 1974, Carlton et al. 1992, Thom and Perks 1990, Rochelle et al. 2000b). However, by using NKCC1(-/-) mouse, it has been shown that NKCC1 may be only the rate-limiting factor for fetal lung fluid production and other anion channels may maintain the fluid secretion to near normal levels (Rochelle et al. 2000a). KCC3 and KCC4 have been reported on airway epithelial cells recently. Their roles in lung fluid homeostasis are not clear. It is proposed that the expression of KCC may provide a route to resolve the hyperpolarity across the membranes that is produced by NKCC (Lee et al. 2003).

There are at least two important questions need to be answered. (i) What other Cl^- channels work in the fetal lung, so that the fetal lung can secrete fluid independent on the current Cl^- channels? (ii) How are ASF and ASL regulated by Cl^- channels? The study on the ionotropic GABA receptors on alveolar epithelial cells may provide answers to both questions.

1.7 Specific aims and significance

Ionotropic GABA receptors have been widely studied as the main inhibitory mediators for neurotransmitter release in neuronal systems. Relative little is known regarding their functions in non-neuron system. In peripheral organs including pancreas, uterus and pituitary, GABA receptors have been shown to play important roles. It is a brand new field to study the function of ionotropic GABA receptors in alveolar epithelial cells. The unique expression patterns and pharmacological characteristics of the $GABA_A$ receptors π subunit indicate that it may be a special modulator of cell activities. However, only a few studies have been performed since its discovery in 1997 and no information has been reported in pulmonary system. Based on the previous studies on ionotropic GABA receptors and alveolar epithelial cells, we hypothesize that ionotropic GABA receptors may play important roles in the lung. Although those receptors may have various effects on alveolar epithelial cells, the functional study will be focused on whether they are involved with lung fluid transport, due to the fact that fluid homeostasis and chloride channels are critical for pulmonary functions but are relatively less documented. To test our hypothesis, we proposed the following

objectives:

Specific Aim I: Characterize the $GABA_A$ receptors π subunit in cultured alveolar epithelial cells.

Specific Aim II: Characterize all the ionotropic GABA receptor subunits in developing lungs and type II cells and in hyperoxia-exposed lungs.

Specific Aim III: Determine the roles of ionotropic GABA receptors in alveolar epithelial cells.

This proposed research would open a new aspect in the field of alveolar epithelial cell biology. In addition, if ionotropic GABA receptors are proved to regulate lung fluid transport, GABA receptor agonist, many of which are widely used drugs, may become novel candidates to resolve pulmonary edema and cystic fibrosis in the future.

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Chapter 2

CHARACTERIZATION OF THE IONOTROPIC GABA RECEPTOR PI SUBUNIT IN CULTURED ALVEOLAR EPITHELIAL CELLS[1,2]

2.1 Abstract

Although type A γ-aminobutyric acid (GABA) receptors (ligand-gated Cl^- channels) have been extensively studied in the central nervous system, no information is available on this receptor in lung cells. We have examined the expression of $GABA_A$ receptor π-subunit (GABRP) during the trans-differentiation between rat alveolar epithelial type II cells and type I cells. Rat alveolar type II cells, when cultured on plastic plates, gradually trans-differentiated into type-I-like cells and lost their GABRP mRNA expression. However, the GABRP mRNA was partially retained in the type II cells cultured on Matrigel. Keratinocyte growth factor (a mitogen of type II cells) increased GABRP expression. A detached collagen gel maintained the GABRP mRNA to a level close to that of the freshly

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[2] Contributions of co-authors: Telugu Narasaraju and Kolliputi Narsaiah performed Western blot; Jiwang Chen performed some cell isolation. Lin Liu is the principal investigator.

isolated type II cells. An air–liquid interface culture system, mimicking in vivo conditions in the lung, significantly up-regulated the expression of GABRP mRNA and protein. mRNAs of the $GABA_A$ receptor α1-, α3-, β2-, γ2-, and γ3-subunits were also detected in rat type II cells. These results suggest that GABRP expression is differentially regulated by culture substrata, growth factor, detached gel, and an air-apical surface.

2.1 Introduction

Receptors for γ-aminobutyric acid (GABA) are widely distributed in the central nervous system (CNS). Based on their pharmacological characteristics, GABA receptors can be classified into three subtypes: $GABA_A$, $GABA_B$, and $GABA_C$ receptors. The $GABA_A$ receptor is a ligand-gated Cl^- ion channel, whereas the $GABA_B$ receptor is a G-protein-coupled receptor. The $GABA_C$ receptor is an ionotropic Cl^- channel but has unique ligand-binding characteristics different from $GABA_A$ and $GABA_B$ receptors (Bormann *2000*). $GABA_A$ receptors mediate most of the rapid inhibitory transmissions in the CNS. They may also be involved in ion homeostasis (Limmroth et al. *1996*), cell proliferation and differentiation (Chen et al. *2004*; Mong et al. *2002*), and hormone secretion (Mayerhofer et al. *2001*; Park and Park *2000*). Although $GABA_A$ receptors are also found in several peripheral tissues (Akinci and Schofield *1999*; Glassmeier et al. *1998*; Park and Park *2000*), the functions of $GABA_A$ receptors in these tissues are not well documented.

The $GABA_A$ receptor π-subunit (GABRP) is abundant in the uterus but rarely detected in the brain. Transcripts of GABRP mRNA have been detected in the

lung (Hedblom and Kirkness *1997*). The co-assembly of the GABRP with α, β, and γ subunits can form functional receptors (Neelands and Macdonald *1999*). The expression of GABRP throughout pregnancy contributes to maintaining the uterus in quiescence status before parturition (Fujii and Mellon *2001*).

Alveolar epithelial type II cells are crucial for the maintenance of normal functions of the alveoli. They regulate the surface tension of the alveoli by synthesizing, secreting, and recycling lung surfactant. They proliferate and differentiate into type I cells and thus play a central role in the turnover of normal alveolar epithelium and in the repair of damaged alveolar epithelium. Type II cells also regulate fluid transport in the lung. In addition, they secrete cytokines and growth factors and serve as an interconnection for other cells in the alveoli (Fehrenbach *2001*).

The trans-differentiation from type II cells to type I cells has been studied in vitro by using cultured type II cells (Paine and Simon *1996*; Williams *2003*). Type II cells lose their phenotype and trans-differentiate into type-I-like cells when cultured on plastic dishes. Surfactant proteins B and C (SP-B and SP-C) and T1α have been used as markers for type II and type I cells. Several culture conditions have been developed to maintain the type II cell phenotype. These include culturing cells on Matrigel (Shannon et al. *1992*), on a detached floating collagen gel (Borok et al. *1998a*; Danto et al. *1995*), at an apical-air surface (Dobbs et al. *1997*), or in the presence of keratinocyte growth factor (KGF; Borok et al. *1998b*; Mason et al. *2002*; Xu et al. *1998*). The efficiencies and reproducibility of these culture conditions are variable. We have previously identified GABRP in alveolar

epithelial type II cells (Chen et al. *2004*). In the present study, we have examined GABRP expression during the trans-differentiation of type II cells to type I cells by using various culture conditions that promote or depress the trans-differentiation, including culture substrata, growth factor, detached gel, and an air-apical surface.

2.3 Materials and Methods

2.3.1 Materials

Minimal essential medium (MEM), fetal bovine serum (FBS), and M-MLV reverse transcriptase were purchased from Invitrogen (Calsbad, Calif.). DNase I, phenylmethanesulfonyl fluoride, rat immunoglobulin G (IgG), and rabbit anti-β-actin antibodies were from Sigma (St Louis, Mo.). Bovine dermal collagen was from Cohesion (Palo Alto, Calif.). Pancreatic elastase was from Worthington (Lakewood, N.J.). Matrigel was from Becton Dickinson (Bedford, Mass.). Human recombinant KGF was from R&D systems (Minneapolis, Minn.). Millicell-CM filter inserts and collagen-coated costar filter inserts (30 mm diameter) were from Millipore (Bedford, Mass.). TRI reagent was from Molecular Research Center (Cincinnati, Ohio). DNA-free DNase was from Ambion (Austin, Tex.). Random hexamer primers were from Promega (Madison, Wis.). Primers for real-time polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) were synthesized by MWG Biotech (High Point, N.C.). Faststart *Taq* DNA polymerase, PCR nucleotide mix^{PLUS}, and uracil–DNA glycosylase were purchased from Roche Molecular Biochemicals (Indianapolis, Ind.). QuantiTech SYBR Green PCR master mix was from QIAGEN (Foster city, Calif.). GENECLEAN Turbo for

PCR was obtained from Qbiogene (Carlsbad, Calif.). Protein concentration assay reagents were from BIO-Rad Laboratories (Hercules, Calif.). Polyclonal goat anti-GABRP antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif.). Monoclonal anti-LB180 antibodies were from Covance (Richmond, Calif.). Horseraddish peroxidase (HRP)-conjugated anti-goat IgG, HRP-conjugated anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, and Alexa 568-conjugated anti-goat IgG were from Molecular Probes (Eugene, Ore.). The enhanced chemiluminescence (ECL) Western blot kit was from Amersham Biosciences (Piscataway, N.J.).

2.3.2 Isolation of alveolar epithelial type II cells

Alveolar epithelial type II cells were isolated according to the method of Dobbs et al. (*1986*) as previously described (Liu et al. *1996*). Briefly, male Sprague-Dawley rats (180–200 g) were anesthetized by intraperitoneal injection with ketamine and xylazine. Lungs were perfused, lavaged, and digested with elastase (30 U/ml). The lung lobes were chopped into small pieces of approximately 1 mm^3 in size. The minced lung tissue was treated with DNase I (1 mg/ml) for 2 min and then filtered sequentially through 160-, 37-, 15-, and 15-size filters. Cells were harvested by centrifugation at 1,100 rpm for 10 min, resuspended in 10 ml MEM, and panned in two bacteriological dishes coated with 3 mg rat IgG for 30 min each. The purity of freshly isolated type II cells was approximately 85% as assessed by modified Papanicolaou staining. The yield of type II cells from each rat varied from 20×10^6 to 30×10^6. Viability was greater than 95% as determined by trypan blue exclusion.

2.3.3 Isolation of alveolar macrophages

The cells in lavage were harvested by centrifugation at 1,100 rpm for 10 min, resuspended in 10 ml MEM, and panned in a bacteriological dish coated with 3 mg rat IgG for 30 min. The medium, together with the floating cells, was discarded. The attached cells were collected as alveolar macrophages with a purity of >95%.

2.3. 4 Cell culture

Type II cells were cultured on various matrices for a variable number of days in MEM supplimented with 10% FBS, except for the cells cultured on Matrigel, in which no FBS was used. The medium was changed 24 h after seeding and every 48 h thereafter for all culture conditions.

Cell culture on plastic dishes Type II cells (5×10^6) in 10 ml MEM plus 10% FBS were seeded into 100-mm diameter plastic dishes and cultured for 1 or 7 days.

Cell culture on Matrigel An equal volume of Matrigel (11.6 mg/ml) and MEM were mixed on ice with a pre-cooled pipette. The mix (1 ml) was transferred to 6-well cluster plates and allowed to solidify at 37℃ for 30 min. Type II cells ($5\times10^5/cm^2$) in 2.5 ml medium were gently placed onto the surface of the Matrigel and cultured for 7 days.

Cell culture on collagen gel with KGF To prepare collagen gel, eight volumes of the collagen stock (3.2 mg/ml in 0.012 N HCl) were mixed with 1 volume of 10× MEM and 1 volume of sterile 0.4 M NaOH on ice. The mixture (3 ml) was placed in 6-well cluster plates or filter inserts and allowed to solidify at

37℃ for 2 h. The collagen gel was rinsed thoroughly with MEM before use. Type II cells ($5\times10^5/cm^2$) in 2.5 ml medium were cultured on the collagen gel for 8 days. KGF (10 ng/ml) was added to the medium at day 4 after plating.

Cell culture on a detached or attached collagen gel Type II cells were cultured on a detached or attached collagen gel according to the method of Danto et al. (*1995*) and Borok et al. (*1998a*). Type II cells in 3 ml culture medium were cultured at a density of $5\times10^5/cm^2$ for 24 h in 6-well cluster plates coated with collagen gel. The collagen gel was then detached from the edge of the dishes and floated in the culture medium. The cells were cultured on the detached gels for 1 or 2 weeks. This condition is referred to here as detached gel (DG). The cells cultured on an attached collagen gel (AG) were used as a control. In some experiments, cells were cultured on AG for 8 days and then on DG for an additional week (AG–DG).

Cell culture on an apical-air surface Type II cells were cultured on an apical-air surface as described by Dobbs et al. (*1997*). The cells were seeded in the filter insert coated with collagen gel at a density of $5\times10^5/cm^2$. Culture medium (2 ml) was added to both the inside and outside of the insert. The cells were allowed to attach for 24 h, and the apical surface was then exposed to air by removing the culture medium from the insert. The cells were cultured for an additional 2 weeks. The apical surface was rinsed with culture medium every 48 h to prevent it from drying out. This condition is referred to as the air–liquid interface (AL). Cell cultures without exposure of the apical surface to air are referred to as liquid–liquid interface (LL). For the purpose of microscope

observation and immunostaining, type II cells were cultured in collagen-coated costar inserts for 7 days.

2.3. 5 Isolation of total RNA and synthesis of cDNA

Total RNA was isolated with TRI reagents from the cultured cells according to the manufacturer's instructions. Residual DNA was removed by treating the RNA with DNA-free DNase. Lack of DNA contamination was confirmed by PCR of the RNA samples by using 18S rRNA primers. The integrity of the RNA samples was checked by visual evaluation of the 28S and 18S ribosomal RNA bands by using agarose gel electrophoresis and by measurement of the absorbance at 260 nm and 280 nm. The $A_{260\ nm}/A_{280\ nm}$ ratios were between 1.9 and 2.1 for all the RNA samples. Total RNA (1 μg) was reverse-transcribed into cDNA at 42℃ for 1 h in a total volume of 20 μl by using 200 U M-MLV reverse transcriptase and 0.5 μg random hexamer primers. The cDNA was diluted five times with water and stored at –20℃ until use.

2.3.6 Real-time quantitative PCR

The primers were designed with Primer Express software and are listed in Table 1. The specificity of the primers was confirmed by BLAST search. No other sequences in the Genebank matched the primer sequences. An absolute standard method was used in this study (Yin et al. *2001*). To construct a standard curve, conventional PCR was performed, and PCR products were purified with a GENECLEAN Turbo kit. Copy numbers in the purified PCR products were calculated with the following formula:

$$\text{Copies/ml} = \left(6.023 \times 10^{23} \times 5 \times 10^{-5} \times A_{260} \times \text{dilution times}\right) / (\text{base pairs} \times 658)$$

where 6.023×10^{23} is the number of molecules per mole materials, 5×10^{-5} is weight of DNA (in g/ml) when the absorbance at 260 nm is 1, and 658 is the average molecular mass of nucleotides.

PCR standards for real-time PCR were prepared by ten-fold series dilutions of purified PCR products from 10^8 to 10 copies in 5 µl water. Real-time PCR was performed on an ABI 7700 system (Applied Biosystems, Foster City, Calif.) in a 25-µl reaction volume by using SYBR Green detection (Simpson et al. *2000*). SP-B and T1 αwere amplified with Faststart *Taq* DNA polymerase. PCR nucleotide mix^{PLUS} and uracil–DNA glycosylase were included to prevent carry-over contaminations. SP-C, GABRP, and 18S rRNA were amplified with QuantiTech SYBR Green PCR master mix. The PCR mix was optimized according to the protocols provided by the various companies. The thermal conditions include a heat activation of the hotstart DNA polymerase at 95℃ for 15 min, followed by 40 cycles of denaturing at 95℃ for 20 s, annealing at 60℃ for 30 s, extension at 72℃ for 30 s, and a data acquisition for 15 s. Fluorescence signals were collected only at the data acquisition step. Data acquisition temperatures were optimized to 2–6℃ lower than the Tm of the amplicon in order to obtain a highly efficient and specific amplification (Table 2.1). To confirm the specificity of the amplification, a dissociation analysis was performed after each amplification under the following thermal conditions: denaturation at 95℃ for 20 s, annealing at 60℃ for 15 s, ramping slowly to 95℃ over a 20-min period, and holding at 95℃ for 15 s. A dissociation curve was obtained by the software provided by the Applied Biosystems. At least two reactions were run for each standard or

unknown on each plate.

A standard curve was generated by plotting the Ct value versus the log copy numbers of the PCR standards. Ct values were the PCR cycle number required for fluorescence intensity to exceed an arbitrary threshold in the exponential phase of the amplification. The copy numbers of samples were obtained by the software Sequence Detector 1.7 from the standard curve. Copy numbers were normalized with 18S rRNA in order to account for the differences in RNA quality and efficiencies in reverse transcriptions.

Table 2.1 Primers used for real-time PCR and RT-PCR

Gene	Direction	Primer sequence	Starting base	Size (bp)	Amplicon Tm (°C)	Acquisition (°C)
SP-B	Forward	5′-AATGACCTGTGCCAAGAGTGTG-3′	196	336	82	78
	Reverse	5′-AGGACCAGCTTGTTCAGCAGAG-3′	531			
SP-C	Forward	5′-AGCTCCAGGAACCTACTGCTACAT-3′	360	101	83.2	78
	Reverse	5′-AGGACTTGGCCTGGAAGTTCTT-3′	460			
T1 α	Forward	5′-GCCATCGGTGCGCTAGAAGATGATCTT-3′	53	205	79.3	76
	Reverse	5′-GTGATCGTGGTCGGAGGTTCCTGAGGT-3′	257			
GABRP	Forward	5′-AAATTTCTGGCGACAATGTCAACTA-3′	1,160	101	80.7	74
	Reverse	5′-GTAATCAATGATTCTGCCGATCTTT-3′	1,260			
18S rRNA	Forward	5′-TCCCAGTAAGTGCGGGTCATA-3′	1,655	101	81.2	78
	Reverse	5′-CGAGGGCCTCACTAAACCATC-3′	1,755			
GABAR α1	Forward	5′-AAACATATCTGCCATGCATAATGAC-3′	821	303	NA	NA
	Reverse	5′-CTTTCTTTGGCTTTTCTGGAACC-3′	1,123			
GABAR α3	Forward	5′-GTTGTTGGGACAGAGATAATCCG-3′	941	351	NA	NA
	Reverse	5′-TCGCTTGGTGAAGTAGTTGACAG-3′	1,291			
GABAR β1	Forward	5′-AACTGGAGATGAACAAAGTCCAGG-3′	1,135	301	NA	NA
	Reverse	5′-GACCACTTGTCTATGGAGTTCACGT-3′	1,435			
GABAR β2	Forward	5′-ATGCCATCCATTCTGATTACCATC-3′	827	303	NA	NA
	Reverse	5′-CTCGTTGTTGGCATTAGCAGC-3′	1,129			
GABAR β3	Forward	5′-GACATGTACCTG ATGGGTTGCTT-3′	989	301	NA	NA
	Reverse	5′-TGCTCTGTTTCCTATACTGGATTCCT-3′	1,289			
GABAR γ3	Forward	5′-AACATGGTGGGTTTGATATGGATA-3′	537	351	NA	NA
	Reverse	5′-TGCAGATGTTGTCACGATTTCTG-3′	887			

SP-B surfactant protein B, *SP-C* surfactant protein C, *GABRP* GABA receptor π-subunit, *GABAR* GABA receptor, *NA* not applicable.

2.3.7 RT-PCR

The primers used for RT-PCR are listed in Table 1. RT-PCR was performed under the following thermal conditions: 95℃ for 2 min; 35–45 cycles of 95℃ for 20 s, 55℃ for 30 s, 72℃ for 30 s; a final extension at 72℃ for 6 min. PCR products were separated on 1.2% agarose gel and visualized with UV light.

2.3.8 Western blot

Lavaged rat lungs and freshly isolated or cultured type II cells were immediately homogenized with lysis buffer (250 mM sucrose, 1% Triton X-100, 10 mM EGTA, 2 mM EDTA, 50 mM TRIS–HCl pH 7.4, 200 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride). Protein concentrations were measured by the BIO-Rad protein assay. The lysate (30 μg) was separated by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto a polyvinylidine difluoride membrane. After being blocked with 5% non-fat milk in a buffer containing 20 mM TRIS–HCl pH 7.4, 150 mM NaCl, and 0.05% Tween 20 (blocking solution), the membrane was incubated with goat anti-GABRP antibodies (1:200 dilution) in the blocking solution at 4℃ overnight. After being washed, the blot was incubated with HRP-conjugated anti-goat IgG (1:4,000 dilution) for 1 h. The membrane was reprobed with anti-β-actin antibodies (1:1,000 dilution) and HRP-conjugated anti-rabbit IgG (1:5,000 dilution) in order to normalize protein loading. Immunoreactive bands were visualized with the ECL Western blot kit.

2.3.9 Cell morphology and immunocytochemistry

Type II cells were maintained on various matrices and under various culture

conditions as described above. At the indicated times, the cells were washed with phosphate-buffered saline and observed with an inverted Nikon microscope. The cells from the DG, AL, and AG cultures were released with trypsin and cytospun onto coverslips. Immunocytochemistry was carried out as previously described (Chen et al. *2004*). The dilutions for anti-GABRP and anti-LB180 antibodies were 1:100 and 1:200, respectively. The dilutions for both FITC-conjugated anti-mouse IgG and Alexa 568-conjugated anti-goat IgG were 1:200.

2.3.10 Statistics

Results are expressed as means ± SE. A statistic analysis was performed with SAS Window V8 by one-way analysis of variance (ANOVA). Multiple comparisons (Dunnett's or Fisher least significance difference) were used to analyze the data further if a significant difference was indicated by ANOVA. Significance was taken when $P<0.05$.

2.4 Results

2.4.1 Absolute GABRP mRNA abundance in rat lung and type II cells

Real-time PCR is a highly sensitive, accurate, reproducible quantitative method compared with traditional semi-quantitative methods such as RT-PCR (Freeman et al. *1999*; Ginzinger *2002*). To quantitate GABRP gene expression in type II cells, we first developed a real-time PCR protocol by using the SYBR Green detection and absolute standard method (Simpson et al. *2000*; Yin et al. *2001*). The standard curve was linear over a wide range of PCR product standard inputs (10 to 10^8 copies/25 μl reaction) with a correlation coefficient (R^2) value of

0.997. The amplification was specific because only a single peak was detected by dissociation analyses. The abundance of GABRP mRNA in the rat lung and type II cells was 26±5 and 88±19 copies per 1 million 18S rRNAs, respectively, indicating a 3.33-fold enrichment in type II cells.

2.4.2 Gradual decrease in GABRP mRNA expression of type II cells cultured on plastic plates

The amounts of mRNA for SP-B and SP-C (type II cell markers) were dramatically decreased after cells were cultured on plastic plates, whereas T1 α mRNA (a type I cell marker) increased (Fig. 2.1). These results indicated that the type II cells cultured on plastic plates rapidly lost their phenotype and converted to type-I-like cells. Similar to the expression of mRNAs for SP-B and C, GABRP mRNA expression decreased in type II cells after culture on plastic plates. The rate of GABRP mRNA decrease was slower than these of SP-B and SP-C. Approximately 74% of GABRP mRNA was retained after overnight culture, whereas both SP-B and SP-C mRNA expression levels decreased more than 90%.

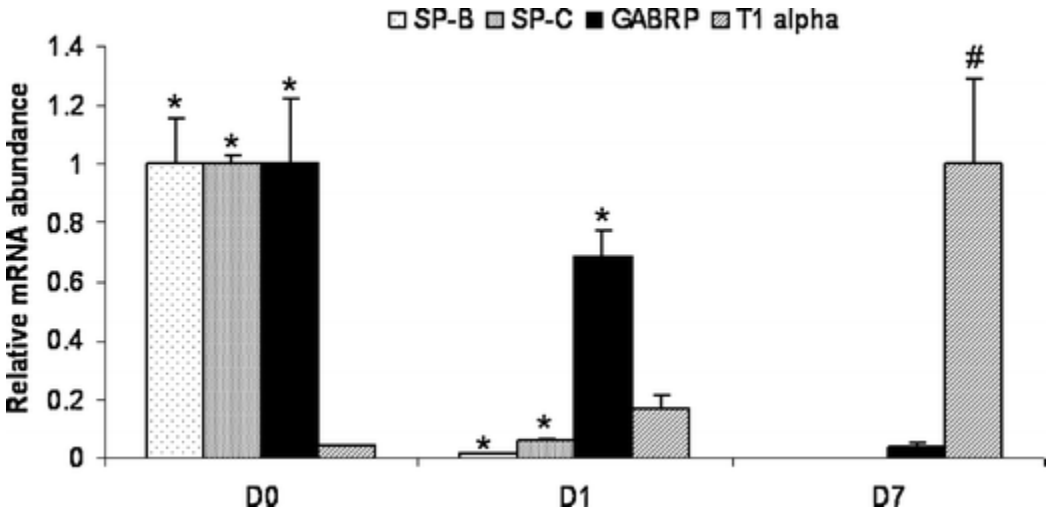

Fig. 2.1 Time course of GABRP mRNA expression in type II cells cultured on plastic plates. Freshly isolated type II cells (*D0*) were cultured on plastic plates for 1 day (*D1*) or 7 days (*D7*). The mRNA levels of *GABRP*, *SP-B*, *SP-C*, and *T1* α were quantified by using real-time PCR and normalized with 18S rRNA. The results were presented as a percentage of *D0* for *GABRP*, *SP-B*, and *SP-C* and as a percentage of *D7* for T1 α (*T1 alpha*). Data shown are means±SE from ≥ 3 independent cell preparations, each assay being performed in duplicate. **P*<0.05 vs. *D7*; #*P*<0.05 vs. *D0*. ANOVA, Dunnett's analysis

2.4.3 Partial retention of GABRP mRNA expression in the presence of Matrigel and KGF

Culture with Matrigel maintains type II cell characteristics, including cuboidal morphology and type II cell markers (Mason et al. *2002*; Shannon et al. *1987*). We therefore wanted to determine whether the GABRP expression level was retained in type II cells cultured on Matrigel. We found that type II cells retained 45% of GABRP mRNA expression after 7-day culture on Matrigel (Fig. 2). In comparison, only 31% of SP-B and 8% of SP-C were retained under the same conditions. The amount of T1 α mRNA did not increase when type II cells were cultured on Matrigel.

KGF is a mitogenic growth factor that specifically acts on epithelial cells. KGF inhibits the appearance of type I cell phenotype and favors the preservation of type II cell phenotype (Borok et al. *1998a*, *1998b*; Xu et al. *1998*). GABRP mRNA was expressed at a low level when type II cells were cultured on collagen gel for 8 days. However, when KGF was added to the culture medium at day 4 and the culture was continued for an additional 4 days, 49% of the GABRP mRNA expression was retained. Similar results were obtained for SP-B and opposite results for T1 α (Fig. 2.2).

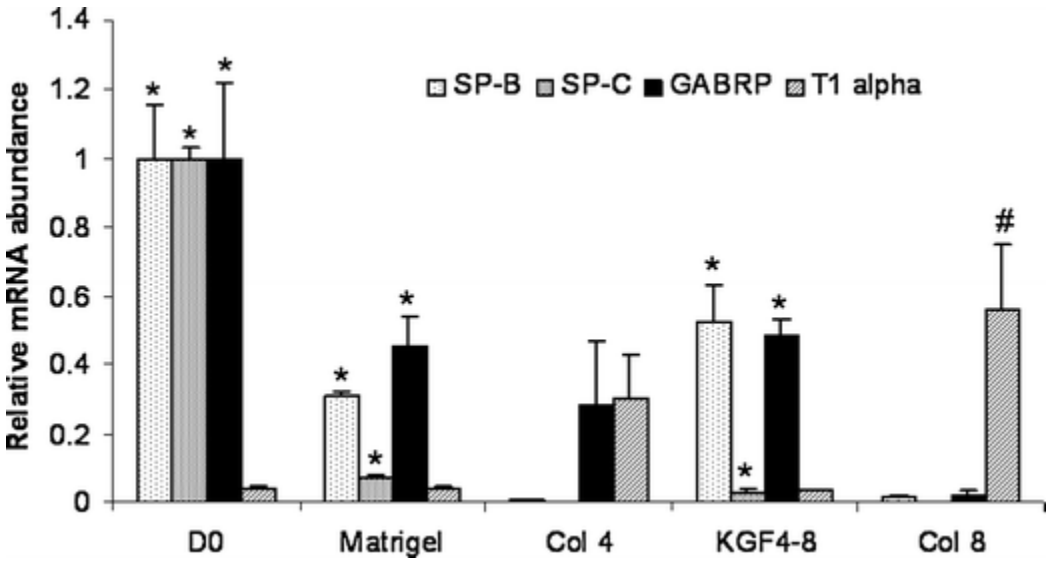

Fig. 2.2 Effects of Matrigel and KGF on GABRP mRNA expression in cultured type II cells. Freshly isolated type II cells (*D0*) were cultured on Matrigel for 7 days or on collagen gel for 4 days (*Col 4*) or 8 days (*Col 8*). For *KGF4-8*, type II cells were cultured on collagen gel for 4 days, then 10 ng/ml of *KGF* was added, and the culture was continued for additional 4 days. The mRNA levels of *GABRP*, *SP-B*, *SP-C*, and T1 α (*T1 alpha*) were quantified by using real-time PCR and normalized with 18S rRNA. The results were presented as a percentage of *D0* for *GABRP*, *SP-B*, and *SP-C* and as a percentage of *D7* for T1 α (*T1 alpha*). Data shown are means±SE from ≥ 3 independent cell preparations, each assay being performed in duplicate. $^{*}P<0.05$ vs. *Col 8*; $^{\#}P<0.05$ vs. *D0*. ANOVA, Dunnett's analysis

2.4.4 Maintenance of GABRP mRNA in type II cells cultured on DG

DG was reported to improve type II cell phenotype and inhibit the expression of the type I cell marker, T1 α (Borok et al. *1998a*; Danto et al. *1995*), possibly because of the effect of mechanical stretching on cells (Dobbs and Gutierrez *2001*; Edwards *2001*; Gutierrez et al. *2003*; Sanchez-Esteban et al. *2001*). When type II cells were cultured on AG for more than 1 week (AG8 and AG14), the expression level of GABRP mRNA was low (Fig. 3). In contrast, type II cells cultured on DG for 8 days (DG8) expressed GABRP mRNA at a significantly higher level. Culture of the cells on DG for a longer time further increased the expression of GABRP mRNA. On DG at day 14, the relative expression level of GABRP was even higher than that of the freshly isolated type II cells. DG also induced the re-expression of GABRP mRNA in the cells that had undergone trans-differentiation to type-I-like cells on AG (AG-DG). The expression of both SP-B and SP-C only retained 3%–18% of that of the freshly isolated type II cells on DG. The expression of T1 α was higher on AG and lower on DG.

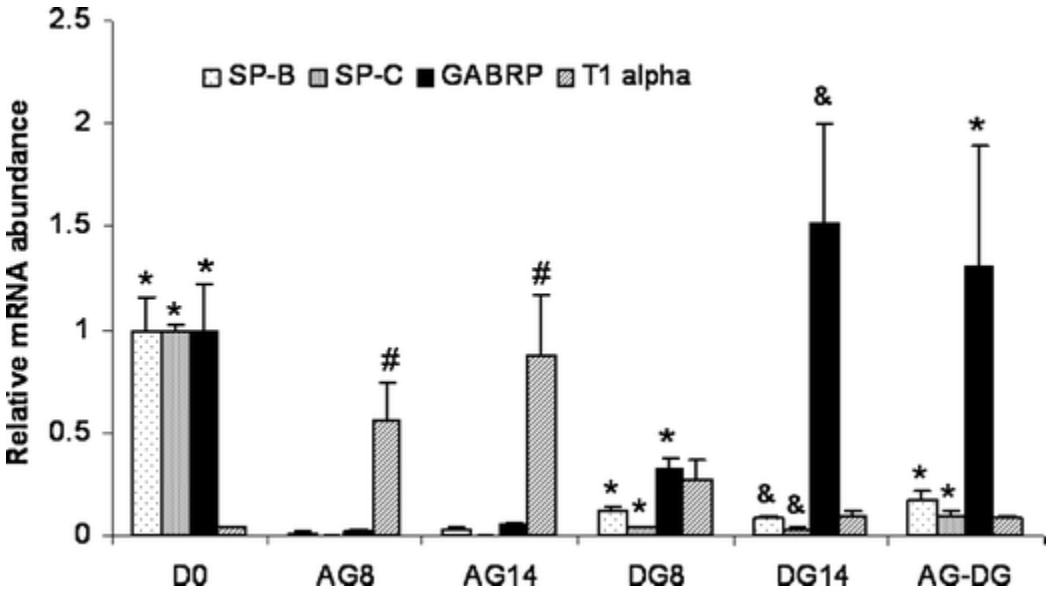

Fig. 2.3 Effects of detached gel (DG) and attached gel (AG) on GABRP mRNA expression. Type II cells (*D0* day 0) were cultured on AG for 8 days (*AG8*) or 14 days (*AG14*) or on DG for 8 days (*DG8*) or 14 days (*DG14*). For *AG–DG*, type II cells were cultured on AG for 8 days and then on DG for an additional 6 days. The mRNA expression levels of *GABRP*, *SP-B*, *SP-C*, and T1 α (*T1 alpha*) were quantified by using real-time PCR and normalized to 18S rRNA. The results were presented as a percentage of *D0* for *GABRP*, *SP-B*, and *SP-C* and as a percentage of *D7* for T1 α (*T1 alpha*). Data shown are means±SE from ≥ 3 independent cell preparations, each assay being performed in duplicate. $^{*}P<0.05$ vs. *AG8*; $^{\#}P<0.05$ vs. *D0*; $^{\&}P<0.05$ vs. *AG14*. ANOVA, Dunnett's analysis.

2.4.5 Up-regulation of GABRP mRNA expression in type II cells cultured on an apical-air surface

Cultivation of type II cells on an apical-air surface has been reported to maintain the type II cell phenotype (Dobbs et al. *1997*). This culture system simulates a normal alveolar environment in vivo. We examined the GABRP mRNA expressions of type II cells in this culture system. As expected, the GABRP mRNA expression level was low when type II cells were cultured LL for 14 days (Fig. 2.4). However, GABRP mRNA expression in the type II cells cultured on AL for 14 days was 12-fold higher than that of the freshly isolated type II cells. Under the same conditions, SP-B and SP-C mRNA expression levels were only 50% and 41% of the freshly isolated type II cells, respectively. T1 αmRNA was lower on AL than LL.

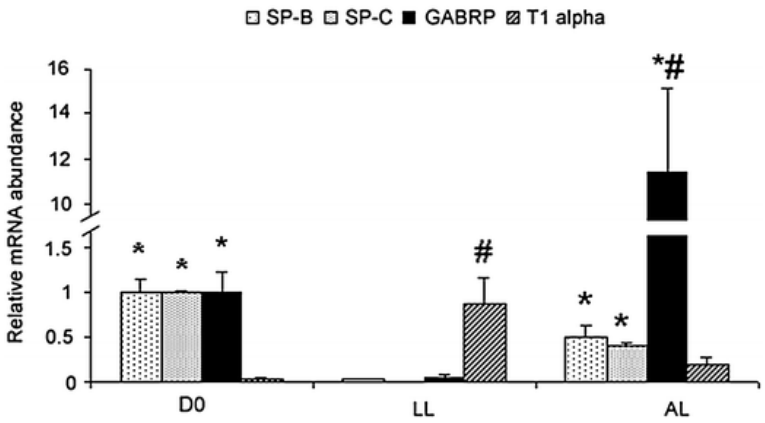

Fig. 2.4 Effects of air–liquid (*AL*) and liquid–liquid (*LL*) interface on GABRP mRNA expression. Type II cells (*D0* day 0) were cultured for 14 days. The mRNA expression levels of *GABRP*, *SP-B*, *SP-C*, and T1 α (*T1 alpha*) were quantified by using real-time PCR and normalized to 18S rRNA. The results were presented as a percentage of *D0* for *GABRP*, *SP-B*, and *SP-C* and as a percentage of *LL* for T1 α (*T1 alpha*). Data shown are means±SE from 4 independent cell preparations, each assay being performed in duplicate. $^{*}P<0.05$ vs. *LL*, $^{\#}P<0.05$ vs. *D0*. ANOVA, Dunnett's analysis.

2..4.6 Up-regulation of GABRP protein in type II cells cultured on an apical-air surface

Western blot was performed to determine the GABRP protein level in type II cells. The expected 50-kDa band was observed in the lung and type II cells (Fig. 2.5a). The GABRP band was then quantitated by densitometry and normalized to β-actin. GABRP protein was enriched in type II cells compared with whole lung tissue (Fig. 2.5b). Macrophages are the main source of contaminations in isolated type II cells. However, little GABRP protein was observed in macrophages. No GABRP protein was detected in type II cells cultured on plastic plates for 7 days. When type II cells were cultured on AL for 14 days, the GABRP protein level increased 4.3-fold, indicating that the AL system also up-regulates GABRP protein expression (Fig. 2.5c, d).

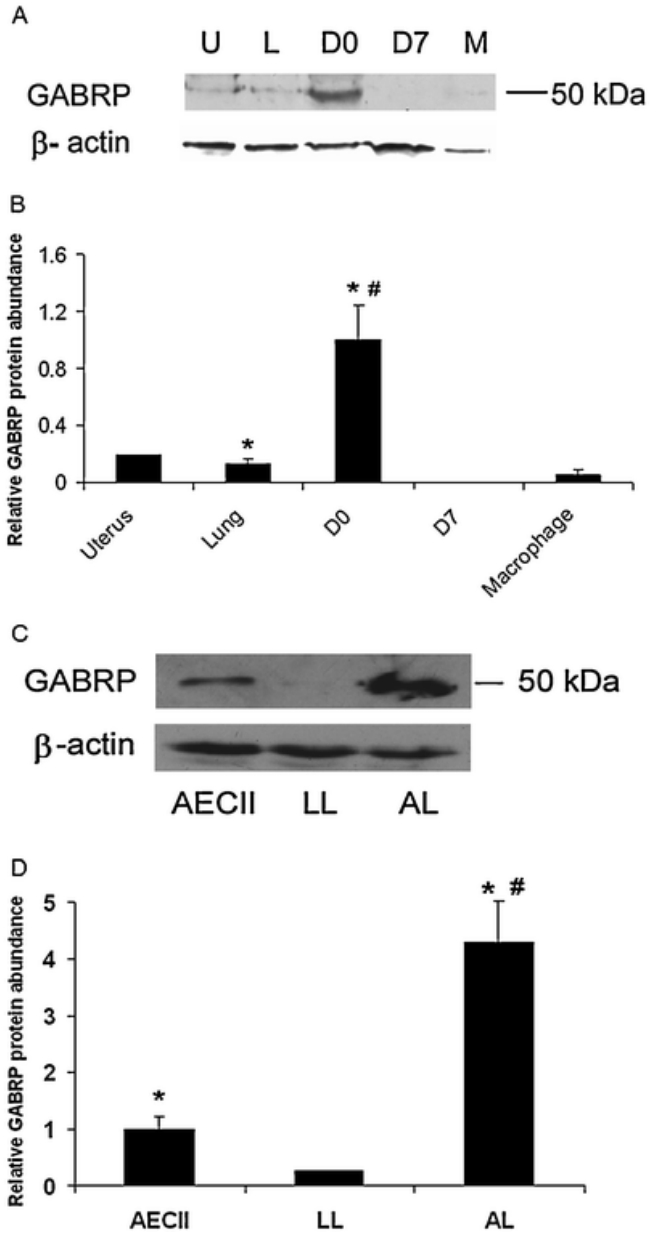

Fig. 2.5 GABRP protein expression in the lung and in freshly isolated and cultured type II cells. **a** Representative Western blot of *GABRP* protein expression in the uterus (*U*), lung (*L*), freshly isolated type II cells (*D0*), type II cells cultured on plastic plates for 7 days (*D7*), and macrophages (*M*). The blot was reprobed with β-actin as a sample loading control. **b** GABRP protein was quantitated by densitometry and normalized to β-actin. The results are expressed as a percentage of *D0*. Data shown are means±SE ($n \geq 3$) except the uterus ($n=1$). $^{*}P<0.05$ vs. *D7*; $^{\#}P<0.05$ vs. lung. ANOVA, Fisher least significance difference analysis.

c Representative Western blot of *GABRP* in freshly isolated type II cells (*AECII*) or cultured on liquid–liquid (*LL*) or air–liquid interface (*AL*). **d** Quantitation of GABRP proteins by densitometry. The results were normalized to β-actin and expressed as a percentage of *AECII*. Data shown are means±SE ($n \geq 4$ for all conditions). $^{*}P<0.05$ vs. *LL*; $^{\#}P<0.05$ vs. *AECII*. ANOVA, least significance difference analysis

2.4.7 Co-localization of GABRP protein with LB-180 in cuboid-shaped type II cells

Cell morphology under various culture conditions was observed with an inverted microscope. Type II cells became squamous in shape after being cultured on AG [Fig. 2.6a(a)], consistent with their phenotypic change to type I-like cells. Type II cells cultured on Matrigel maintained their cuboidal shape but formed clusters [Fig. 2.6a(b)]. The addition of KGF to the medium [Fig. 2.6a(c)] or DG [Fig. 2.6a(d)] slowed down the trans-differentiation from type II cells to type-I-like cells, and the cells assumed a more cuboidal shape than those on AG. On AL, the cells exhibited typical type II cell morphology [Fig. 2.6a(e)]. Double-labeling showed that GABRP protein was co-localized with a type II cell marker, LB-180, on the cuboid-shaped type II cells from AL or DG cultures (Fig. 2.6b, arrows). However, a few type II cells (approximately 20%) on the DG trans-differentiated into type-I-like cells and lost both LB-180 and GABRP protein expression (Fig. 2.6b, arrowheads). The negative control without primary antibodies gave no signals on both channels. The cells from AG did not show staining for either GABRP or LB-180.

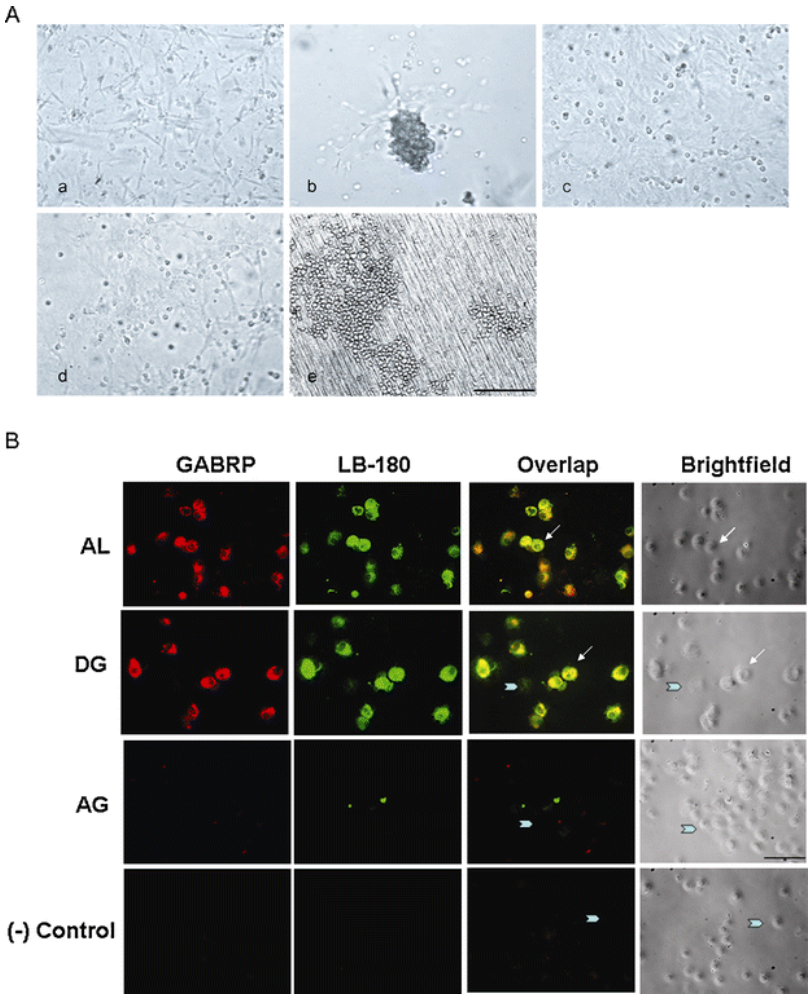

Fig. 2.6 Cell morphology and immunocytochemistry of cultured type II cells. a Freshly isolated type II cells were cultured on AG for 7 days (*a*), Matrigel for 7 days (*b*), AG for 4 days and an additional 4 days with 10 ng/ml KGF (*c*), DG for 8 days (*d*), or AL for 7 days (*e*). Cell morphology was observed with an inverted microscope. *Bar* 50 μm. **b** Cultured type II cells were released from *AL*, *DG*, and *AG*, and cytospun to coverslips. Double-labeling was performed with anti-GABRP (*red*) and anti-LB-180 (*green*) antibodies (*arrows* co-localization of GABRP and LB-180 in cuboid-shaped cells, *arrowheads* no GABRP or LB180 expression on the trans-differentiated type-I-like cells on *DG* and *AG*, *(–)Control* negative control with no primary antibodies on the cells from *AL*). *Bar* 20 μm

2.4.8 Detection of other $GABA_A$ receptor subunit mRNAs in lung and type II cells

Because GABRP has to be co-assembled with other subunits to form functional channels, we performed RT-PCR to examine whether other $GABA_A$ receptor subunits were expressed in lung or type II cells. The mRNAs of α1, α3-, β2-, γ2-, and γ3-subunits were detected in both lung tissue and type II cells (Fig. 2.7). Neither β1-subunits nor β3-subunits were expressed in the lung and type II cells.

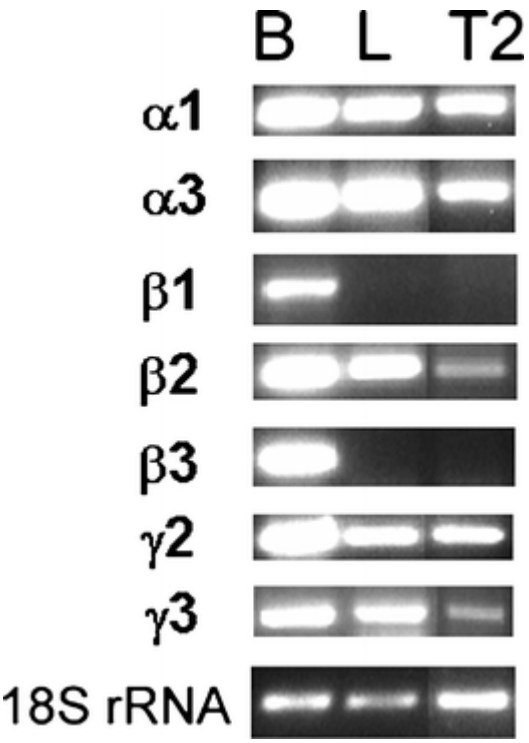

Fig. 2.7 Detection of $GABA_A$ receptor subunits in type II cells. RT-PCR was performed to detect mRNA expression of $GABA_A$ receptor α1-, α3-, β1-, β2-, β3-, γ2, and γ3 in brain (*B*), lung (*L*), and freshly isolated type II cells (*T2*). *18S rRNA* was used as a control.

2.5 Discussion

In the present study, we have demonstrated, by quantitative real-time PCR and Western blot, that GABRP expression is regulated by culture conditions and that expression levels are correlated with alveolar epithelial cell phenotypes. The culture conditions that maintain a type II cell phenotype favor the expression of GABRP, whereas culture conditions that promote trans-differentiation into type-I-like cells decrease the expression of GABRP. An AL interface markedly up-regulates the GABRP expression in type II cells. Furthermore, the mRNAs of $GABA_A$ receptor α1-, α3-, β2-, γ2-, and γ3-subunits have been detected in type II cells.

Culturing on plastic dishes is an established method to induce type II cells to trans-differentiate into type-I-like cells (Borok et al. *1998a*, *1998b*; Shannon et al. *1992*). Some of the functionally important genes related to ion transport are differentially expressed during this process, such as those for Na^+–K^+-ATPase subunits (Ridge et al. *1997*). A decrease in GABRP expression during the trans-differentiation of type II cells to type-I-like cells indicates reduced Cl^- transport in type I cells. This property can also be used to reveal the differentiation status of type II cells (Chen et al. *2004*). A change in the composition of GABA receptors has been observed during the differentiation of human teratocarcinoma NT2 into neuron-like cells (Neelands et al. *1999*).

KGF is an epithelial cell-specific mitogen that stimulates the proliferation of alveolar epithelial type II cells in vitro and in vivo (Panos et al. *1993*; Ulich et al. *1994*). It protects the lung from injury caused by hyperoxia, bleomycin, and other

insults (Mason et al. *1996*; Panos et al. *1995*; Yano et al. *1996*; Yi et al. *1996*). Consistent with these previous reports, we have found that KGF increases SP-B mRNA levels more than SP-C levels (Borok et al. *1998a*, *1998b*). Furthermore, KGF also enhances GABRP expression. The physiological significance of the regulation of GABRP expression by KGF is unclear. In the fetal lung, KGF stimulates fluid secretion via Cl^- secretion that is independent of the cystic fibrosis transmembrane conductance regulator (Zhou et al. *1996*). The effects of KGF on Cl^- transport in the lung may be attributable to the GABA receptors. The effect of KGF on GABRP expression is probably exerted through the stabilization of mRNA, similar to the KGF regulation of CLC-2 in fetal distal lung epithelial cells (Blaisdell et al. *1999*).

Maintenance of type II cells on DG reduces cell spreading compared with culturing these cells on AG (Danto et al. *1995*; Shannon et al. *1992*). However, this system appears to generate a heterogeneous cell population, which may be a result of the uneven contraction of the collagen gel. GABRP protein has only been detected in the cells that retained a type II cell phenotype and not in the few trans-differentiated cells (approximately 20%) as indicated by double-labeling [Fig. 6a(d)]. This result reinforces our previous conclusion of GABRP as a novel type II cell marker (Chen et al. *2004*). The effects of DG on gene expression may occur at the transcriptional and post-transcriptional levels and may also involve the cytoskeletal network (Shannon et al. *1992*; Shannon et al. *1998*).

Culturing type II cells on an apical-air surface system mimics the in vivo conditions expected in the lung (Dobbs et al. *1997*; Mason et al. *2002*). The type

II cells in this system not only retain their typical cell morphology, but also markedly up-regulate the expression of GABRP mRNA and protein. The enhanced surfactant protein mRNA expression on AL compared with LL appears to involve cytoskeletal elements (Dobbs et al. *1997*). However, because GABRP mRNA expression on AL is 12-fold higher than that of freshly isolated type II cells, the up-regulation may not be simply attributable to the maintenance of the type II cell phenotype but may involve transcriptional regulation, since the GABRP protein level is also up-regulated in type II cells cultured on AL. AL has been shown to affect ion channels in type II cells. A low-conductance, highly Na^+-selective channel has been observed in type II cells maintained on AL, whereas a high-conductance, non-selective cation channel is present in type II cells cultured on plastic plates (Jain et al. *2001*). Basal amiloride-sensitive Na^+ transport in type II cells on an apical-air surface increases compared to that on LL (Liu and Mautone *1996*). Whether the differences observed are attributable to the regulation of Na^+ channel gene expression or the modulation of channel activity is unknown.

In addition to mediating rapid inhibitory transmission in the CNS, $GABA_A$ receptors may have roles in the regulation of cell volume and the maintenance of fluid balance (Hill et al. *1998*; Limmroth et al. *1996*; Panek et al. *2002*). The functions of GABRP in rat alveolar type II cells are unknown. Because we have also detected other necessary $GABA_A$ receptor subunits in rat type II cells (Fig. 7), GABRP may co-assemble with those subunits to form functional $GABA_A$ receptors and contribute to surfactant secretion or fluid transport in the lung. The

physiological ligand activating the channel is probably GABA, which has been found in the proximal and middle portion of the lung (Chapman et al. *1993*). Whether type II cells synthesize GABA is unclear. In addition to the CNS, endocrine organs also synthesize GABA locally, including pancreatic islets and the pituitary (Braun et al. *2004*; Mayerhofer et al. *2001*).

In summary, the present study indicates that GABRP is abundantly expressed in alveolar type II cells and is regulated by culture substrata, growth factor, DG, and an air-apical surface. The functional roles of GABRP in type II cells require further investigations.

2.6 Acknowledgement

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Chapter 3

CHARACTERIZATION OF IONOTROPIC GABA RECEPTOR SUBUNITS IN DEVELOPING LUNGS AND TYPE II CELLS AND HYPEROXIA-EXPOSED LUNGS

3.1 Abstract

Cl^- transport is essential for fetal lung development and adult lung fluid homeostasis. Ionotropic GABA receptors act as the most important inhibitory Cl^- channels in the central nervous system but their roles in the lung are not clear. To examine their functions in the lung, we studied the ontogeny and hyperoxic regulation of GABA receptors in rat lungs and type II cells. By using real-time PCR, we identified 17 GABA receptor subunits (α1-6, β1-2, γ 1-3, θ, ε, π, and ρ1-3) in fetal lungs and type II cell. According to their expression patterns during the development, these subunits can be grouped into 4 clusters in both lungs and type II cells. In the lung, the subunits in one of the 4 clusters (α2, α5, α6, β1, γ3, and ρ3) were relatively high during fetal stages and low in the adult. Another cluster (α3, γ1, γ2, θ, and ρ1) was steadily expressed throughout fetal to adult stages and had a slight up-regulation at birth. In type II cells, one of the 4 clusters (including subunit α1, α2, α4, β1, β2, γ1, θ, ρ1, and ρ3) exhibited a peak at birth.

Hyperoxia exposure down-regulated almost all the subunits except for the π-subunit in adult lungs. In summary, the dynamic changes of the expression of GABA receptors in rat lungs and type II cells during lung development and hyperoxia-exposure suggest their roles in pulmonary fluid homeostasis.

3.2 Introduction

Cl^- channels play important roles in the lung throughout an animal's life. During the fetal stages, the lung is a fluid-secreting organ. The main driving force for fluid secretion is the active secretion of Cl^- into the developing lungs. Therefore, Cl^- channels are indispensable for fetal lung development and morphogenesis (Folkesson et al. 1998;Olver et al. 2004;Bland and Nielson 1992). In adults, the lung maintains a "dry" state in which the apical surface is covered by only a thin layer of alveolar surface fluid. Cl^- channels are essential for maintaining the composition and volume of the alveolar surface fluid which is necessary for the proper air exchange and airway defenses (Brochiero et al. 2004).

Several Cl^- channels have previously been studied in the fetal lung. Cystic fibrosis transport regulator (CFTR) (Harris et al. 1991), the voltage-gated Cl^- channels, ClC2 (Thiemann et al. 1992), ClC3 (Lamb et al. 2001), and ClC5 (Edmonds et al. 2002), and Na^+-K^+-Cl^- cotransporter (NKCC) (Rochelle et al. 2000) were detected in the fetal lungs, distal airway epithelial cells, and alveolar epithelial cells. CFTR and ClC have been shown to be involved in fluid homeostasis in the fetal lungs (Brochiero et al. 2004;Fang et al. 2002;Blaisdell et

al. 2000). In a previous study, these two channels appeared in early gestation and were maintained relatively high throughout the fetal stage. The lung undergoes a change from fluid secretion to absorption at birth. It has been shown that the level of some Cl^- channels drop from late gestation and remain low during adult stages (Harris et al. 1991;Murray et al. 1996;Lamb et al. 2001;Murray et al. 1995).

The lung is sensitive to hyperoxia exposure. We, along with others, have shown that the exposure of rats to >95% O_2 for 48-60 hrs leads to mild to moderate lung injury, whereas 72 h-exposure results in severe injury and pulmonary edema (Narasaraju et al. 2003). An increase in the expression and activity of Na^+ transporters is essential for the re-absorption of edema fluid (Yue et al. 1995;Johnson et al. 1998;Carter et al. 1997). Efficient removal of Na^+ from the alveoli requires the coordination of Cl^- channels (O'Grady et al. 2000). Though CFTR has been shown to be important in alveolar fluid clearance in pathological conditions, such as acute volume-overload pulmonary edema (Fang et al. 2002), the relationship between other Cl^- channels and alveolar fluid clearance is not clear.

More and more Cl^- channels are being identified in the lung and alveolar epithelial cells. However, the CFTR and NKCC knock-out mice were not fatal (Gillie et al. 2001;Snouwaert et al. 1992), suggesting that other Cl^- channels may compensate for those channels. Ionotropic γ-aminobutyric acid (GABA) receptors are the most important Cl^- channels in the central nervous system (CNS) and their expression was also found in peripheral organs. These receptors mediate a

fast inhibitory neurotransmission in CNS but their functions in peripheral organs were not extensively studied (Akinci and Schofield 1999). Ionotropic GABA receptors can be categorized into $GABA_A$ and $GABA_C$ receptors based on their subunit compositions and pharmacological properties (Bormann 2000). Nineteen GABA receptor subunits have been cloned from rats, which include α1-6, β1-3, γ1-3, ρ1-3, δ, θ, ε, and π (Whiting et al. 1999). We have recently identified the $GABA_A$ receptor π-subunit as a novel alveolar epithelial type II cell marker (Chen et al. 2004b). The expression of α1-, α3-, β2-, γ2-, and γ3-subunit were also detected in adult type II cells (Jin et al. 2005). In this study, we investigated the developmental and hyperoxic regulation of all the ionotropic GABA receptor subunits in the lung and type II cells. Our results provide the first documentation on the dynamic changes of GABA receptor subunits in the lung and type II cells under two conditions which are closely related to the alveolar fluid balance.

3.3 Materials and Methods

3.3.1 Materials

Pregnant Sprague-Dawley (SD) and adult male rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). Oklahoma State University Animal Use and Care Committee approved all the animal protocols used in this study. Nylon meshes (160, 37, and 15 μm) were from Tetko (Elmsford, NY). Minimum essential medium (MEM), trypsin, collagenase V, DNase I, rat IgG were from Sigma-Aldrich (St Louis, MO). M-MLV reverse transcriptase was purchased from Invitrogen (Calsbad, CA). Polyclonal goat anti-

GABA receptor π subunit (anti-GABRP) was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-rat leukocyte common antigen antibodies (anti-LC) were from Accurate (Westbury, NY). Polyclonal anti-rat T1 α antibody was raised in rabbits by Custom Service of Affinity Bio-reagents (Golden, CO) and affinity-purified. Goat anti-rabbit IgG BioMag® beads were from QIAGEN (Valencia, CA, USA). Sheep anti-rat IgG magnetic beads and goat anti-mouse IgG Dynabeads were from Dynal Biotech (Lake Success, NY). TRI reagent was from Molecular Research Center (Cincinnati, Ohio). DNA-free™ DNase was from Ambion (Austin, Texas). Random hexamers primers were from Promega (Madison, WI). Primers for real-time PCR were synthesized by MWG Biotech Inc (High Point, NC). QuantiTech™ SYBR Green PCR master mix was from QIAGEN (Foster city, CA). GENECLEAN Turbo for PCR was obtained from Qbiogene (Carlsbad, CA).

3.3.2 Collection of fetal lung tissues and isolation of fetal alveolar type II cells

Fetal lung tissue collection and type II cell isolation were carried out as described previously (Fraslon-Vanhulle et al. 1991). Briefly, fetal lungs were removed from pregnant S.D. rats at gestational day of 18, 19, 20, and 21.The fetal lungs from the same mother were pooled as one group. After removing the trachea, bronchi, thymus, and hearts, 3-4 fetal lungs were frozen in liquid nitrogen for RNA isolation; the other lungs were used for fetal type II cell isolation. The fetal lungs were chopped and digested with an enzyme solution (1 mg/ml of collagenase V, 0.2 mg/ml of trypsin, and 20 μg/ml of DNAse I in MEM)

for about 20~60 min until no tissue was left. The cell mixture was filtered sequentially through 160 and two 37 nylon mesh. Thereafter, the cells were further purified by panning on a bacterial culture plastic dish for four times (40 min each time) at a density of 35 million cells per 100 mm dish for removing fibroblasts. And then a hypotonic solution (75 mM KCl) was applied to the cell suspension for 2-5 min to remove red blood cells. The protocol for type II cell isolation from neonate rats was the same as that for fetal lungs, except that 0.036 units/ml elastase was included in the digestion enzyme solution. The purity of the isolated fetal type II cells was 90-95% as determined by alkaline phosphatase staining (Miller and Hook 1990) and the viability was >90% as determined by Trypan blue exclusions.

3.3.3 Isolation of highly pure adult type II cells

Highly pure type II cells were isolated from the lungs of adult male SD rats (180-200 g), according to our recently improved methods (Chen et al. 2004a). The perfused lungs were lavaged, digested with 3 units/ml elastase, chopped, and filtered through 160-, 37-, and two 15-size nylon meshes. The cell mixture was panned twice on rat IgG-coated plates (40 min each). The cell suspension was further incubated with 40 μg/ml of rat IgG at room temperature for 15 min, and then sheep-anti-rat IgG magnetic beads (100 μl/rat) were applied to remove macrophages. The cells were again incubated with anti-LC (40 μg/ml) and anti-rat T1 α (40 μg/ml), followed by incubation with goat anti-mouse Dynabeads (100 μl/rat) and goat anti-rabbit IgG BioMag® beads (500 μl/rat) to remove leukocytes and type I cells. The purity of the resulting type II cell preparation was 95~98%

and viability was >98% in average.

3.3.4 Exposure of animals to hyperoxia and collection of lung tissues

Male rats (250-275 g) were exposed to hyperoxia, as previously described (Narasaraju et al. 2003). Rats were housed for 1 week and then exposed to >95% oxygen in a sealed Plexiglas chamber for 48, 60, and 72 h. The control rats were exposed to room air. All the animals were allowed free access to food and water. At the end of each exposure time, animals were sacrificed. The lungs were perfused and lavaged with normal cold saline and immediately homogenized with TRI reagent for RNA isolation.

3.3.5 Absolute quantitative real-time PCR

Total RNA was extracted from tissue or cells with TRI reagents. Absolute quantitative real-time PCR was performed, as previously described (Jin et al. 2005). Briefly, the total RNA was digested with DNAase I to remove genomic DNA. Absence of DNA contamination in the total RNA samples was proven by that no PCR product was found from the amplification of the non-reverse transcribed RNA with 18S rRNA primers for 40 cycles. The integrity of the RNA was determined by running the RNA samples on a 1.2% agarose gel, which showed that the intensity of the 28S rRNA bands were twice of those of the 18S rRNA bands. The total RNA was reverse-transcribed into cDNA with M-MLV and random hexamer. The primers for real-time PCR were designed with Primer Express 1.5 and are listed in Table 3.1. The standards used for constructing the standard curves were amplified by traditional PCR and purified with GENECLEAN Turbo. Real-time PCR was performed on an ABI 7700 system

(Applied Biosystems, Foster city, CA) using SYBR Green detection. The copy numbers of target genes in cDNA samples were normalized with18S rRNA and expressed as log values.

Table 3.1 Primers used for real-time PCR for GABA receptor subunits.

Subunit	Direction	Primer sequence	Start base	Amplicon Tm (^{o}C)	Data acquisition (^{o}C)
α1	Forward	5'-AGAAGTCAAGCCTGAGACAAAACC-3'	1248	78.7	76
	Reverse	5'-CAAATAGCAGCGGAAAGGCTATT-3'	1348		
α2	Forward	5'-TCTGGACTCCTGACACCTTCTTT-3'	305	78.6	76
	Reverse	5'-CAATGTTCCGTCATCCTGGATT-3'	405		
α3	Forward	5'-GTGTCAGCAAGGTTGACAAAATTT-3'	1551	78.7	76
	Reverse	5'-AGCGGATTCCCTGTTCACATAT-3'	1651		
α4	Forward	5'-CAGGAGTCTTCTGAAACCACTCCT-3'	1340	80.2	76
	Reverse	5'-GACCTCTTGCTGCTGCAGAGATA-3'	1442		
α5	Forward	5'-TTTCAATCTAGTTTACTGGGCAACA-3'	1604	76.2	76
	Reverse	5'-CATTTCTGTGCAAGTATGGTTTCC-3'	1704		
α6	Forward	5'-CTCTACCCAAAGTGTCCTATGCAA-3'	1244	78.0	76
	Reverse	5'-GTAGTTGACAGCTGCGAATTCAAT-3'	1344		
β1	Forward	5'-ATACCCTCTGGATGAGCAAAACTG-3'	578	77.7	76
	Reverse	5'-TTACTGCTCCCTCTCCTCCATTC-3'	678		
β2	Forward	5'-CTTCTGGAATATGCTTTGGTCAACT-3'	1034	78.3	76
	Reverse	5'-ATCTTCTCGTTGTTGGCATTAGC-3'	1134		
β3	Forward	5'-TCAACACTCACCTTCGAGAGACTCT-3'	936	78.4	78
	Reverse	5'-CAGAAGTGCCAGGAATACAAAGAC-3'	1036		
γ1	Forward	5'-AGACGGATGGGCTATTTCACAAT-3'	901	77.2	76
	Reverse	5'-GC AGGTACAGCATCTTTGTTGATC-3'	1001		
γ2	Forward	5'-ATGGACTCCTATGCTCGGATCTT-3'	1362	77.5	76
	Reverse	5'-ATAAAACCCAAACCTCCTCACAGA-3'	1462		
γ3	Forward	5'-AAAATTCAGTTGAGGCAGCTGAT-3'	790	78.9	76
	Reverse	5'-ACCTGCAGATGTTGTCACGATT-3'	890		
δ	Forward	5'-ATGCTGACTACAGGAAGAAACGG-3'	1031	80.5	76
	Reverse	5'-AGCAGAGAGGGAGAAGAGGACAA-3'	1131		
θ	Forward	5'-GTCCTGACTACCATCGACTCACAT-3'	901	78.7	76
	Reverse	5'-CTTGGCTGAAGAAAAGGTAGTTGAT-3'	1051		
ε	Forward	5'-CTCAATTTTCCAATGGATTCTCACT-3'	2070	75.0	73
	Reverse	5'-TTGAGTTTGAAATTCTCCCACTTG-3'	2170		
π	Forward	5'-AAATTTCTGGCGACAATGTCAACTA-3'	1160	77.2	74
	Reverse	5'-GTAATCAATGATTCTGCCGATCTTT-3'	1260		
ρ1	Forward	5'-TGGTCATAAAAAGAGCTATTTGCAA-3'	2991	74.6	73
	Reverse	5'-GAACGGTCATTTTGAAAATCAATG-3'	3142		
ρ2	Forward	5'-CATCTACCTCTGGGTCAGCTTTG-3'	1143	79.7	76
	Reverse	5'-GCTTCCGTTCCTTCTGTTCCT-3'	1243		
ρ3	Forward	5'-TTATCTCAGGCACTATTGGAAGGAT-3'	510	76.7	73
	Reverse	5'-GCACCCAAATCTTTTGAATCAATC-3'	610		
18S rRNA	Forward	5'-TCCCAGTAAGTGCGGGTCATA-3'	1655	80.7	78
	Reverse	5'-CGAGGGCCTCACTAAACCATC-3'	1755		

3.3.6 Cluster analysis

The absolute mRNA expression levels of GABA receptor subunits in developing lungs and type II cells were saved as text files with gene name and sample name associated with the log copy numbers. Those subunits were then grouped according to their similarity of the expression patterns by using Clusfavor software (Peterson 2002) (http://condor.bcm.tmc.edu/genepi/clusfavor.html). The clustering was based on both attributes and objects and the method was centroid with distant functions as euclidean.

3.3.7 Statistics

The relative expression levels were expressed as ratios to D18 lungs/cells or control lungs (room air exposure). Data were means ± SE in all the graphs. Statistics analysis was performed using SigmaStat 3.2 by one-way ANOVA, followed with the Least Significance Difference (LSD). Significance was taken when $P<0.05$.

3.4 Results

3.4.1 Quantification of the mRNA levels of GABA receptor subunits in fetal rat lungs and type II cells

By using absolute quantitative real-time PCR, we determined the mRNA abundance of 19 GABA receptor subunits (α1-6, β1-3, γ1-3, δ, θ, ε, π, and ρ1-3) in lungs and type II cells on a gestational day 18 (D18) which was selected to represent the late pseudoglandular stage of lung development. Brain tissue from

adult rats was used as a positive control and contained more than 50 copies per 10^8 copies of 18S rRNA for all the subunits (Fig. 3.1). The most abundant subunit in the brain was α1 (>10^5 copies per 18^8 copies of 18S rRNA). α2-, α4-, α5-, α6-, β2-, γ1-, γ2-, and δ-subunits were also enriched in the brain (10^4~10^5 copies per 18^8 copies of 18S rRNA). In the lung, α5-, α6-, ε, π-, ρ1, and ρ3-subunits were highly expressed (>100 copies per 10^8 copies of 18S rRNA). α1-, α2-, α3-, β2-, γ3-, and ρ2-subunits were also expressed in the lung (10-100 copies per 10^8 copies of 18S rRNA). In type II cells, the subunits with the highest expression were α1, α3, α4, β2, π, ρ1, and ρ3 (>100 copies per 10^8 copies of 18S rRNA). Some of those subunits (α1, α3, α4, β2, and π) appear to be enriched in type II cells in comparison with lung tissue. α5- and ε-subunits were also expressed in type II cells at a lower level. As a whole, the mRNA levels of GABA receptor subunits in the lung and alveolar epithelial cells were much lower than that in brains, especially for α-, β-, and γ-subunit families. However, ε-, π-, ρ1-, ρ2- and ρ3-subunits had a similar abundance of mRNA in lungs and type II cells as that in brains (Fig. 3.1).

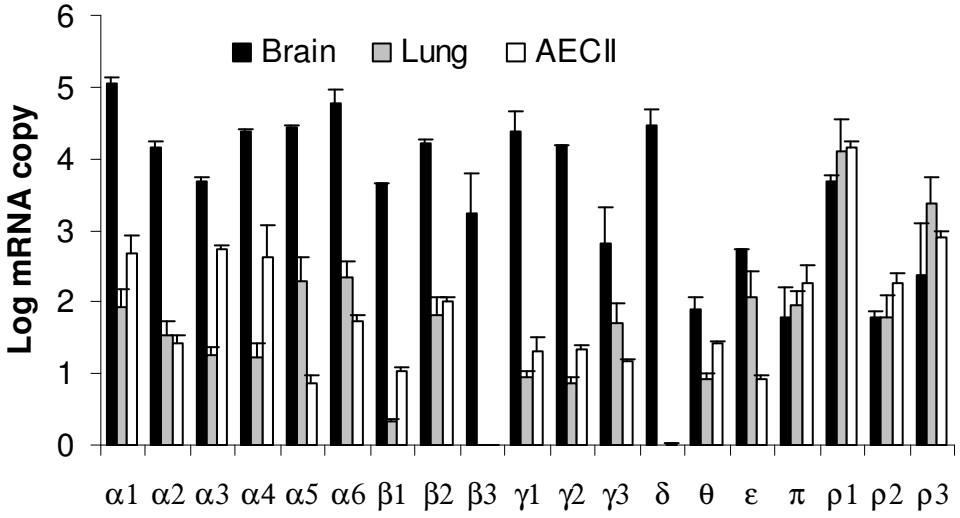

Fig. 3.1 Expression of GABA receptor subunits in fetal rat lungs and type II cells. Total RNA was isolated from the rat fetal lungs and type II cells (AEC II) with a gestational day 18, and reverse transcribed into cDNA. The mRNA abundance of 19 ionotropic GABA receptors was quantified by using real-time PCR, normalized to 18S rRNA, and expressed as log copy number per 10^8 copies of 18S rRNA. The adult brain was used as a positive control. Data shown are means ± SE (n ≥3 independent cell preparations).

3.4.2 Developmental alteration of GABA receptor mRNA in rat lungs

Cluster analysis indicated that the expression pattern of the D18 fetal lung was more similar to that of D21, whereas the pattern of the newborn lung (NB) was clsoer to those of the fetal D20 and the adult lungs (AD). The 18 subunits could be classified into 2 big groups: ρ3-, ρ1-, ρ2-, ε-, β2-, π-, α1-, α5-, and α6-subunits (1-9) belonged to a group, whereas θ-, γ2-, γ1-, α3-, α2-, α4-, γ3-, and β1-subunits (10-17) to another (Fig. 3.2A). Those subunits could be furher

grouped into 4 clusters according to their relative expression patterns (ratios to D18) during lung development (Fig. 3.2B-E). Cluster 1 was composed of 6 subunits (α2, α5, α6, γ3, β1, and ρ3). The genes in this cluster exhibited a decreasing trend from D18 to adult. The representative genes in this cluster are α5-, and α6-subunits. Both subunits had significantly lower mRNA expression in adult lungs in comparison with the fetal or newborn lungs (Fig. 3.2 C). Cluster 2 had 5 subunits (α3, γ1, γ2, θ, and ρ1). The mRNA expression in this group was relatively steady from the fetal to the adult lungs, but was slightly up-regulated at birth. The representative gene in this cluster is γ1-subunit, whose mRNA level in the newborn lung was significantly higher than D18 fetal and the adult lung. Another subunit, ρ1, showed a peak at D19, in addition the one at birth (Fig. 3.2 B). Cluster 3 included 3 subunits, α1, β2, and π. The genes in this cluster showed an increasing trend from fetal D18 to adult. The representative subunit in this cluster is α1, whose expression in adult lungs was significantly higher than that of the D18 fetal lung (Fig. 3.2 D). Cluster 4 was composed of 3 subunits (α4, ε, and ρ2). The genes in this cluster exhibited irregular fluctuation and no significant difference was found among all the time points (Fig. 3.2E).

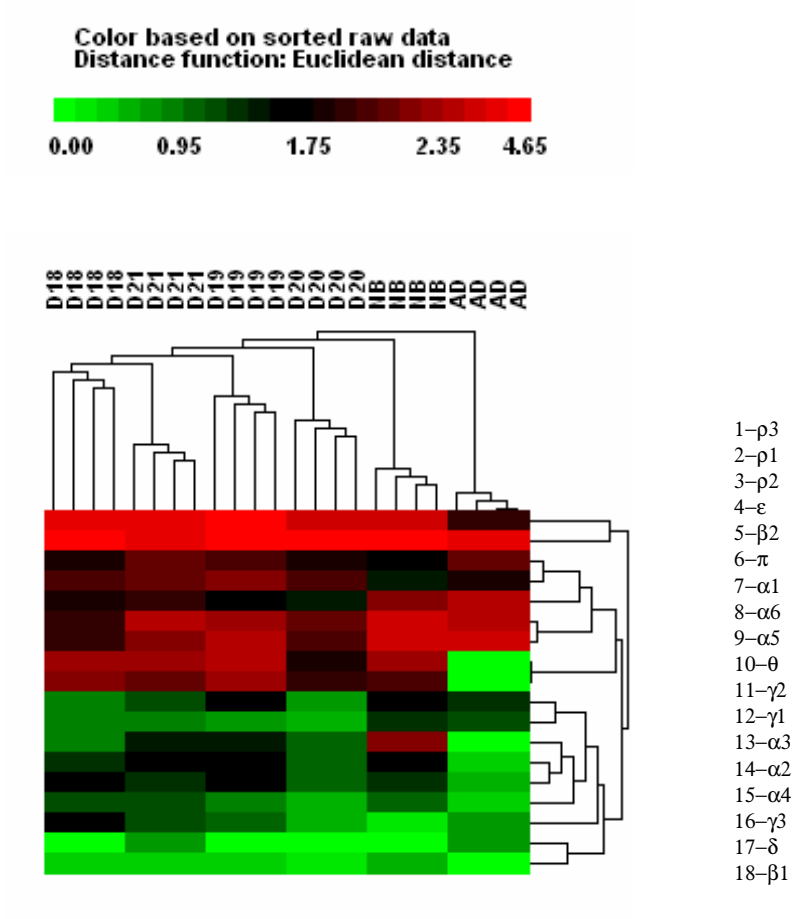

Fig. 3.2 Cluster anaysis of GABA receptor subunits in developing rat lungs. Total RNA was isolated from the fetal lungs at gestational day 18 (D18), day 19 (D19), day 20 (D20) and day 21 (D21), or from newborn rat lungs (NB) and adult rat lungs (AD). The RNA was reverse-transcribed into cDNA and the mRNA levels of GABA receptor subunits were determined with real-time PCR and expressed as log copy number per 10^8 copies of 18S rRNA. The log copy numbers were then applied for cluster analysis with X-axis showing the similarity of samples and Y-axis the similarity of genes.

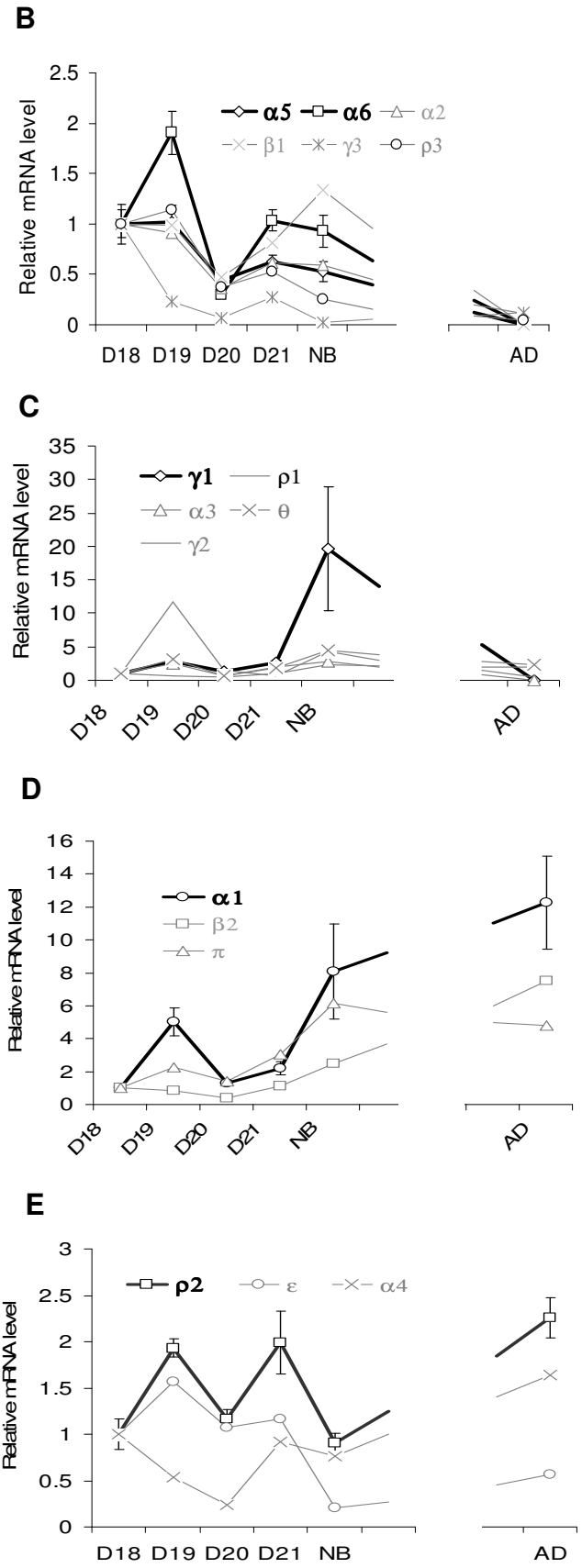

Fig. 3.2 Developmental alteration of GABA receptor mRNA in rat lungs. Total RNA was isolated from the fetal lungs at gestational day 18 (D18), day 19 (D19), day 20 (D20) and day 21 (D21), or from newborn rat lungs (NB) and adult rat lungs (AD). The mRNA levels of GABA receptor subunits were determined with real-time PCR and expressed as relative levels to D18. Those subunits were classified into 4 clusters according to their expression patterns. Cluster 1, α2, α5, α6, β1, γ3, and ρ3 (B); cluster 2, α3, γ1, γ2, θ, and ρ1 (C); cluster 3, α1, β2, and π (D); cluster 4, α4, ε and ρ2 (E). Data shown are means ± SE (n ≥ 3 independent preparations).

3.4.3 Developmental alteration o GABA receptor mRNA in type II cells

With the realization of the potential importance of type II cells in fluid transport of the fetal lungs, we further determined the mRNA abundance of GABA receptor subunits in rat type II cells during fetal lung development. Similar to the fetal lung tissue, we clustered the GABA receptor subunits based on their mRNA expression patterns (Fig 3.3 A). The ontogeny alteration of those subunits in type II cells was more regular than those in fetal lungs and similarity of expression was always present in the connected time points. Those subunits could be classified into 3 groups: subunits ρ3, ρ2, π, α3, β2, α4 and α1 (1-7) in one group, subunits ε, θ, β1, δ, α5, γ3, γ2, γ1, α2, α6, and (8-17) in another group, and ρ1 in an individual group (Fig. 3.3A). They could also be grouped into 4 clusters based on their relative mRNA expression patterns (to D18). Cluster 1 contained 9 subunits: α1, α2, α4, β1, β2, γ1, θ, ρ1, and ρ3. All of them had a peak expression at birth. The representative gene in cluster 1 was subunit ρ3. Its mRNA level in newborn rat type II cells was 6-fold higher than that in D18 and was significantly higher than all the other time points (Fig. 3.3B). Cluster 2 included α3-, ε-, π-, and ρ2-subunits (Fig. 3.3D). Those genes either kept a relative stable expression or showed an increasing trend during development. The representative gene in this cluster was π-subunit. Its mRNA levels in new born and adult were significantly higher than those of D18 and D19 (Fig. 3.3C). Cluster 3 was composed from γ2- and γ3-subunits. Both genes showed a relative low expression on D20 and D21 in comparison with D18 and adult (Fig. 3.3D).

α5- and α6-subunits constituted cluster 4, both genes had a lower expression in adult type II cells than in all other days (Fig. 3.3E).

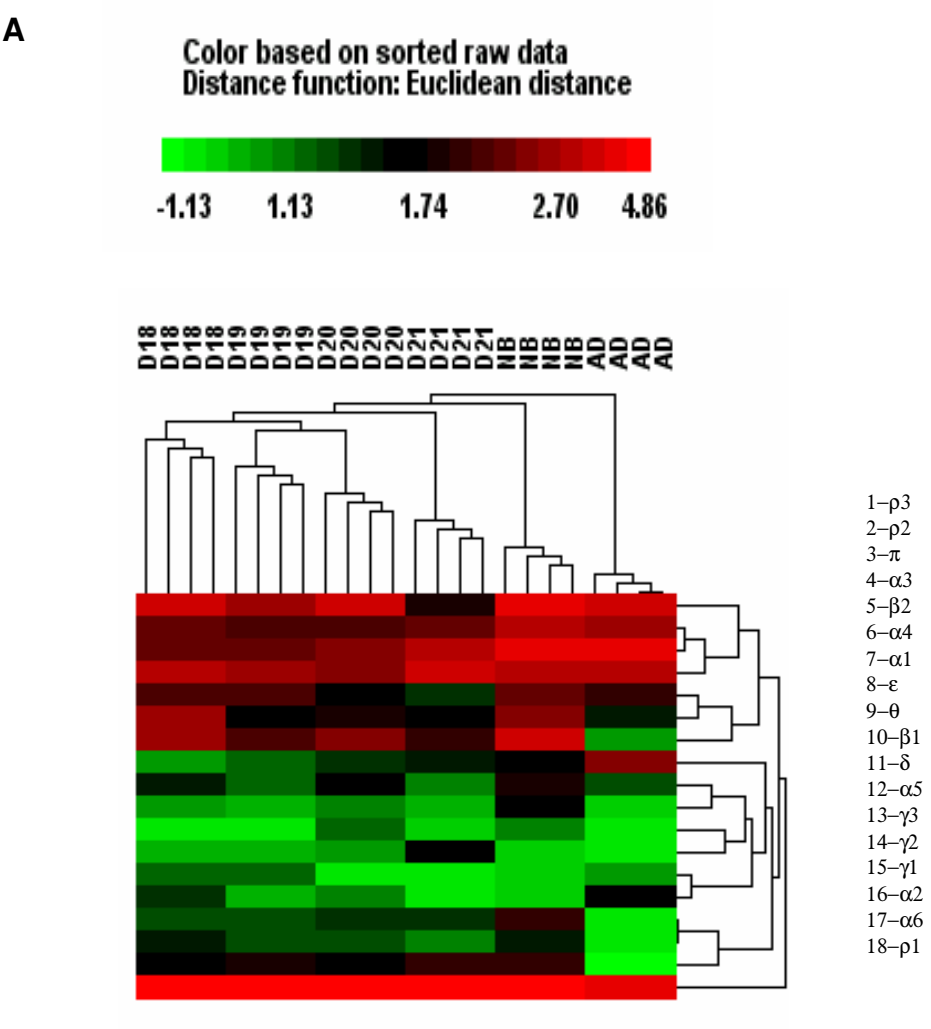

Fig. 3.3 Cluster analysis of GABA receptor subunits in developing rat type II cells. Alveolar epithelial type II cells were isolated from the fetal rats at gestational day 18 (D18), day 19 (D19), day 20 (D20), and day 21 (D21), or from newborn rats (NB) and adult rats (AD). Total RNA was extracted and reverse-transcribed into cDNA. The mRNA levels of GABA receptor subunits were determined with real-time PCR and expressed as log copy number per 10^8 copies of 18S rRNA. The log copy numbers were then applied for cluster analysis with X-axis showing the similarity of samples and Y-axis the similarity of genes.

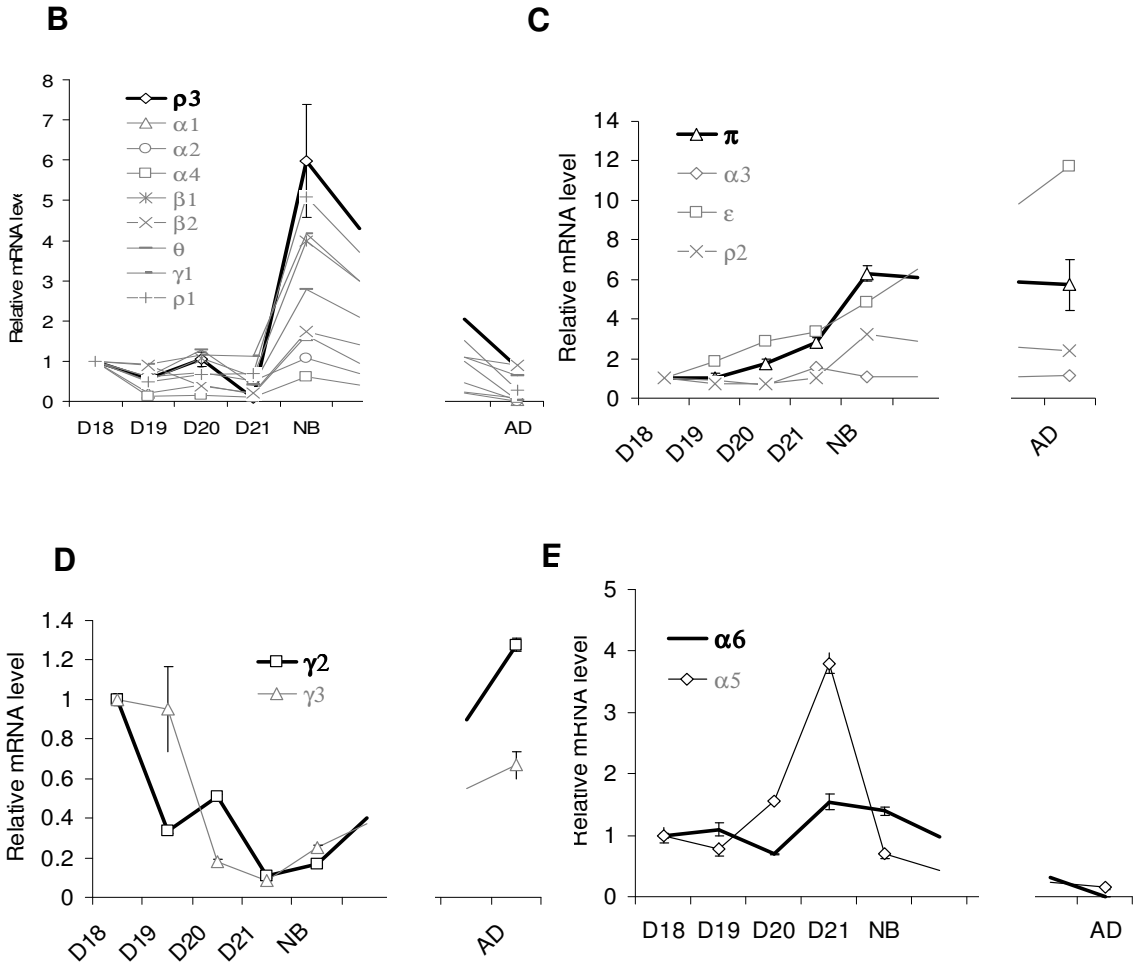

Fig. 3.3 Developmental alteration of GABA receptor mRNA in rat type II cells. Alveolar epithelial type II cells were isolated from the fetal rats at gestational day 18 (D18), day 19 (D19), day 20 (D20), and day 21 (D21), or from newborn rats (NB) and adult rats (AD). Total RNA was extracted and reverse-transcribed into cDNA. The mRNA abunance of GABA receptor subunits were determined with real-time PCR and expressed as relative levels to D18. Those subunits were classified into 4 clusters according to their expression patterns. Cluster 1, α1, α2, α4, β1, β2, γ1, θ, ρ1, and ρ3 (B); cluster 2, α3, ε, π, and ρ2 (C); cluster 3, γ2 and γ3 (D); cluster 4, α5 and α6 (E). Data shown are means ± SE (n ≥3).

3.4.4 Hyperoxic regulation of GABA receptor subunits in adult rat lungs

To determine the effects of hyperoxia on GABA receptor mRNA expression, rats were exposed to >95% O_2 for 48, 60, and 72 hrs and mRNA levels in the lungs were measured by real time PCR. For the sake of clarity, the GABA receptor subunits were graphed in 3 groups based on their amino acid homologies, as described by Whiting et al (Whiting et al. 1999). For α-subunit family (group 1), a 48 hrs-hyperoxia exposure had little effect on α1- and α2-subunits, but a longer exposure time (60 and 72 hrs) resulted in a significant down-regulation of these two subunits (Fig. 3.4A). All other subunits in group 1 (α3, α4, α5, and α6) and all of the subunits in group 2 (β1, β2, γ2, γ3, ε, and θ) were down-regulated as a function of time starting at 48 hrs (Fig. 3.4A and B). Group 3 (π and ρ1-3) appeared to be more resistant to hyperoxia (Fig. 3.4C). Hyperoxia exposure up to 72 hrs had no obvious effects on the mRNA expression of π-subunit. ρ1-3-subunits displayed a decreasing trend in the mRNA expression after hyperoxia exposure, but the changes were lower than the subunits in other two groups. ρ3-subunit mRNA expression returned to the control at 72 hr-exposure.

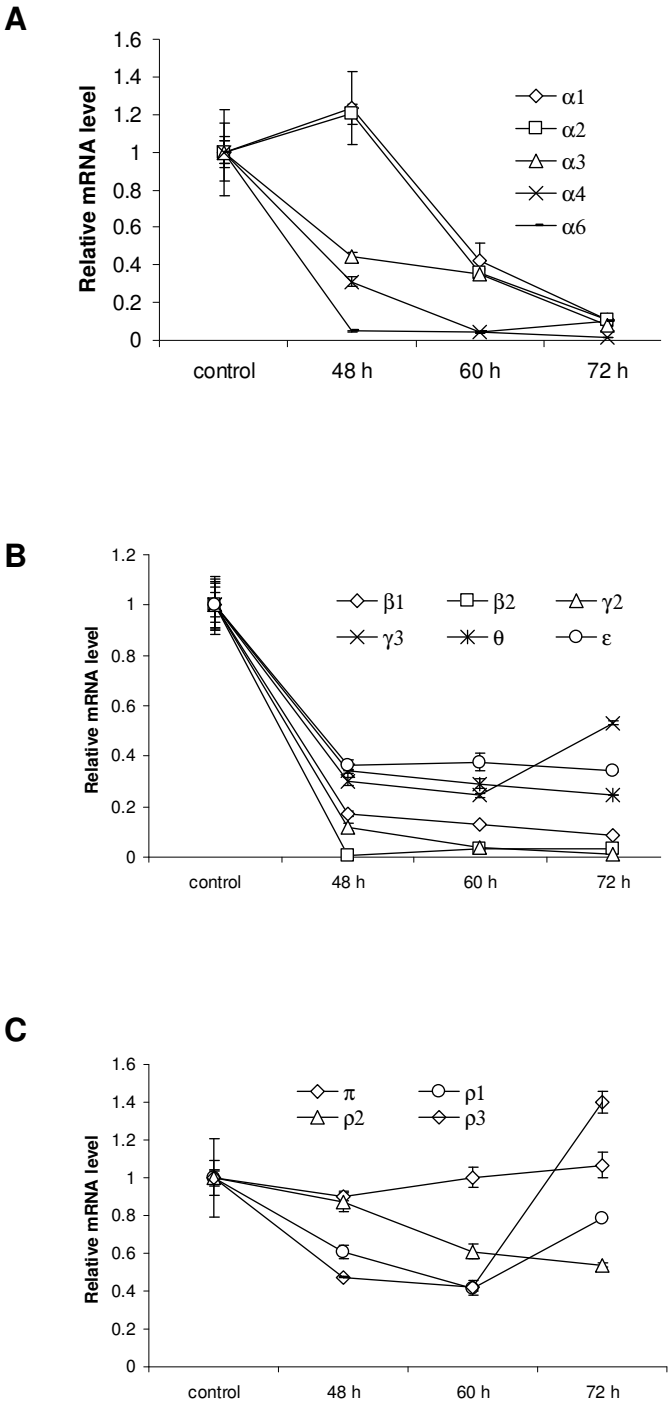

Fig.3.4 Hyperoxic regulation of GABA receptor mRNA in adult rat lungs. Adult rats were exposed to >95% O_2 for 48, 60, and 72 h and total RNA was isolated from the lung tissues and then reverse-transcribed into cDNA. The mRNA levels of the GABA receptor subunits were determined with real-time PCR and expressed as ratios to control. The subunits were categorized into 3 groups based on the amino acid homologies: group 1, α1, α2, α3, α4, and α6 (A); group 2, β1, β2, γ2, γ3, θ, and ε (B); and group 3, π, ρ1, ρ2, and ρ3 (C). Data shown are means ± SE (n=3).

3.5 Discussion

In the current study, by using real-time PCR, we demonstrate that multiple ionotropic GABA receptor subunits are expressed in fetal rat lungs and type II cells. The expression of those subunits is developmentally and hyperoxically regulated in the lungs and in type II cells, indicating that GABA receptors may contribute to Cl^- and alveolar fluid homeostasis in the lung throughout fetal and adult stages.

Ionotropic GABA receptor expression in rat fetal lungs and type II cells

A functional $GABA_A$ or $GABA_C$ receptor requires five subunits (Whiting et al. 1999). While $GABA_A$ receptors are assembled from at least 3 different subfamilies of the subunits, $GABA_C$ receptors can be formed from only the ρ-subunit family. The most common combination of a $GABA_A$ receptor is αβγ, in which α- and β-subunits appear to be essential and γ-subunit can be replaced by other subunits (Sieghart et al. 1999). In addition to those common subunits, “rare” subunits such as θ, ε, δ, and π can assemble with αβγ (Sieghart and Sperk 2002;Sieghart et al. 1999). We have previously detected α1-, α3-, β2-, γ2-, γ3-, and π-subunits in rat adult lungs and type II cells by RT-PCR (Jin et al. 2005). In this study, we systematically quantified the expression levels of all the currently known subunits in the fetal rat lung tissue and type II cells using absolute quantitative real-time PCR which is a more sensitive and accurate method. We identified more subunits in fetal lungs and in type II cells.

Essentially, all the GABA receptor subunits were expressed in the brain. The heterogeneous GABA receptors may reflect the complexity in controlling

nervous network. Many of the receptors were also expressed in the fetal lung. Some of them were enriched in type II cells, but others were expressed higher in the lung tissue than in type II cells, suggesting that GABA receptors may also be present in other lung cells rather than type II cells. Our cluster analysis indicated that subunit α1, α3, α4, β2, and π belonged to the same cluster in fetal type II cells (Fig. 2.3A). They may form a functional αβγ GABA receptor, or the π-subunit may co-assemble with them. The π-subunit was found to assemble with α1β3γ2 or α5β3γ3 in the co-transfected cell lines (Hedblom and Kirkness 1997;Neelands and Macdonald 1999). Subunit β1, γ1, γ2, and θ, although belonged to the other group as indicated by the cluster analysis, they may also constitute functional receptors in type II cells, because they were more enriched in type II cells than in the lung (Fig. 2.1). The most abundant GABA receptor subunits in type II cells were ρ1 and ρ3. ρ2 was also enriched. They could constitute the $GABA_C$ receptors, which could be homo- or hetero-pentameric. The ρ-subunit family was abundantly expressed in retina (Enz et al. 1995). Functional $GABA_C$ receptors were also found in several hormone secretory organs, such as the pancreas and the pituitary glands (Gamel-Didelon et al. 2003;Jansen et al. 2000). Therefore, the necessary subunits needed to assemble a functional $GABA_A$ or $GABA_C$ receptor are present in fetal type II cells.

Developmental regulation of GABA receptors in the lung and type II cells

The expression patterns of GABA receptors during fetal lung development are much more complicated than the channels encoded by a single gene because they are composed of multiple subunits and each subunit has multiple

isoforms. Similar to CFTR and ClC in the developing lungs (Brochiero et al. 2004;Blaisdell et al. 2000;Harris et al. 1991;Murray et al. 1996;Lamb et al. 2001;Murray et al. 1995), the mRNA of some GABA receptor subunits (cluster 1, Fig. 3.2B) were relatively high in mid-gestation, but decreased in late gestation and remained low in adult. Interestingly, some of those subunits, such as α2 and α5, were also less abundant in the adult brain than in the fetal brain (Poulter et al. 1992). Some subunits in the lung from cluster 2, 3, and 4, were constitutively expressed or only slightly fluctuated from mid-gestation to adult (Fig. 3.2C-E). This suggests that these subunits are ontogeny-"conserved" in the lung. Many of these subunits, including α3, α4, β2, and γ2, were also more developmentally "conserved" than other subunits in the brain (Huang and Dillon 2002;Poulter et al. 1992). These subunits may be the basic components for forming functional GABA receptors in both embryonic and postnatal brains and lungs. ρ1-3-subunits were only detected in the postnatal brains and their expression was up-regulated one week after birth. This is consistent with the results from the visual system (Alakuijala et al. 2005;Young and Cepko 2004). In the lung, ρ1-subunit mRNA was up-regulated at gestational day 19 and at birth (Fig. 3F). Other two ρ-subunits had a different expression pattern than that of ρ1-subunit, suggesting that $GABA_C$ receptors may have distinct roles during lung development from the brain.

The expression patterns of GABA receptor subunits in type II cells displays a "clearer" pattern than those in whole lung tissue, probably because of the heterogeneous nature of the lungs, which contains more than 40 types of cells.

The most obvious expression characteristics for many of the subunits are that their mRNAs are relatively unchanged during fetal stages, but markedly up-regulated at birth (clusters 1, Fig. 3.3 B). It is unexpected for up-regulation of GABA receptors to occur in the lung at birth if GABA receptors are involved in Cl^- and fluid secretion, since the lung switches from fluid-secreted to fluid-absorbed at birth. There are two possibilities for an explanation: first, ionotropic GABA receptors are a pentameric structure whose electrophysical properties are determined by all the subunits, but are not completely dependent on the absolute abundance of a single subunit (Whiting et al. 1999); second, the direction of Cl^- flux of GABA is dependent on the electrochemical gradient. It was reported the Cl^- channels were required for terbutaline-stimulated Na^+ absorption (O'Grady et al. 2000). It is possible that newborn rat type II cells absorb Cl^- via GABA receptors and, thus, help the re-absorption of fluid.

Hyperoxic regulation of GABA receptors in adult rat lungs

In the adult lungs, Cl^- secretion is still needed for the thin layer of fluid covering the alveolar surface. Re-absorption of edema fluid is essential for the recovery of hyperoxia-injured lungs. The ability of alveolar fluid clearance in mild-to-moderate lung injury was enhanced. This may be due to the increased expression level and activity of Na^+ transporters (Yue et al. 1995;Johnson et al. 1998;Carter et al. 1997). In this research, we found that hyperoxia exposure down-regulated the mRNA expression of almost all the GABA receptor subunits in the adult lungs. This may be an adaptive response to reduce fluid secretions.

In summary, our research documented the dynamic change of GABA

receptor subunits in lung development and hyperoxia, which suggest ionotropic GABA receptors may play a role in lung fluid transport.

3.6 Acknowledgments

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Chapter 4

FUNCTIONAL STUDY OF IONOTROPIC GABA RECEPTORS IN ALVEOLAR EPITHELIAL CELLS

4.1 Abstract

We have previously shown the expression of ionotropic GABA receptor pi subunit in alveolar epithelial type II cells. In this study, we revealed the expression of multiple subunits in both type I and type II cells. GABA and glutamate acid decarboxylase were found in type II cells as well. By co-immunoprecipitation, we identified one $GABA_A$ receptor complex, α1α3β2γ2π in type II cells. Using cell membrane protein biotinylation, we demonstrated that this receptor was localized on the apical plasma membrane, but not on the basolateral membrane. When GABA was added to the freshly isolated type II cells, Cl^- efflux rate was increased dose-dependently, which was blocked by pre-incubation with picrotoxin, an ionotropic GABA receptor antagonist. The GABA-stimulated Cl^- efflux was not due to CFTR, Na^+-K^+-Cl^- co-transporter and Ca^{2+}-activated Cl^- channel because glybenclamide, bumetanide, and BAPTA-AM did not have effects on this process. When type II cells were cultured on the air-liquid interface, GABA inhibited the basal and isoproterenol-stimulated apical-to-

basolateral Cl^- transport across the monolayer. Knock-down of the π-subunit using RNA interference eliminated the GABA-dependent Cl^- efflux. In anesthetized rats, GABA inhibited both basal and isoproterenol-stimulated alveolar fluid clearance. In summary, we have identified a novel fluid transport pathway in adult rat alveolar epithelial cells involving the ionotropic GABA receptors.

4.2 Introduction

Alveolar fluid homeostasis is essential for pulmonary functions. Fluid is actively secreted in fetal lungs but absorbed in adult lungs for the purpose of breathe. Although in a “dry” state, the adult alveoli are covered with a thin layer of fluid on the inner surfaces, namely alveolar surface liquid (ASL) or alveolar subphase fluid (AVSF). The volume of the ASL is tiny, but it is precisely and dynamically regulated (Scarpelli 2003, Ng et al. 2004). On one hand, the ASL volume has to be maintained low for an efficient gas-exchange (Matthay et al. 2002); on the other hand, sufficient volume and proper composition are required for the circulation of the surfactant “bubbles” as well as the exchange of electrolytes between the ASL and the alveolar epithelium (Scarpelli 2003, Ng et al. 2004).

It has long been established that Na^+ transporters play an important role in alveolar fluid clearance (Matthay et al. 2002, Olver et al. 2004, Berthiaume et al. 2002). The epithelial Na^+ channel (ENaC) and the Na^+-K^+-ATPase have been identified in both type I and type II cells (Schneeberger and McCarthy 1986, Yue

et al. 1995, Johnson et al. 2002, Borok et al. 2002), In addition, the cyclic nucleotide-gated cation channels (CNG) and a cGMP-evoked Na^+ transporter contribute to a large part of the amiloride-insensitive alveolar fluid clearance (Norlin et al. 2001). Cl^- was previously thought to move passively after Na^+; however, active Cl^- transport began to be recognized recently, which is indispensable for the alveolar fluid homeostasis (Olver et al. 2004, Reddy et al. 1999, O'Grady et al. 2000, Jiang et al. 1998a, Fang et al. 2002).

The regulation of alveolar fluid homeostasis via Cl^- transport has been studied through pharmacological methods (O'Grady et al. 2000, Jiang et al. 1998a, Lee et al. 2003). Relatively little is known at the molecular level on how fluid tranport regulation occurs. When cAMP was administered to type II cell monolayer, the Cl^- current appeared in precede the Na^+ current (Jiang et al. 1998a). Terbutaline (β-agonist) directly increased the apical-to-basolateral net Cl^- transport on type II cell monolayer (Lee et al. 2003). In an animal model, when Cl^- free solution was instilled into the rabbit lungs, a significant reduction in liquid clearance from the distal airspaces was noted (Nielsen et al. 1998). Those studies indicate that the efficiency of Cl^- transport directly affects the alveolar fluid clearance. Several groups reported the expression of CFTR on freshly isolated or cultured type II cells (Lee et al. 2003, Brochiero et al. 2004). Very recently, it was shown that CFTR was a rate-limiting factor for cAMP agonist-stimulated fluid clearance on type II cells (Fang et al. 2005). Although they have the intact Na^+ transporters, the CFTR (-/-) mice failed to increase the alveolar fluid clearance upon activation with cAMP agonists, because of a reduced Cl^- uptake ability (Fang et al. 2002). In

addition to CFTR, NKCC and KCC were also identified in type II cells but their roles in alveolar fluid transport are unknown (Lee et al. 2003).

The ionotropic GABA receptor is an important Cl^- channel that is widely expressed in both nervous systems and peripheral organs. In mature neurons, these receptors normally result in a hyperpolarizing Cl^- influx. In immature neurons, they exhibit outward Cl^- currents that lead to the depolarization of the membrane (Jentsch et al. 2002a, Ben Ari 2002). In addition to modulation of Cl^-, ionotropic GABA receptors also change the conductance of several cations including K^+, Mg^{2+} and Ca^{2+} in the nervous system (Owens and Kriegstein 2002, Zhang and Jackson 1995, Ganguly et al. 2001). Also, through cross-talking with other channels such as KCC2 and P2X, GABA receptors affect cellular functions via various mechanisms (Ganguly et al. 2001),, (Boue-Grabot et al. 2004). GABA receptors are also involved in pituitary growth factor secretion, pancreatic fluid and insulin secretion, and gonadotropin-releasing hormone (GnRH) release (Gamel-Didelon et al. 2003, Park et al. 2002, DeFazio et al. 2002).

We have recently identified the expression of a $GABA_A$ receptor π subunit (GABRP) and some other subunits in type II cells (Chen et al. 2004b, Jin et al. 2005). This suggests that a novel type of Cl^- channel may exist on type II cells. In this study, we found the expression of functional ionotropic GABA receptors on the apical plasma membrane of alveolar epithelial type II cells. By using freshly isolated and cultured type II cells, as well as anesthetized rats, we demonstrated that the ionotropic GABA receptors contribute to alveolar fluid homeostasis via luminal secretion of Cl^-. Our discovery may provide a new mechanism of alveolar

fluid regulation, which may have wide clinical implication for diseases involving abnormal pulmonary fluid dynamics.

4.3 Materials and methods

4.3.1 Materials

Materials. Sprague-Dawley (SD) rats were obtained from Charles River Breeding Laboratories (Wilmington, MA, USA). Dulbecco's Modified Eagle Medium (DMEM), 100 × non-essential amino acid solution, 100 × penicillin/streptomycin solution, fetal bovine serum (FBS), M-MLV reverse transcriptase were purchased from Invitrogen (Calsbad, CA). Ketamine and zylazine were from LLOYD Laboratory (Shenandoah, IO). Pancreatic elastase was from Worthington (Lakewood, NJ). Keratinocyte growth factor (KGF), dexamethasone, DNase I, rat IgG, GABA, picrotoxin, bucuculine, tetrahydrofuran (TPMPA), glybenclamide, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), bumetanide, BAPTA-AM, and dinonyl phthalate were purchased from Sigma-Aldrich (St Louis, MO). Corning transwell collagen-coated membrane inserts and Millicell-CM filter inserts were from Fisher Scientific (Houston, TX). Bovine dermal collagen was from Cohesion (Palo Alto, CA) and Matrigel was from Becton Dickinson (Bedford, MA). Silicone fluid DC 550 was from PolyScience (Niles, IL).

Bovine serum albumin (BSA), normal goat serum, goat anti-mouse IgG (H+L, heavy and light chains), and Cy3-conjugated goat anti-mouse IgG (H+L) were from Jackson ImmunoResearch Laboratories (West Grove, PA);

Horseraddish peroxidase (HRP)-conjugated anti-goat IgG, HRP-conjugated anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-goat IgG and Alexa Fluor 633-conjugated rabbit anti-mouse IgG were from Molecular Probes (Eugene, OR, USA). Monoclonal antibodies against lamellar body membrane protein (LB180) were from Berkeley antibody company (Richmond, CA). Monoclonal anti-rat leukocyte common antigen antibodies (anti-LC) were from Accurate (Westbury, NY). Monoclonal hamster anti-mouse T1α antibody was from Developmental Studies Hybridoma Bank (University of Iowa). Polyclonal rabbit antibodies against GABA receptor α1-, γ2-, and δ-subunit, GABA and GAD65/67, were from Abcam Inc (Cambridge, MA). Polyclonal goat antibodies aginst GABA receptor α3- and π-subunits and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibodies against GABA receptor β2-subunit were from Chemicone (Temecula, CA). Polyclonal Monoclonal antibody against zonula occludens (ZO)-1 was from Zymed Laboratories (San Francisco, CA). EZ-Link Sulfo-NHS-Biotin reagents, Streptavidin-agarose beads, and protein G plus agarose beads were from Pierce Biotechnology (Rockford, IL). Protein D*c* assay reagents were from Bio-Rad Laboratories (Hercules, CA).

TRI reagent was from Molecular Research Center (Cincinnati, Ohio). DNA-free™ DNase and monoclonal anti-GAPDH antibody were from Ambion (Austin, Texas). Random hexamers primers were from Promega (Madison, WI). Primers for real-time PCR were synthesized by MWG Biotech Inc (High Point, NC). QuantiTech™ SYBR Green PCR master mix was from QIAGEN (Foster city,

CA). GENECLEAN Turbo for PCR was obtained from Qbiogene (Carlsbad, CA).

All isotopes including [^{36}Cl]NaCl, [^{14}C]sucrose and [^{14}C]mannitol were from Amersham Biosciences (Piscataway, NJ).

4.3.2 Cell isolation

Highly pure alveolar type I and type II cells were isolated from adult rat lungs according to our modified methods (Chen et al. 2004a). Type II cell were isolated by elastase digestion of rat lungs (60-70 units per rat), followed by negative selection by using rat IgG, anti-LC, and monoclonal anti-T1 alpha antibodies to remove macrophages, lymphocytes, and type I cells, respectively. The resulting cells had a purity of >95% and a viability of >98%. Type I cells were isolated by incubating rat lungs with a larger amount of elastase (135 units per rat). After removing macrophages and lymphocytes with rat IgG and anti-LC antibody, the cells were positively selected with monoclonal anti-T1 alpha antibody. The resulting type I cells had a purity of >90% and a viability of >95%. Those cell preparations were used for the identification of GABA receptor subunits.

4.3.3 Cell culture

Type II cells were isolated according to the normal procedure and the purity was ~85% (Martin-Martin et al. 2000). Those cells were grown on two air-liquid culture models to maintain the type II cell phenotype as previously described (Lee et al. 2003, Mason et al. 2002). For studies that a confluent cell monolayer was required, the cells were plated in transwell collagen-coated inserts at a density of 1.5×10^6 cells/cm^2 in DMEM containing 10% FBS, 0.1 mM

non-essential amino acid, and 100 U/ml penicillin, and 100 μg/ml streptomycin. After overnight culture at 37°C in humidified 95% air and 5% CO_2 incubator, non-adherent cells were removed and fresh medium was added only to the outside of the inserts. The cells were cultured in such condition for additional 3-4 days until a confluent monolayer was formed. The transepithelial electrical resistance (TER) was determined with an EVOM epithelial voltometer and a chopstick electrode (World Precise Instrument, Sarasota, FL). Only those wells that had a TER of >800 ohms.cm^2 were used for further studies. For studies in which a confluent monolayer was not a prerequisite, the cells were plated on 30 mm filter inserts coated with rat-tail collagen and matrigel (4:1, v/v) in a density of 3 × 10^6 cells/well. DMEM containing 5% rat serum, 10 ng/ml KGF, 10 nM dexamethasone, 1× non-essentail amino acid, and 1× penicillin/streptomycin was used in the culturing. Twenty-four hours after plating, non-attached cells were removed. Fresh medium, 1.5 ml and 0.4 ml, were added to the outside and the inside of the insert, respectively. The plates were shaken on a rocking rotator inside the humidified incubator for 4-5 more days. The resulting cells were used for immunostaining and RNA isolation.

4.3.4 Adenoviral RNAi vector construction

Adenoviral vectors were designed and constructed as previously described (Gou et al. 2004). Briefly, small hairpin RNA (shRNA) sequences against rat GABRP including the 19 to 21-nt antisense (AS), followed by a 9-nt loop (5'-TTCAAGAGA-3'), and the sense (S) sequence, were designed by free software (Ambion, Austin, TX) and synthesized by MWG Biotech (High Point,

N.C.). The name, the sequences with SalI and EcoR I overhangs, as well as the starting and ending site of each shRNA are as follows: virus 2510, from 276 to 294, top 5'-TCGACGTAACATGGACTACACAGCTTCAAGAGAGCTGTGT AGTCCATGTTACTTTTTT-3', and bottom 5'-AATTA AAAAAGTAACATGGA CTACACAGCTCTCTTGAAGCTGTGTAGTCCATGTTACG-3'; virus 2511, from 352-370, top 5'-TCGACGAGCTT TACTCTAGATGCCTTCAAGAGAGGCATCTA GAGTAAAGCTCTTTTTT-3', and bottom 5'-AATTAAAAAAGAGC TTTACTCTAGATGCCTCTCTTGAAGGCATCTAGAGTAAAGCTCG-3'; virus 2512, from 479 to 491, top 5'-TCGACGGCACAGTCTTGTAT GCACTTCAAGAGAGTGCATACAAGACTGTGCCTTTTTT-3', and bottom 5'-AATTAAAAAAGGCACAGTCTTGTATGCACTCTCTTGAAGTGCATACAAGACT GTGCCG-3'. The shRNA with the poly T overhangs were directly cloned into pENTER vector behind the U6 promoter (Invitrogen, Carlsbad, CA) through the SalI–EcoRI sites. The inserts were subsequently switched into the adenoviral vector, pAd/PL-DEST, through the Gateway technique (Invitrogen, Carlsbad, CA). The resulted adenoviral plasmids were linearized by PacI, purified with GENECLEAN Turbo kits, and transfected into 293A cells. The titers of the amplified viruses were determined according to the standard protocols of Ambion and stored at -80^{o}C before use.

4.3.5 Adenoviral infection

Type II cells were isolated and plated in inserts as described in “Cell culture”. After overnight culture, non-adherent cells were removed. One ml fresh media containing adenoviruses (2.5-100 multiplicity of infection or MOI per cell)

were added to the inside of the inserts and allowed to slowly leak to the outside by gravity. Twenty four hours later, the virus-containing medium was removed and the cells were maintained at the air-liquid interface with fresh medium. The cultures were continued for 3 more days and the resulting cells were used for immunostaining, RNA isolation, immunoprecipitation, western blot, and and Cl^- efflux study. For unidirectional Cl^- transport studies, transepithelial resistance of the cultures were measured and only the wells with a TER of >800 ohms.cm^2 were used.

4.3.6 Absolute quantitative real-time PCR

Real-time PCR was carried out as previously described (Chen et al. 2004b, Jin et al. 2005). Briefly, total RNA was extracted from freshly isolated type I cells and type II cells, cultured type II cells, and whole rat lung and brain tissues. Trace amount of genomic DNA was removed with DNase I. Thereafter, 1 µg of RNA was reverse-transcribed into cDNA in a total volume of 20 µl with 200 U M-MLV reverse transcriptase in the presence of 0.5 µg random hexamers primers. Primers for real-time PCR were designed with Primer Express 1.5 (Appliedbiosystems, Foster, CA) and are listed in Table 3.1. The absolute quantitative real-time PCR was performed on an ABI 7700 system (Applied Biosystems, Foster city, CA) by using SYBR Green detection. Standard curves were constructed by purified PCR products. The following thermal conditions were used: denaturing at 95 ^oC for 15 min, followed by 40 cycles of 95 ^oC 20 s, 60 ^oC 30 s, 72 ^oC 30s and data acquisition for 15 s. Data acquisition

temperatures were optimized to 2~5 ^{o}C lower than the Tm of the amplicon and listed in Table 3.1. A dissociation analysis was performed after each run to confirm the specificity of the amplifications. Quantity of mRNA of the GABA receptor subunits was normalized to 18S rRNA.

4.3.7 Immunoprecipitation

Freshly isolated type II cells (~10^7 cells) were immediately homogenized on ice with 100 µL IP buffer (20 mM sodium phosphate, pH 7.5, 500 mMNaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate and 0.02% sodium azide). After centrifuging the lysate at 16,000 *g* for 15 min at 4^{o}C, 30 µg of the protein was incubated with goat anti-$GABA_A$ receptor γ2-subunit (3 µg) at 4^{o}C overnight with rotation. The protein-anitbody mix was further incubated with protein A/G agarose gel (35 µL) for 2 hrs. After being washed with IP buffer, the beads were boiled in 50 µL SDS-PAGE sample buffer (10 mM Tris-HCl, pH 8.0, 40 mM DTT, 1 mM Na_2EDTA and 10% glycerol) for 10 min. The supernatant was then analyzed with western blot to detect the protein of α1-, α3-, β2-, γ2-, γ3-, π-, and δ-subunits.

4.3.8 Membrane protein biotinylation

To assess the polarity of the identified $GABA_A$ receptors on type II cell membrane, the biotinylation of cell membrane proteins were performed as previously described (Roux et al. 2005). Briefly, type II cells were maintained on an air-liquid culture system for 5-6 days until a monolayer was formed. After washing the inserts gently with cold PBS buffer, EZ-Link Sulfo-NHS-Biotin reagents was added to either the apical or basolateral side of the inserts. The

cells were incubated on ice for 1 h, harvested and resuspended in lysis buffer. The biotinylated proteins were collected by incubating the lysate with Streptavidin-agarose beads at 4^{o}C overnight. The beads were boiled in SDS-PAGE sample buffer for 5 min. The supernatant containing the biotinylated proteins were analyzed by western blot using anti-GABRP antibody. To make sure that the biotinylation was free from cytosolic proteins, β-actin, a cytoskeleton protein was used as a control.

4.3.9 Western blot

Protein concentrations were determined by the protein D*c* assay. Equal amount of proteins were separated with 10% SDS-PAGE and electrophoretically transferred to a polyvinylidine difluoride membrane. The target proteins were detected by incubating the membranes with the following antibodies: goat anti-$GABA_A$ receptor π subunit (1:100) or goat anti-$GABA_A$ receptor γ2 subunit (1:200) antibodies and HRP-conjugated donkey anti-goat IgG (1:1000); rabbit anti-$GABA_A$ receptor α1-, α3-, and β2-subunit antibodies (1:200) or rabbit anti-GAD65/67 antibody (1:1000) or rabbit anti-GABA antibody (1:200) and HRP-conjugated goat anti-rabbit IgG (1:2000); rabbit anti-β-actin antibody (1:1000) and HRP-conjugated goat anti-rabbit IgG (1:4000); monoclonal anti-GAPDH antibody (1:4000) and HRP-conjugated rabbit anti-mouse IgG (1:5000). The immunoreactive bands were visualized with enhanced chemiluminescence reagents.

4.3.10 Immunostaining

Immunohistochemistry and immunocytochemistry were performed as

previously described (Chen et al. 2004b, Narasaraju et al. 2003). To reveal the localization of GABA, the lung tissue slides were double-labeled with rabbit anti-GABA and monoclonal anti-LB180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and FITC-conjugated anti-mouse IgG; to show the localization of GAD, the lung tissue slides were double-labeled wtih rabbit anti-GAD65/67 and anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and Alexa Fluor 633-conjugated anti-mouse IgG. To show the silencing effects of various adenoviruses, type II cells cultured on the inserts were double labeled with goat anti-GABRP and anti-LB-180 antibodies, followed by incubation with Alexa 568-conjugated anti-goat IgG and FITC-conjugated anti-mouse IgG. To show whether tight junctions were formed on the siRNA treated cells, type II cells on the inserts were labeled with monoclonal anti-ZO1 antibody and FITC-conjugated anti-mouse IgG. The dilutions of all the antibodies were 1:200 except for that the anti-GABRP antibody was 1:100.

4.3.11 $^{36}Cl^-$ efflux

The Cl^- efflux rate from freshly isolated type II cells was assessed as previously described (Kemp et al. 1994). Twelve million cells were incubated at 37^oC for 20 min with Ringer's solution (126.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 15 mM HEPES, 5.55 mM glucose, and 0.075 mM dextran 40, pH 7.4). And then 5 μCi/ml [^{36}Cl]NaCl was added and the cells were further incubated for 30 min (The final Cl^- concentration increased 10 mM upon addition of the [^{36}Cl]NaCl). After centrifugation and removing the supernatant, the cells were rapidly washed with cold Ringer's solution for 3 times.

The cells were then resuspended with warm Ringer's solution (basal) in the presence of various drugs. The aliquot of cells (200 µl) was removed at different intervals. The cellular aliquot was quickly centrifuged through 200 µl of silicone oil (40% silicone fluid DC 550 plus 60% dinonyl phthalate) at 1,000 *g* for 20 s. The tubes were then frozen at -20^{o} C. The tips containing the cell pellets were cut off from the microtubes and the cells were lyzed with 200 µl of 1% SDS. Protein amount and $^{36}Cl^{-}$ radioactivity of the cells were determined by *D*c assay and liquid scintillate analysis, respectively. The specific cellular $^{36}Cl^{-}$ was expressed as nmole/mg protein. The cellular $^{36}Cl^{-}$ at different time points were expressed as a percentage of 0 time and their logarithmatic values vs. time was plotted. The $^{36}Cl^{-}$ efflux rate coefficient (*k*; in min^{-1}) was determined by linear regression analysis. The concentrations of drugs in those experiments were as follows: GABA, 3 - 1000 µM; picrotoxin, 100 µM; glybenclamide, 100 µM; bumetanide, 100 µM; and NPPB, 60 µM.

Cl^{-} efflux study on the cultured cells was carried out as follows. By the end of the RNAi treatment, type II cells on the inserts were washed with warm DMEM. The cells were loaded with $^{36}Cl^{-}$ by incubating with DMEM containing 5 µCi/ml [^{36}Cl]NaCl at 37^{o}C for 30 min. After removing the "hot" media, the cells were rapidly washed with 3 ml ice-cold DMEM for 4 times. Washing solutions were removed rapidly by aspiration and warm DMEM or DMEM containing GABA was added immediately. Ten min later, the medium was aspirated. The cells were lysed with 1% SDS and specific radioactivity was determined as described above.

4.3.12 Unidirectional Cl^- transport

The Cl^- transport across the type II cell monolayer was determined on 0.33 cm^2 transwell insert according to the reported method (Roux et al. 2005). Briefly, the cells were incubated with warm Ringers solution on both sides (apical 150 µl and basolateral 800 µl). Isotopes were added to only one side of the insert with a final radioactivity of 1 µCi/ml (both $^{36}Cl^-$ and ^{14}C). Fifty µl of solution was removed every 10 min from the other side and the same volume of fresh media was added back. Radioactivity of $^{36}Cl^-$ and ^{14}C in the collections was determined simultaneously with liquid scintillate counter and the accumulations of isotopes against time were estimated by linear regression analysis. The transepithelial $^{36}Cl^-$ transport was calculated by subtracting the ^{14}C (the paracellular transport) from the total ^{36}Cl transport. For some experiments, samples were only collected at 10 or 30 min. The following drugs were used in this set of experiments: GABA (100 µM), picrotoxin (100 µM), and glybenclamide (100 µM) to the apical side; isoproterenol (100 µM) to the basolateral side.

4.3.13 Synthesis of conjugated GABA

Conjugation of GABA to BSA was carried out as previously described (Meyer et al. 1991) with glutaradehyde (GA) as the cross-linker. GABA (515 mg) in 3 ml 1 M sodium acetate was incubated with 2 ml of 25% GA solution at room temperature with slow stirring for 30 s. BSA (500 mg) in 5 ml 1 M sodium acetate was added and the mixture was stirred for 10 min. Fifty mg of $NaBH_4$ was then added to saturate the double bonds and the resulting products were dialyzed

against 10 mM sodium acetate at 4°C overnight with several changes. For the control, BSA conjugate was generated by the same reaction except for omitting GABA.

4.3.14 Alveolar fluid clearance in anesthetized rats

All the experiments on animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Oklahoma State University. Alveolar fluid clearance (AFC) was measured as previously described (Charron et al. 1999). Adult male Sprague-Dawley Rats (270 - 350 g) were housed for one week before the experiment. The rats were anesthetized with intraperitoneal injection of ketamine/xylazine (90 and 10 mg/kg, respectively). A tracheotomy was performed and the lungs were ventilated (CWE-inc., Ardmore, PA) with 100% oxygen for 30 min. The tidal volume was maintained at 8 ml/kg by adjusting the flow rate at 1.7 ~ 2.5 ml/min. The respiratory rate was 50 times/min. The rat body temperature was maintained at 37°C with an external heating lamp and a warming pad. After the equilibration period, a 22-gauge catheter was inserted through the incised trachea into the left bronchia and then the ventilation was continued. The rats were changed to a left-side laying position and the bodies were elevated to an upright angle of 45°C. Pre-warmed 5% BSA in normal saline (3 ml/kg) were delivered through the catheter into the left lungs with a syringe pump (Kent sciences, Torrington, CT) at a rate of 66 µl/min. In some experiments, drugs were included in the instillate and listed as follows: 100 µM of picrotoxin, bicuculine, TPMPA, or glybenclamide, and/or 300 µM of GABA. In some other experiments, GABA conjugate or BSA conjugate was included in the 5% BSA instillate and

their volume was ~15 μl per rat. Upon the completeness of the instillation, the ventilation was continued for 1 hour. The rats were then exsanguinated by transaction of the renal artery. The chests were opened and the right bronchia were tightened. A sample of the remaining alveolar fluid was removed from the left lung with a syringe. The fluid was briefly centrifuged to remove cell debris. Protein concentration of the instillate (C*i*) and the final alveolar fluid (C*f*) were determined by using D*c* protein assay. The AFC% was calculated by:

$$AFC\% = [(C_f - C_i)/C_f] \times 100\%$$

4.3.15 Statistics

All the data shown are Mean ± SE from at least 3 independent experiments. Variances among different groups were analyzed by student *t* test or one-way ANOVA followed with Dunnett's or Fisher's Least Significance Difference (LSD) multiple comparison methods. Significance were taken when $P<0.05$.

4.4 RESULTS

4.4.1 Alveolar epithelial cells express GABA receptors and their ligand

To identify which subunits were expressed in alveolar epithelial cells, we systematically determined the mRNA level of 19 subunits of the ionotropic GABA receptors (α1-6, β1-3, γ1-3, δ, θ, ε, π, and ρ1-3) by using quantitative real-time PCR. As shown in Fig.4.1A, the mRNAs of multiple GABA receptor subunits are expressed in rat lungs, type I cells and type II cells, including α1-, α3-, α4-, α6-, β2-, γ3-, δ-, ε-, π-, and ρ1-3 subunits. α2- and β1-subunits were only detected in

type I cells but not type II cells, whereas γ2-subunit only in type II cells but not in type I cells. The abundance of the π-subunit is ~10 times higher in type II cells than type I cells and ~3 times higher than in the lung tissue. All the subunits detected previously by RT-PCR including α1, α3, β2, γ2-3, were detected by real-time PCR (Jin et al. 2005).

Next we determined whether the physiological ligand of GABA receptors is synthesized locally in the lung. As indicated by the immunostaining in Fig. 4.1B, GABA was co-localized with LB-180, the type II cell marker, on the cuboidal cells at the corners of the alveoli. The squamous type I cells do not express GABA. To find out the source of GABA in alveolar epithelial cells, we examined the expression of glutamic acid decarboxylase (GAD), which is responsible for the synthesis of GABA, in alveolar epithelial cells. As shown in Fig. 4.1C, GAD65/67 was also expressed specifically in type II cells but not in type I cells. No signals were detected if the primary antibodies were omitted (data not shown). The GAD expression in type II cells was also confirmed by western blot. The GAD65/67 was enriched in type II cells in comparison with the whole lungs (Fig. 4.1D).

A

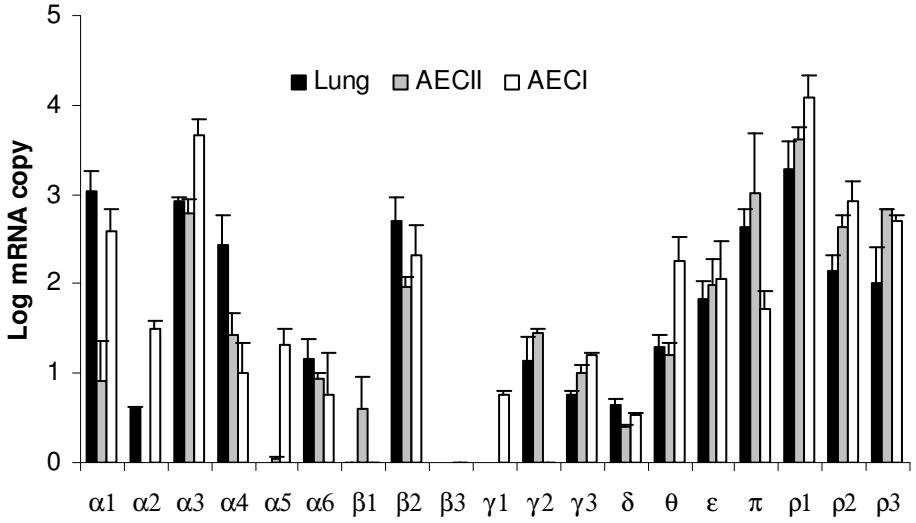


Fig.4.1 The mRNA expression of GABA receptor subunits in rat lungs and alveolar epithelial cells. Total RNAs were isolated from rat lungs, isolated alveolar type II cells and type I cells. The mRNA abundance of the GABA receptor subunits were determined using absolute real-time PCR and expressed as a log copy number per 10^8 copies of 18S rRNA. The results shown were means ± S.E. of at least three independent cell preparations, assayed in duplicate.

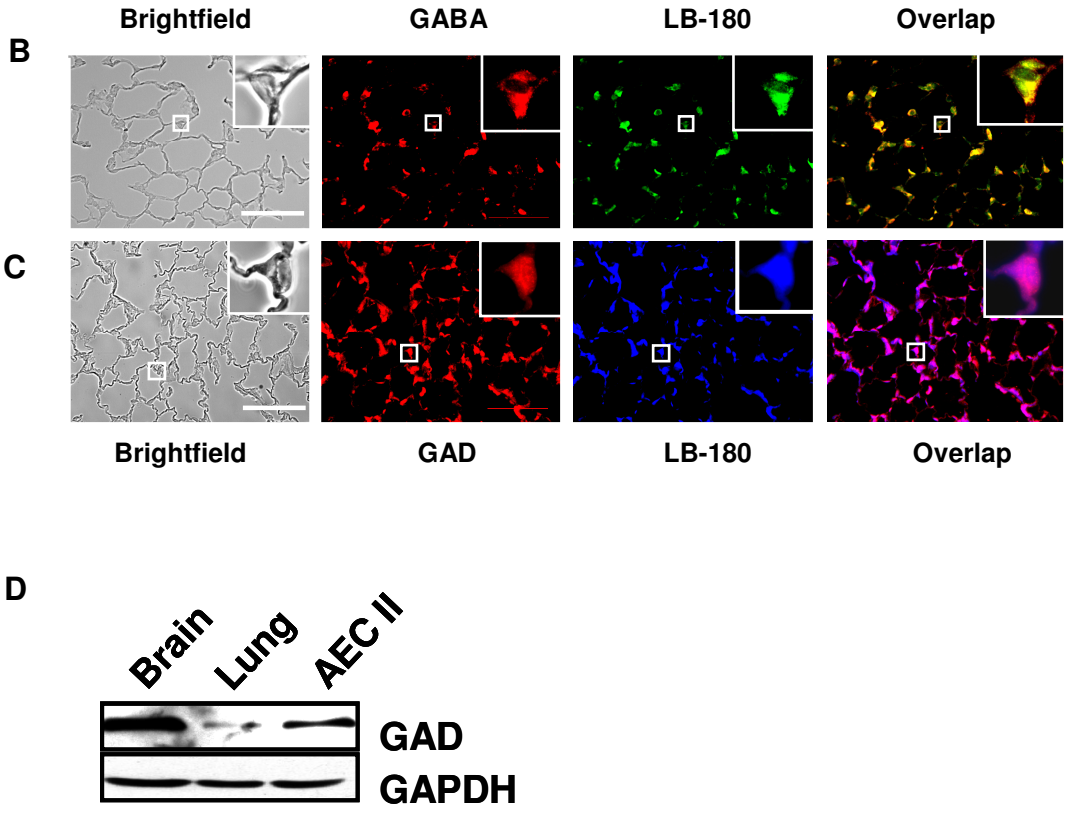


Fig 4.1 The synthesis of the physiological ligand of GABA receptor in alveolar type II cells. (B) & (C) Presence of GABA and glutamic acid decarboxylase (GAD). Lung tissue sections were double-labeled with anti-GABA antibodies (red) and anti-LB180 antibodies (green), or anti-GAD antibodies (red) and anti-LB180 antibodies (blue). Overlap (yellow for GABA and pink for GAD) indicated that GABA and GAD were only expressed in the cells that were stained with the type II cell marker LB-180. Scale bar: 30 µm; insert: an enlarged type II cell. (D) Western blot of GAD. Equal amount of brain, lung and freshly isolated type II cell (AECII) lysate were separated on SDS-PAGE and detected for the presence of GAD65/67. GAPDH was used as a loading control.

4.4.2 A native $GABA_A$ receptor is localized on the apical membrane of type II cells

The existence of the necessary GABA receptor subunits, the physiological ligand GABA, and the GABA-synthesizing enzyme GAD in alveolar epithelial cells does not mean the formation of functional GABA receptors in those cells. Therefore, we used co-immunoprecipitation to determine whether there are native receptors. Type II cell lysate was immunoprecipitated with anti-γ2 subunit antibodies. The presence of other subunits in the immunoprecipitate was detected by western blot. α1-, α3-, β2-, and π-subunits were co-precipitated with γ2-subunit; however, δ-subunit was absent in the immunoprecipitate even after a prolonged exposure up to 30 min. The immunoprecipitation was specific, since pre-immune serum did not pull down any subunits (Fig. 4.2A). The results suggest a native GABA receptor composed of α1-, α3-, β2-, γ2- and π-subunits in type II cells.

Cellular localization of Cl^- channels may provide important clues on their potential functions. Therefore, in the next experiment, we determined the localization of the π-subunit on the plasma membrane. Type II cells were cultured on permeable supports until forming monolayer. The apical or basolateral surface membranes were biotinylated. The π-subunit was only detected on the apical but not the basolateral membranes. The biotinylation was specific to the membrane proteins because β-actin was only detected in the whole cell lysate but not in the biotinylated proteins (Fig. 4.2B).

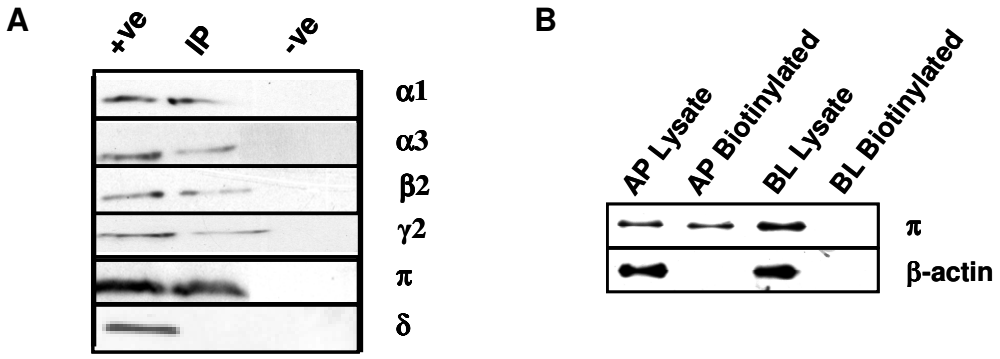


Fig. 4.2 Identification of a native $GABA_A$ receptor α1α3β2γ2π on the apical membrane of type II cells. (A) Freshly isolated type II cell lysate (+ve) was immunoprecipitated with anti-$GABA_A$ receptor γ2-subunit antibodies (IP) and detected for the presence of α1-, α3-, β2-, γ2-, π-, and δ-subunits using western blot. The same cell lysate immunoprecipitated with normal goat IgG was used as a negative control (-ve). The blot shown is a representative from three experiments with comparable results. (B) Type II cells were cultured on air-liquid interface for 5 days until the formation of a confluent monolayer. The apical (AP) or basolateral (BL) membrane proteins were biotinylated and detected using antibodies against π-subunit. β-actin showed that the cytosolic proteins were not biotinlylated. The blot shown was a representative from three experiments with comparable results.

4.4.3 GABA increase Cl^- efflux in freshly isolated type II cells

The polarity distribution of the $GABA_A$ receptor ($\alpha1\alpha3\beta2\gamma2\pi$) is similar to CFTR and CaCC on airway epithelial cells. We speculated that ionotropic GABA receptors may contribute to Cl^- secretion in type II cells. Therefore, we determined the effect of GABA on the efflux of Cl^- by using freshly isolated type II cells. At 100 µM, GABA significantly increased the $^{36}Cl^-$ efflux rate from 0.0623 ± 0.0088 to 0.103 ± 0.0139 nmole/min/mg. Pretreatment of the cells with picrotoxin restored the $^{36}Cl^-$ efflux rate to the basal level (Fig. 4.3A & B). The effect of GABA on type II cells was dose-dependent and saturated at 300 µM (Fig. 4.3C). The GABA-mediated $^{36}Cl^-$ efflux was not due to other channels since bumetanide, the NKCC inhibitor, glybenclamide, an inhibitor of the CFTR, and BAPTA-AM, the intracellular calcium chelator to inhibit CaCC, and NPPB, a general inhibitor of anion channels, had no effect on the GABA-mediated $^{36}Cl^-$ efflux (Fig. 4.3 D).

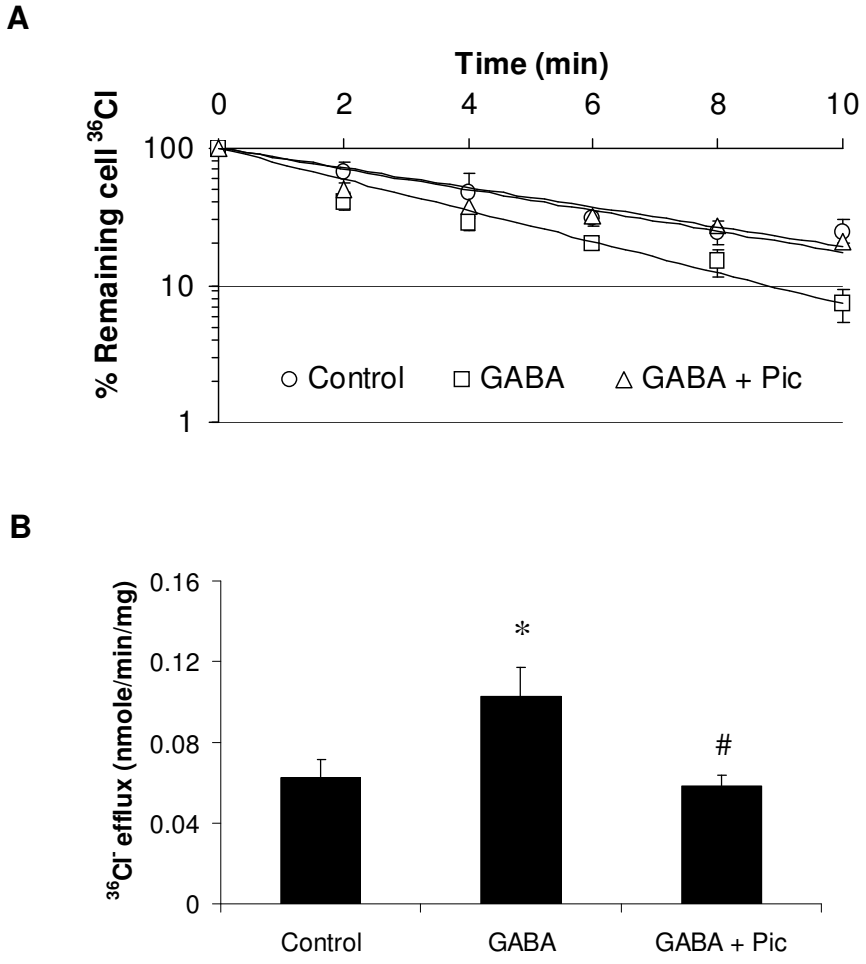
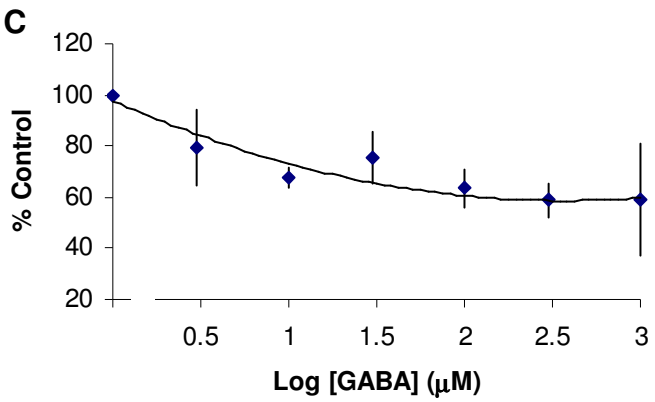


Fig. 4.3 GABA increases $^{36}Cl^-$ efflux in freshly isolated type II cells. (A) Type II cells were loaded with $^{36}Cl^-$ and then incubated for 0-10 min with Ringers solution in the absence (control) or the presence of 100 µM GABA and/or 100 µM picrotoxin (Pic). The cellular $^{36}Cl^-$ (nmole/mg) at different time points were determined. A percentage of remaining cellular $^{36}Cl^-$ was plotted against time and analyzed by linear regression. (B) Cl^- efflux rate in type II cells calculated from panel (A). Data shown are means ± S.E. *P<0.05 vs. control and #P<0.05 vs. GABA by LSD analysis (n=4).



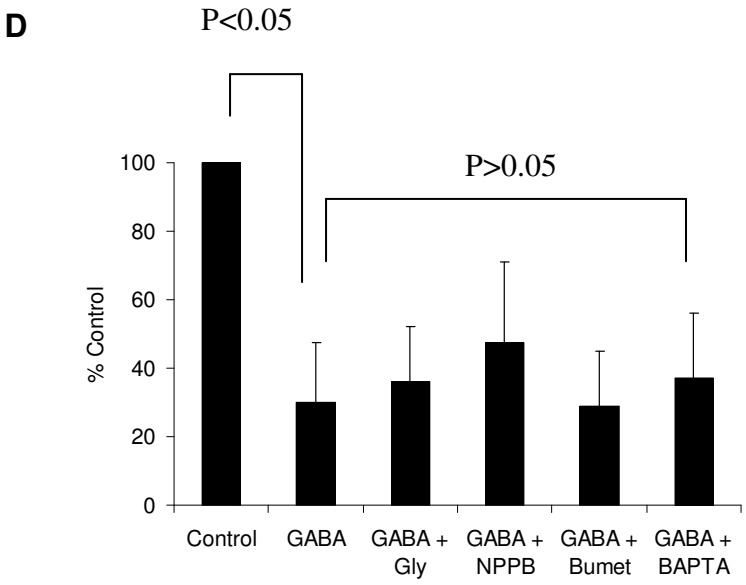


Fig. 4.3 GABA increases $^{36}Cl^-$ efflux in freshly isolated type II cells. (C) A dose-dependence of $^{36}Cl^-$efflux to GABA. Type II cells were loaded with $^{36}Cl^-$ and then incubated for 10 min with Ringers solution in the absence (control) or the presence of 0-1000 μM GABA. The percentage of remaining cellular $^{36}Cl^-$ to control was plotted vs. log concentration of GABA. (D) The GABA-dependent $^{36}Cl^-$ efflux was analyzed in the presence of 100 μM bumetanide (Bumet), or 100 μM glybenclamide (Gly), or 60 μM NPPB, or 25 μM BAPTA-AM (BAPTA) and expressed as a percentage of remaining cellular $^{36}Cl^-$ to control (no GABA). Data shown are means ± S.E. Connected line, $P<0.05$ vs. control or $P>0.05$ vs. GABA by LSD analysis (n=4).

4.4.4 GABA inhibits the apical-to-basolateral Cl^- transport on the type II cell monolayer

The effect of GABA on the Cl^- transport across the type II cell monolayer was examined, which is more comparable to the *in vivo* conditions. The Cl^- transport from apical to basolateral or from basolateral to apical side was linear within 1 h (Fig. 4.4 A&C). The basal transport rate of transcellular Cl^- was 0.18 ± 0.04 nCi/h from the apical to the basolateral side and 0.08 ± 0.04 nCi/h from the opposite direction. The paracellular Cl^- transport rate, as determined by ^{14}C mannitol, was< 10% of the total Cl^- transport (transcellular plus paracellular) from both directions. The addition of GABA to the apical side of the cells did not change the paracellular Cl^- transport; however, the transcellular Cl^- transport was inhibited by 37% in comparison to the control (P=0.004). When the cells were pre-incubated with picrotoxin, GABA failed to decrease the apical to basolateral Cl^- transport (P=0.046). Picrotoxin alone slightly increased the Cl^- transport but was not significant (Fig. 4.4 B). GABA had no effect on the basolateral-to-apical Cl^- transport (Fig. 4.4 C&D). Isoproterenol was known to elevate cellular cAMP and to increase whole cell Cl^- conductance. The addition of isopreterenol to the basolateral side of type II cell monolayer increased the apical-to-basolateral Cl^- transport by 62%. Glybenclamide, the known CFTR inhibitor, efficiently blocked the effect of isopreterenol and resulted in a Cl^- transport rate even lower than the basal level. GABA also significantly counteracted the isoproterenol-stimulated apical-to-basolateral Cl^- transport (P=0.045 from 6 paired groups). Interestingly, when GABA and glybenclamide were applied to the cells together, the inhibition

on the isoproterenol-elevated Cl^- absorption was not significant.

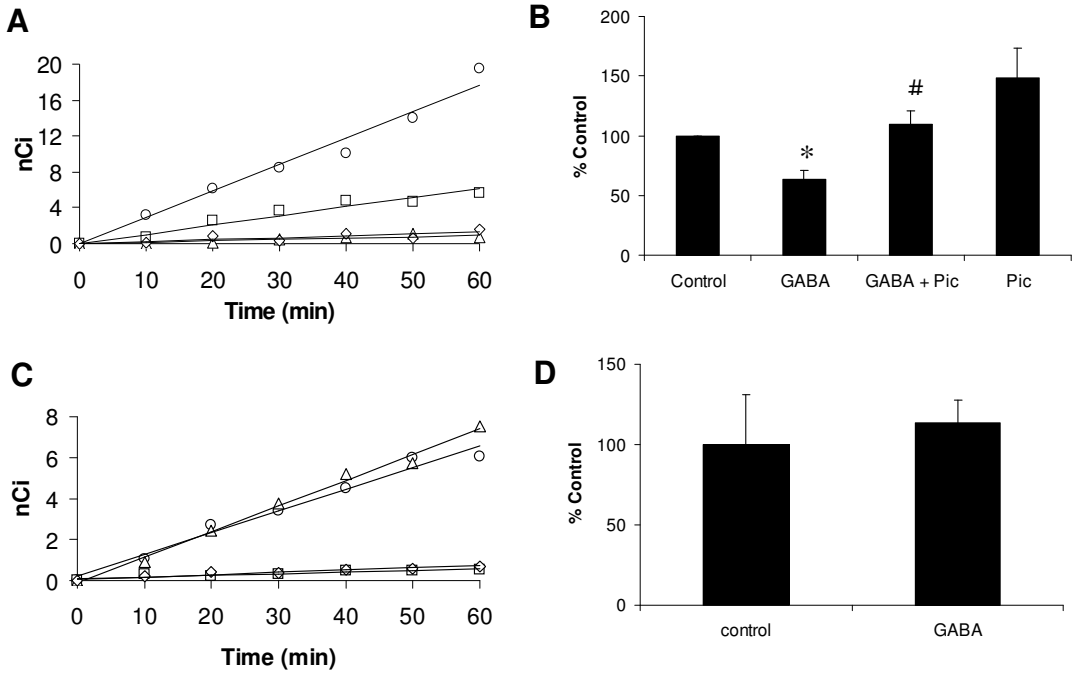


Fig. 4.4 GABA inhibits the basal apical-to-basolateral Cl^- transport on type II cell monolayer. Type II cells were cultured on the air-liquid interface until a monolayer formed. [^{36}Cl]NaCl and [^{14}C]mannitol were added to the apical or basolateral side of the monolayer in the presence or absence of GABA (100 μM) and/or other inhibitors. Isotope accumulation (nCi) was measured on the opposite side. Transcellular $^{36}Cl^-$ transport rate was calculated by subtracting [^{14}C]mannitol transport from total $^{36}Cl^-$ transport. The transport of $^{36}Cl^-$ and ^{14}C (nCi) across the monolayer was plotted vs. time. (A) Apical-to-basolateral and (C) Basolateral-to-apical. Circle: total ^{36}Cl transport in the control group; square: total $^{36}Cl^-$ transport in the GABA group; triangle: paracellular $^{36}Cl^-$ transport in the control group; diagram, paracellular $^{36}Cl^-$ transport in the GABA group. (B) & (D). The net $^{36}Cl^-$ transport rate was expressed as a percentage of the control as calculated from (A) and (C). Pic: 100 μM picrotoxin. Data shown are means ± SE. *P<0.05 vs. control and #P<0.05 vs. GABA by LSD analysis (n≥4).

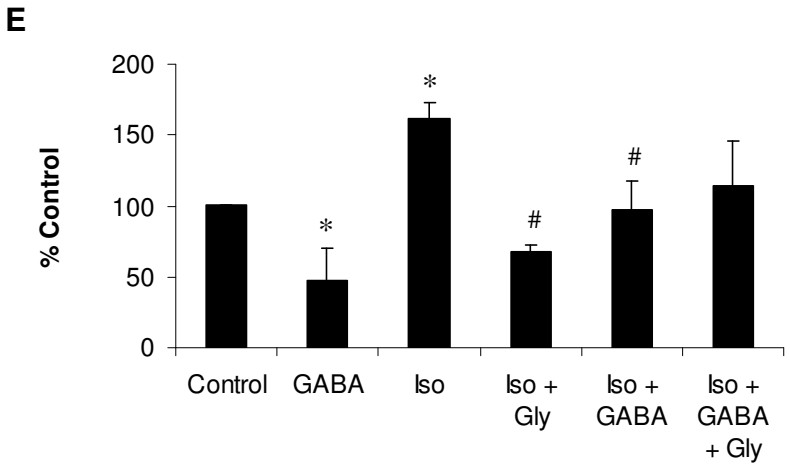


Fig. 4.4 GABA inhibits the isoproterenol-stimulated apical-to-basolateral Cl^- transport on type II cell monolayer. Apical-to-basolateral $^{36}Cl^-$ transport rate was determined in the presence of isoproterenol (iso), isopreternol and Glybenclamide (iso+Gly), isoproterenol and GABA (iso+GABA), isoproterenol and Glybenclamide and GABA (iso+Gly+GABA). The concentration of all the compounds used are 100 μM. Data shown are means ± SE. *P<0.05 vs. control, #P<0.05 vs. iso, n≥5.

4.4.5 Silencing of the π-subunit eliminated the GABA-dependent Cl^- efflux in type II cells

To confirm the functions of the ionotropic GABA receptors in type II cells, we constructed adenoviral vectors containing 3 siRNA sequences target to the π-

subunit under the control of the U6 promoter using the method we developed previously (Gou et al. 2004). We first tested the efficiency of siRNA sequences by using immunostaining. As shown in Fig. 4.5A, 2510 efficiently silenced the expression of $GABA_A$ receptor π subunit while maintaining the cell morphology; 2511 also efficiently silenced the π-subunit expression; 2512 had no significant effect on the expression of the π-subunit. Using 2510, we further optimized the conditions for efficient silencing and using minimal adenoviruses. We added the viruses at day 0, 1, 2, 3, and 4. After totally 8 days culture, the mRNA level of the π-subunit was determined. The addition of the viruses at day 1-3 resulted in >95% reduction of the π-subunit mRNA. However, when the viruses were added on day 0 or day 4, the mRNA of the π-subunit only decreased 90% and 80% (Fig. 4.5 B). A dose-dependent study revealed that a dose ≥10 MOI/cell efficiently knocked down the π-subunit mRNA expression (Fig. 4.5 C). Based on these results, we chose the following conditions: a dose of 10 MOI/cell viruses (2510) addition at day 1 in the subsequent experiments. Western blot analysis further showed that the π-subunit protein was markedly reduced (Fig. 4.5D). The silencing of the π-subunit did not affect the formation of cell monolayer because the tight junctions were still intact in the π-subunit-silenced cells as indicated by immunostaining of ZO-1 (Fig. 4.5E).

The consequence of losing the π-subunit on the $^{36}Cl^-$ efflux was determined. The virus 2512 was used as a control. The 2512-infected cells still maintained their ability responding to GABA for $^{36}Cl^-$ efflux. However, in the 2510-treated cells, GABA did not increase $^{36}Cl^-$ efflux (Fig. 4.5F).

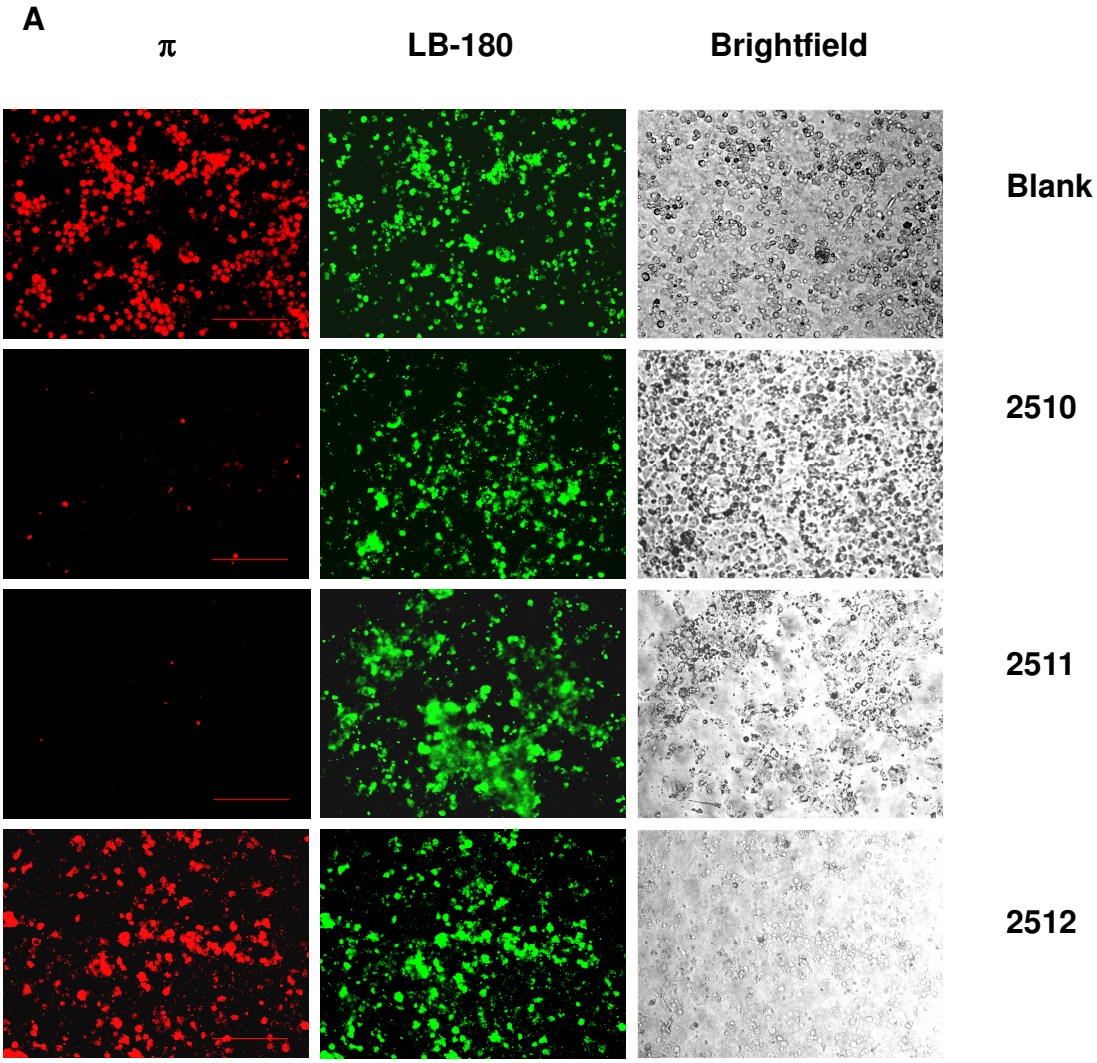


Fig. 4.5 Screening of different siRNA sequences. 50 MOI/cell of adenoviruses carrying U6-driven shRNA targeted to 3 different regions of GABRP: 2510, 2511, and 2512, were transduced to type II cells. The cells were double-labeled with anti-GABRP (red) and anti-LB180 (green) antibodies. Bar: 30 µm.

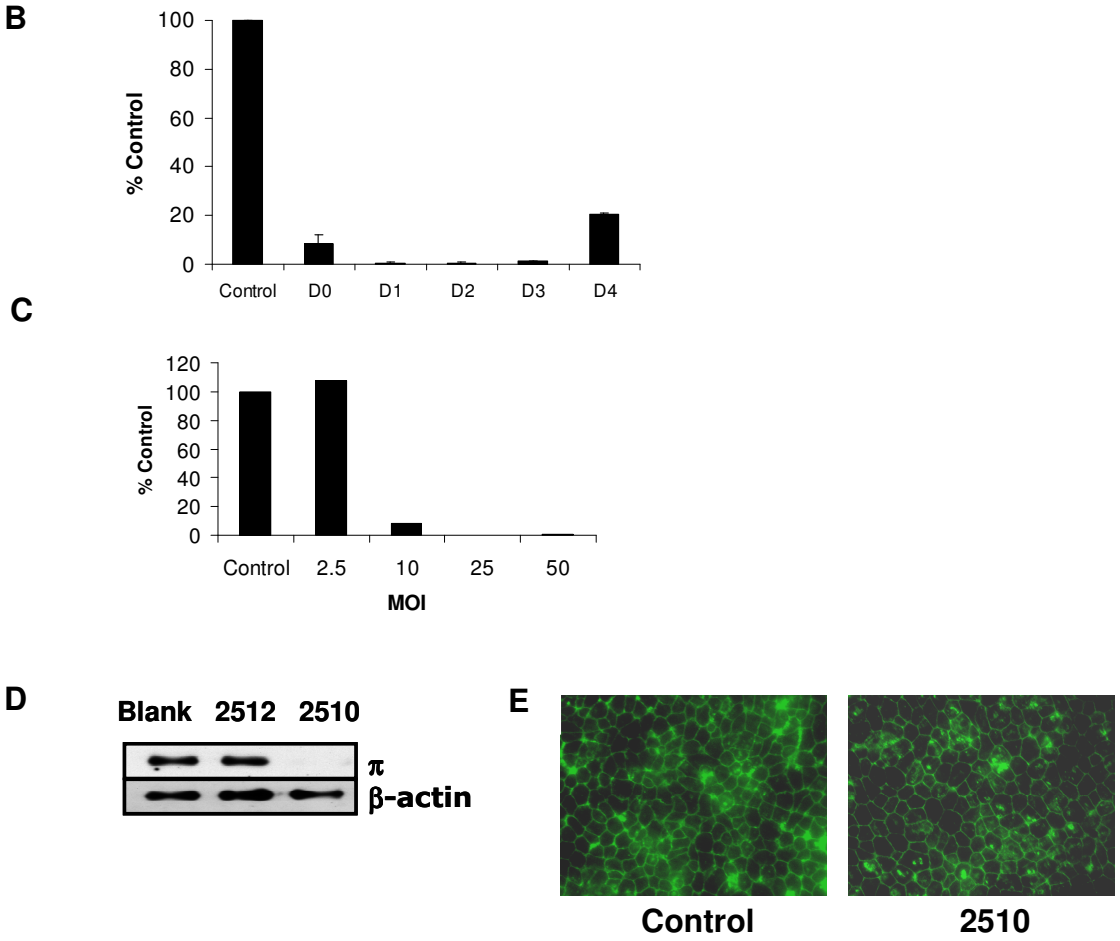


Fig. 4.5 Knocking-down of the π-subunit by RNAi. (B) Time course. Type II cells were transduced with 50 MOI/cell of adenovirus 2510 on the day of isolation, or one, two, three, and four days after isolation (D0-4). All of the cells were harvested at the end of day 8 and the mRNA level of GABRP was analyzed by real-time PCR and normalized with 18S rRNA. The results were expressed as a percentage of control (untreated cells, n=3). (C) Dose-dependence. Type II cells were infected with 2.5, 10, 25, and 50 MOI/cell adenovirus 2510. The mRNA levels of GABRP were determined as described in (C). (D) GABRP protein level. Type II cells were transduced with 10 MOI/cell of adenovirus 2512 or 2510 and the protein level of GABRP was analyzed by western blot. β-actin was used as the loading control. (E) Tight junctions. Type II cells were transduced with 10 MOI/cell of adenovirus 2510 and were stained with anti-ZO1 antibodies.

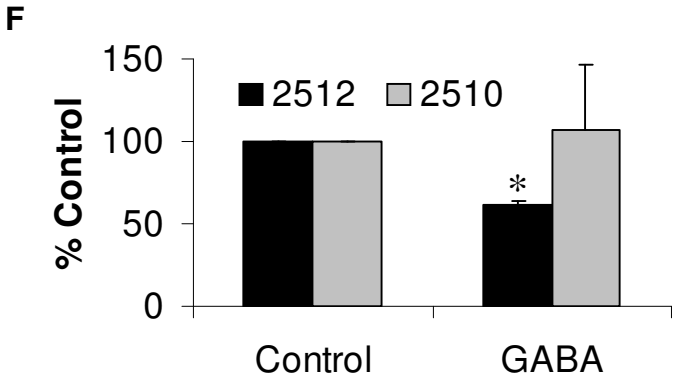


Fig. 4.5 Silencing of the π-subunit eliminated the GABA-dependent Cl^- efflux. $^{36}Cl^-$ efflux was analyzed in type II cells that were transduced with adenovirus 2512 and 2510 in the absence (control) or presence of 100 μM GABA. The remaining cellular $^{36}Cl^-$ in the presence of GABA was expressed as percentage to the control. Data shown in are means ± SE. *P<0.05 vs control (n=3).

4.4.6 GABA inhibits basal and stimulated alveolar fluid clearance in anesthetized rats

The effects of GABA on the Cl^- transport on freshly isolated and cultured type II cells imply a potential role of ionotropic GABA receptors on alveolar fluid homeostasis in the lung. To test the possibility, alveolar fluid clearance were determined on anesthetized rats. The basal alveolar fluid clearance in rat lungs was 24±4 % per hour. GABA (300 μM) inhibited 58% of the basal fluid clearance (Fig. 4.6A). When picrotoxin, an antagonist of ionotropic GABA receptor, was

instilled into rat lungs together with GABA, the alveolar fluid clearance rate was restored to the basal level. Picrotoxin itself only slightly increased the alveolar fluid clearance. However, this increase was not statistically different. Because both $GABA_A$ and $GABA_C$ receptor subunits are expressed in type II and type I cells, it is possible that both type of receptors participate in alveolar fluid clearance. Indeed, bicuculine (a specific $GABA_A$ receptor antagonist) and TPMPA (a specific $GABA_C$ receptor antagonist) partially restored the GABA-mediated inhibition of alveolar fluid clearance. When the two antagonists were combined, the alveolar fluid clearance was restored to the level close to the basal conditions (Fig. 4.6A).

Because GABA is a small molecule, it might cross the epithelial cells and affect the alveolar fluid clearance via the nervous system. To exclude this possibility, we conjugated GABA to a large molecule, BSA protein, which prevents GABA enter the circulation. Western blot showed that the GABA conjugated-BSA specifically reacted with anti-GABA antibodies, and the control BSA without GABA did not (Fig. 4.6B). When the GABA-BSA conjugate was instilled into rat lungs, alveolar fluid clearance was reduced about 59%. The decrease was also restored to the basal levels by instilling picrotoxin together with the GABA-BSA conjugate. BSA conjugate had no effect on the alveolar fluid clearance (Fig. 4.6C). Those results suggest that the effect of GABA on alveolar fluid clearance was only from the alveolar epithelial cells, but not from the nervous system via circulation.

Isoproterenol was known to increase alveolar fluid clearance via

increasing Na^+ and Cl^- absorption (Mutlu et al. 2004, Saldias et al. 1999,Jiang et al. 1998a, Fang et al. 2002). We therefore examined the effects of GABA on the isoproterenol-stimulated alveolar fluid clearance in the anesthetized rats. Isopreternol increased the basal alveolar fluid clearance by 65%. Glybenclamide significantly inhibited the isoproterenol-stimulated alveolar fluid clearance, consistent with previous reports (Fang et al. 2002). When GABA and isopreterenol were instilled together, isoproterenol also failed to stimulate the alveolar fluid clearance. Interestingly, the combination of GABA and glybenclamide did not cause further decrease in comparison with GABA and glybenclamide alone. These data suggest GABA inhibits both basal and β-agonist stimulated fluid clearance in anesthetized rats.

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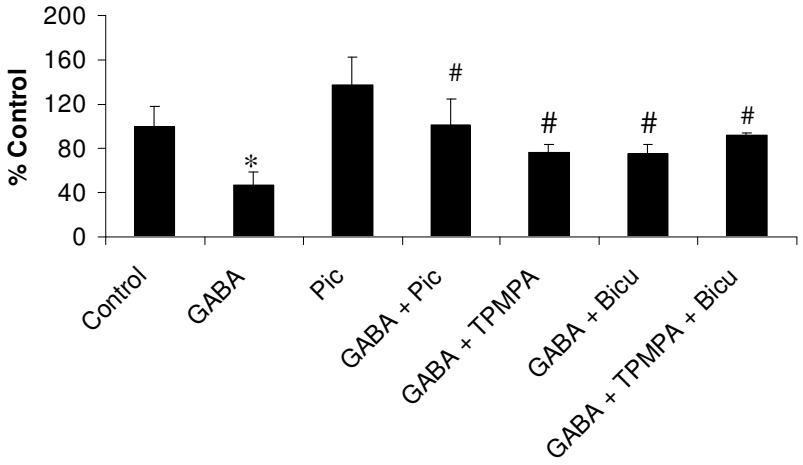

Fig. 4.6 GABA inhibits basal alveolar fluid clearance in anesthetized rats. Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with GABA, picrotoxin (Pic), GABA and picrotoxin (GABA+Pic), GABA and bicuculine (GABA+Bicu), GABA and TPMPA (GABA+TPMPA), GABA and bicuculine and TPMPA (GABA+TPMPA+Bicu). The concentrations used are 300 µM for GABA and 100 µM for other compounds. Alveolar fluid clearance was determined by the concentration change of the instillate. Data shown are mean ± SE (n≥4). *P<0.05 vs. control and #P<0.05 vs. GABA by Dunnett's analysis.

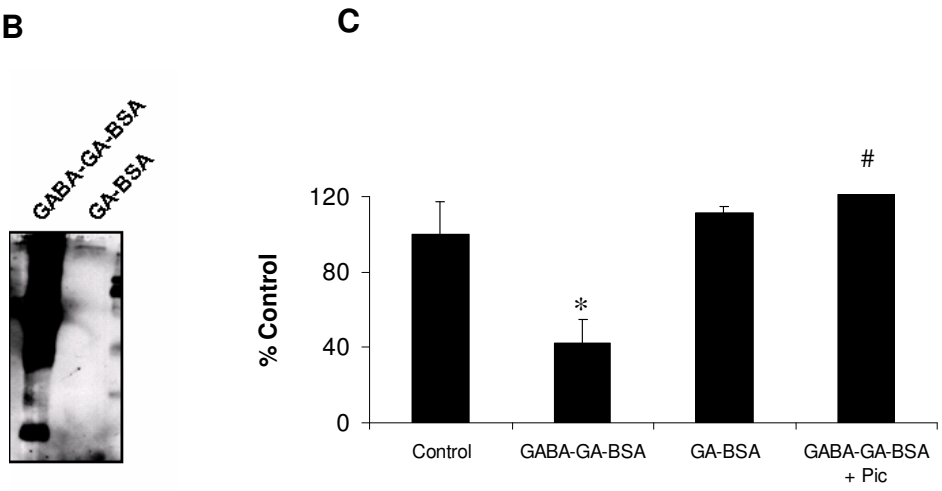

Fig. 4.6 GABA inhibits basal alveolar fluid clearance independent of the nervous system. (B) Conjugation of GABA to BSA. BSA-conjugated GABA (GABA-GA-BSA) was synthesized by using glutaradehyde (GA) as the closslinker. The control was the BSA treated with GA in the absence of GABA (GA-BSA). The conjugates were detected by Western blot by using anti-GABA antibodies. (C) Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with GABA-GA-BSA, GA-BSA, GABA-GA-BSA and 100 μM picrotoxin (GABA-GA-BSA + Pic). Alveolar fluid clearance was determined by the concentration change of the instillate. All of the results were expressed as a percentage of the control. The data shown are means ± SE ($n \geq 4$). *$P<0.05$ vs. control and #$P<0.05$ vs. GABA-GA-BSA by LSD analysis.

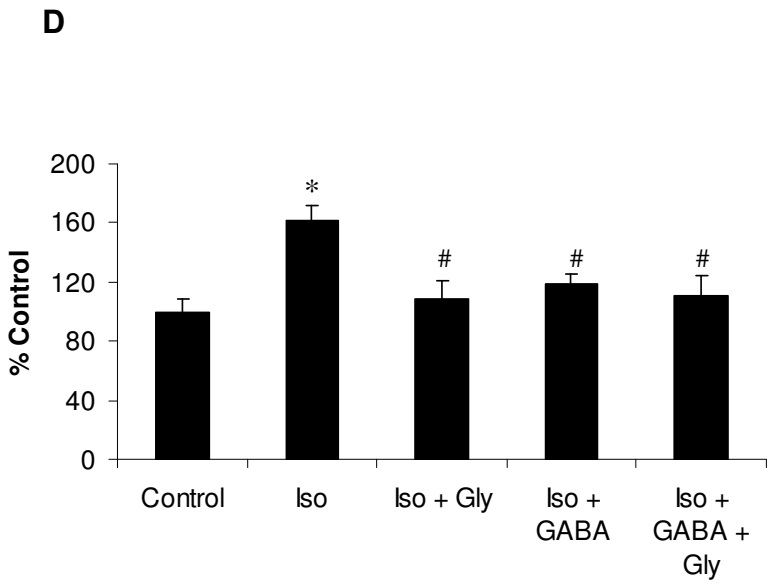

Fig. 4.6 GABA inhibits stimulated alveolar fluid clearance in anesthetized rats. Anesthetized rats were instilled with 5% BSA (control), or 5% BSA with isopreternol (Iso), isopreternol and glybenclamide (Iso + Gly), isopreternol and GABA (Iso + GABA), isopreternol and glybenclamide and GABA (Iso + Gly + GABA). The concentrations used were 100 μM for isopreternol and glybenclamide and 300 μM for GABA. Alveolar fluid clearance was determined. All of the results were expressed as a percentage of the control. The data shown are means ± SE ($n \geq 4$). *$P<0.05$ vs. control and #$P<0.05$ vs. Iso by LSD analysis.

4.5 Discussion

In this study, we found the expression of multiple ionotropic GABA recpetor subunits on adult rat alveolar epithelial cells and the synthesis of the physiological ligand, GABA, in type II cells. We also identified a native receptor on the apical plasma membrane of type II cells. Based on the isotope studies on isolated type II cells and alveolar fluid clearance analysis on anesthetized rats, combining with the RNAi studies, we demonstrate that ionotropic GABA receptors contribute to alveolar fluid homeostasis via a novel Cl^- transport pathway.

The GABA receptor-mediated fluid transport is quite different from CFTR. Although both channels are localized on the apical plasma membranes (Lee et al. 2003, Jiang et al. 1998a, Fang et al. 2005), CFTR does not function at basal conditions (Stutts et al. 1995), while GABA receptors work at both basal and stimulated circumstances. CFTR mediates an apical-to-basolateral Cl^- current in the presence of Cl^- gradient, when activated with cAMP-agonists (Fang et al. 2005). However, GABA receptors mediate an outward Cl^- transport from the luminal surface even there is no Cl^- gradient. CFTR facilitates cAMP-dependent alveolar fluid clearance when activated by the cAMP-agonist (Fang et al. 2002, Fang et al. 2005), but GABA receptors inhibit such effects. On the molecular mechanism of the fluid homeostasis, CFTR provides a Cl^- transport pathway to clear the alveolar fluid, whereas GABA receptors contribute to the pathway to maintain the volume of the alveolar fluid. Although minimum volume of alveolar surface liquid is good for gas-exchange; sufficient liquid is still required for the

circulation of the alveolar surface network as well as electrolyte exchange (Scarpelli 2003, Ng et al. 2004). In addition, CFTR is only distributed on type II cells (Brochiero et al. 2004,Fang et al. 2005), ionotropic GABA receptors may exist on both type I and type II cells (Fig. 4.1A). Type I cells may also play an active role in alveolar fluid homeostasis (Johnson et al. 2002, Borok et al. 2002), therefore, the identification of GABA receptors in both type I and type II cells suggest a role of both cell types in the alveolar fluid homeostasis.

Ionotropic GABA receptors require the presence of ligands to be activated. The discovery of GABA and GAD in type II cells suggest that type II cells are one of the sources of the physiological ligand in alveoli. Such ligand producing pathway has also been found in pituitary and pancreas (Mayerhofer et al. 2001, Franklin et al. 2004). Type II cells are the surfactant-producing cells in alveoli; interestingly, in pituitary and pancreas, GABA or GAD are also predominantly distributed on insulin- or GH-producing cells and stored in synaptic-like microvesicles (SLMVs). Further, the common signals for the exocytosis of insulin, GH, and surfactant, such as Ca^{2+} and cAMP, stimulate the releasing of GABA from pancreas β-cells (Braun et al. 2004). Therefore, those signals may also regulate the secretion of GABA from type II cells. Although lacking the synaptic cleft structure, the exocytosis of GABA was shown to induce transient Cl^- current in the pancreatic β-cells. Similarly, the type II cell-originated GABA may also regulate Cl^- transport on type II cells autocrinally and on type I cells paracrinally.

We have found that GABA increased the Cl^- efflux from freshly isolated type II cells dose-dependently while picrotoxin blocked such effect, suggesting

the efflux was mediated by ionotropic GABA receptors, but not through $GABA_B$ receptors or other non-specific effects of GABA (Bormann 2000). Because the efflux was not dependent on NKCC, Cl^- must be extruded from the apical surface of the cells. In addition, the efflux was not dependent on CFTR, CaCC, and other non-specific Cl^- channels, suggesting the GABA-mediated Cl^- was extruded through the opened receptor pores (Fig. 4.3). GABA-mediated Cl^- secretion was further confirmed by the RNAi study that, after knocking-down the π-subunit expression, type II cells lose the ability to efflux Cl^- in response to GABA (Fig. 4.5).

It is possible that the GABA-mediated Cl^- secretion can inhibit the Cl^- absorption via other Cl^- channels. The addition of GABA to the type II cell monolayer significantly decreased the basal apical-to-basolateral Cl^- transport (Fig 4.4). Isoproterenol activates the cAMP-PKA pathway and increases the conductance of Na^+ and Cl^- channels as well as other channels (Mutlu et al. 2004, Saldias et al. 1999,Jiang et al. 1998a, Fang et al. 2002). Similar with Lee and Jiang's results, we found that isoproterenol significantly increased the apical-to-basolateral Cl^- transport whereas glybenclamide, the CFTR inhibitor, blocked such effect (Jiang et al. 1998a, Lee et al. 2003). GABA significantly inhibited the isoproterenol-stimulated apical-to-basolateral Cl^- transport, suggesting the Cl^- secretion mediated by GABA is enough to counteract against the elevated Cl^- influx by isoproterenol.

The fact that GABA increases Cl^- secretion on fresh type II cells and inhibits Cl^- apical-to-basolateral transport on cultured type II cells suggest that

they may play a role on alveolar fluid homeostasis. To test this possibility, we performed alveolar fluid clearance study on anesthetized rats. Our results was again comparable to the previous observations, that isoproterenol increased the alveolar fluid clearance but glybenclamide inhibited the effect of isoproterenol (Fang et al. 2002). GABA inhibited both basal and isoproterenol-stimulated fluid clearance, which was consistent with the reduction of Cl^- apical-to-basolateral transport on type II cell monolayers (Fig. 4.6). GABA-modulated alveolar fluid homeostasis was through alveolar epithelial cells but not CNS. Because GABA conjugated BSA, which can not enter the circulation, has a similar effect as the free GABA (Fig. 4.6). Therefore, this study demonstrated that GABA receptors were indispensable for maintaining the alveolar fluid homeostasis.

Fluid homeostasis is normally considered to be mediated by “slow” channels such as ENaC, CFTR, ClC, and CaCC (Jentsch et al. 2002b, Matalon et al. 2002). Activation of “fast” channels usually leads to the generation of action potentials or hyperpolarizations (Jentsch et al. 2002a, Whiting et al. 1999). Why ionotropic GABA receptors, a “fast” channel, act like a “slow” channel in alveolar epithelial cells? Probably the receptor density and the concentration of GABA are much lower in alveolar epithelial cells than in the brain (Ong and Kerr 1990, Chapman et al. 1993). As a result, activation of those receptors in alveolar epithelial cells may not enough to generate depolarization or hyperpolarization; instead, the cells regulate fluid homeostasis similar to “slow” channels. Most peripheral organs have lower receptor density and GABA concentration than the brain (Watanabe et al. 2002). Regulation of fluid secretion by $GABA_A$ receptors

was also observed in other peripheral organs. For example, in isolated pancreas, GABA increases the panscreas fluid secretion (Park 2000).

This study may have a great clinical potential. First, an important clinic problem involves how to remove edema fluid from injuried lungs (Berthiaume et al. 2002). Isoproterenol is used clinically to reduce lung edema (Chernow et al. 1982, Persson 1979). Due to the Cl^- secretory property of GABA receptors in alveolar epithelial cells, as well as the fact that GABA counteracts against the effect of isoproterenol, it may be helpful to inhibit the GABA receptor in those cases. Second, most ion channels expressed in alveolar epithelial cells are also expressed on upper airways (Rochelle et al. 2000). If GABA receptors are also expressed on upper airways, they could become a new candidate to resolve CF lung diseases.

In conclusion, we have identified functional ionotropic GABA receptors on alveolar epithelial cells, which contribute to alveolar fluid homeostasis through a novel Cl^- transport pathway. Our study may have a great clinic potential to resolve abnormal lung diseases caused by fluid abnormalities in the future.

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Chapter 5

DISCUSSION

The results of each specific aim have been discussed in chapters 2, 3, and 4. In this part, the significance of the study as a whole is summarized. This dissertation project, started from a single subunit of ionotropic GABA receptor (GABRP) that was identified in alveolar type II cells by DNA microarray analysis. The importance of ionotropic GABA receptors in the lung and alveolar epithelial cells was recognized and examined. Through the characterization of GABRP expression in cultured alveolar epithelial cells, the similar regulatory patterns of the GABRP expression to other Na^+ and Cl^- transporters were demonstrated. In the next step, all the GABA receptor subunits in the fetal lungs and type II cells were identified. By studying their ontogenic expression, a role on fetal lung fluid transport was proposed for those receptors. In addition, through their down-regulation after hyperoxia exposure, their role in adult lung fluid homeostasis was implicated. In the end, functional studies were performed. By using freshly isolated cells, cultured cells, and animals, combining with isotope and RNAi techniques, the role of ionotropic GABA receptors on adult lung fluid transport was confirmed. Those GABA receptors contribute to a novel alveolar fluid

transport pathway. This study has identified a novel function of ionotropic GABA receptors in the lung and broadened the ion channel spectrum of the alveolar epithelium. The results could help researchers to further understand the mechanism of alveolar fluid transport in both physiological and pathological conditions. Furthermore, important clinic strategies may be able to be developed based on this research to improve the clinical practice on lung edema and CF diseases in the future.

Ionotropic GABA receptors are important in both nervous systems and peripheral organs. The roles of ionotropic GABA receptors in peripheral organs including pancreas, pituitary, intestine, and uterus have been studied. They are normally involved with hormone secretion. The roles of GABA receptors on the lung are not known. Current study demonstrates that ionotropic GABA receptors are expressed on alveolar epithelial cells and contribute to alveolar fluid transport. The distinct functions of those receptors in those peripheral organs suggest that ionotropic GABA receptors may have multiple functions. Being a “fast” channel, the role of GABA receptors on fluid transport was largely neglected. This research indicates that “fast” channels can also contribute to fluid transport in addition to “slow” channels.

Cl^- channels are essential for the lung epithelial cells. This study broadens the ion channel spectrum of the alveolar epithelium. The known Cl^- channels on the lung epithelium include CFTR, CaCC, ClC, NKCC, KCC, and purinergic receptors. All those channels can regulate cellular activity via various mechanisms. The discovery of the ionotropic GABA receptors on alveolar

epithelial cells significantly enriches the ion channel spectrum. Not like the single gene-encoded channels, we have identified at least 17 subunits of GABA receptors in the rat lungs. Tens or hundreds of receptor complexes could be formed from those subunits. As a consequence, the discovery of the ionotropic GABA receptors on alveolar epithelial cells introduces a big family of ion channels into the known spectrum. Although we have only identified one complex, $\alpha 1 \alpha 3 \beta 2 \gamma 2 \pi$, other functional channels could also be formed on alveolar epithelial cells. Due to the fact that GABA receptors can modulate their conductance by different mechanisms, such as changing the combination of the subunits and binding with steroids, they may also regulate the alveolar epithelium via those mechanisms. Therefore, this discovery would open a new aspect in the field of alveolar epithelial cell biology.

The importance of Cl^- channels on the regulation of alveolar fluid homeostasis begins to be more and more recognized. However, due to the fact that very few Cl^- channels have been identified on alveolar epithelial cells, very little is known about the molecular mechanisms. To-date, CFTR is almost the only Cl^- channel that has been shown to facilitate alveolar fluid clearance. CFTR may not be the only channels that participate in this process. Further, it is not necessary that the alveolar fluid is always cleared. Our study has shown that ionotropic GABA receptors mediate a “rectified outward” Cl^- transport on type II cells and inhibit the alveolar fluid clearance in rats. The effect is independent of all the known lung epithelial Cl^- channels. Therefore, ionotropic GABA receptors contribute to a novel alveolar fluid transport pathway. This discovery could help

researchers to further understand the mechanisms of the production and regulation of the alveolar fluid. Interestingly, even type I cells are considered to be important for the alveolar fluid homeostasis, only Na^+ transporters have been found in type I cells. Ionotropic GABA receptors may be the first Cl^- channels that have been identified on both type I and type II cells.

Lung fluid secretion is a prerequisite of fetal lung development, whose main driving force is provided via the active Cl^- secretion by the lung epithelium. It is not clear how those fetal lung fluids are formed. A couple of Cl^- channels have been identified on fetal lungs; however, none of them are proved to be indispensable for the fetal lung development. Because type II cell is an important source to produce fetal lung fluid, the identification of the ionotropic GABA receptors on fetal lungs and type II cells has introduced a novel candidate to produce fetal lung fluid. Other researchers in this group have already begun to provide evidence to support the hypothesis. Therefore, this study may finally answer the curious question in the lung field: why CFTR is so important in the lung, but lung development is not hampered in the CF animals?

Lung fluid homeostasis is essential at both physiological and pathological conditions. The roles of Cl^- channels in hyperoxic lung injury are not clear. Almost all the subuits of GABA receptors decrease after the hyperoxia exposure. Because our functional studies have demonstrated the secretory properties of the ionotropic GABA receptors in adult rat lungs, the decreasing of those subunits may be an adaptation response to hyperoxia to reduce the formation of the edema fluid.

The most appealing part of this research is probably its potential application on clinic strategies in the future. Lung edema is a common clinic problem. Lung edema is usually therapied by administration of isoproterenol. Because our study has indicated that GABA counteracts against the effect of isoproterenol on the alveolar fluid clearance, in case of lung edema, the synthesis and releasing of GABA in the lung should be inhibited. However, it may be not a problem in the case of hyperoxic lung injury, because most ionotropic GABA receptors are already down-regulated. Most alveolar ion channels are also found in the airways. If the ionotropic GABA receptors are also expressed in the airway, due to their Cl^- secretory properties, they could become an attracting candidate for resolving CF diseases.

In addition to the significances described above, this study also indicates that DNA microarray is an efficient method to identify target genes. DNA microarray analysis is able to identify thousands of differentially expressed genes, choosing the right target is essential for the microarray follow-up studies. In this research, selection of GABRP as the starting point was not by accident, but because of the importance of GABA receptors in CNS and other systems as well as the essential roles of Cl^- channels in the lung. Furthermore, GABRP is relative new and has distinct distributions and functions in comparison with other subunits. The follow-up characterization and functional study indicated that we have chosen a right target.

This study has provided answers to some questions from previous research; however, more “unknowns” are generated at the same time. We have shown that

ionotropic GABA receptors contribute to alveolar fluid transport in adult lungs, whether those receptors are also expressed in the airway and whether they can restore the fluid secretion in CF lungs is not clear. Therefore, the expression and function of those receptors in the airway should be examined. We have also shown that type I cells express numerous ionotropic GABA receptor subunits, whether functional receptors are formed in type I cell and whether they contribute to fluid transport could be tested. In addition, ionotropic GABA receptors can modulate cellular activities via cross-talking with other ion channels. What the effects of GABA receptors on ENaC, CFTR, and NKCC, could also be examined. Furthermore, ionotropic GABA receptors could be regulated by various phosphorylation pathways, whether those phosphorylations regulate the ASF could be studied.

We still have difficulties to explain some of the expression patterns of the GABA receptor subunits during the development of the lung and type II cells. We have shown that some subunits decrease in the lung from fetal to adult stages, and some of them increase. Whether ionotropic GABA receptors contribute to lung fluid transport throughout the animal's life remains to be determined. It is possible that some subunits play a prominent role in fetal lungs, but other subunits in adult lungs. Therefore, further studies may help us to understand the precise regulation of the Cl^- conductance and the electrophysiological properties of ionotropic GABA receptors as a requirement for the lung development. The roles of Cl^- channels at the late gestational stage and at birth are not well documented in the lung. In the specific aim II, we have shown that 9 subunits of

GABA receptors have a peak expression at birth in type II cells. The observation is contradictionary to the other known Cl^- channels. CFTR and ClC have the highest expression in the lung at mid-gestation but decrease before birth. The changes of CFTR and ClC indicate that the requirement for fluid secretion is reduced at birth. The reason for up-regulation of those GABA receptor subunits is unknown. Probably in addition to the reduced requirement for fluid secretion, there is also a need to increase the Cl^- absorption at birth. If all the Cl^- channels are reduced at birth, the lung will lower its ability to absorb Cl^-, which may slow down the absorption of Na^+. Whether ionotropic GABA receptors are responsible for the fluid absorption in the lung at birth should be further tested.

In summary, we have identified the expression of ionotropic GABA receptors on alveolar epithelial cells throughout the fetal and adult stages, which contribute to adult rat lung fluid transport via a novel pathway and provide a great clinic potential in the future.

Chapter 6

CONCLUSION

1. The $GABA_A$ receptor π subunit (GABRP) is a novel type II cell marker.
2. The expression of GABRP in alveolar epithelial cells is regulated by culture conditions. Plastic plate, attached collagen gel (AG), and liquid-liquid interface (LL) decrease the expression of GABRP, whereas matrigel, keratinocyte growth factor (KGF), detached collagen gel (DG), and air-liquid interface (AL) retain or up-regulate the expression of GABRP.
3. The mRNA of 17 ionotropic GABA receptor subunits are detected in fetal rat lungs and type II cells. Those subunits are differentially expressed during development. Six of them (α2, α5, α6, β1, γ3, and ρ3) significantly decrease in adult rat lungs. Nine of them (α1, α2, α4, β1, β2, γ1, θ, ρ1, and ρ3) are up-regulated at birth in type II cells.
4. Multiple ionotropic GABA receptor subunits, that are sufficient to form both type A and type C GABA receptors, are expressed in rat lungs, type II cells and type I cells.
5. Exposure to a high concentration of O_2 for 48-72 h significantly down-regulates the mRNA expression of most of the ionotropic GABA receptor

subunits in adult rat lungs.

6. GABA and glutamic acid decarboxylase (GAD) are only expressed in type II cells but not type I cells.
7. Adult type II cells express a GABA receptor complex $\alpha 1 \alpha 3 \beta 2 \gamma 2 \pi$, which is located only on the apical plasma membranes but not on the basolateral membranes.
8. GABA increases Cl^- efflux dose-dependently in freshly isolated type II cells, which is independent of the cystic fibrosis transmembrane regulator (CFTR), the Ca^{2+}-activated Cl^- channel (CaCC) and the Na^+-K^+-Cl^- co-transporter (NKCC).
9. GABA inhibits basal and isoproterenol-stimulated apical-to-basolateral Cl^- transport across the type II cell monolayer.
10. Knock-down of GABRP expression with RNAi eliminates the GABA-dependent Cl^- efflux.
11. GABA inhibits basal and isoproterenol-stimulated alveolar fluid clearance in anesthetized rats.
12. Ionotropic GABA receptors constitute a novel fluid transport pathway on alveolar epithelial cells.

Appendices

Appendix A: permission to reprint chapter 2 from Springer

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VITA

Nili Jin

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Doctor of Philosophy

Dissertation: CHARACTERIZATION AND FUNCTIONAL STUDY OF IONOTROPIC GABA RECEPTORS IN ALVEOLAR EPITHELIAL CELLS

Major Field: Physiology

Biographical:

Education:

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M.S. School of Agriculture and Natural Resources, Oklahoma State University, 1999-2001, Forestry Genetics (Advisor: Dr. Yinghua Huang). Thesis: Characterization and Functional Study of An Antimicrobial Protein from Loblolly Pine

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Major Field: Physiology

Scope and Method of Study:

We began this project from a target gene GABRP, identified from DNA microarray. We firstly examined the expression patterns of GABRP in various culture conditions by using real-time PCR, western blot and immunostaining. Then we isolated lung tissue or type II cells from fetal, neonate, and adult rats, as well as hyperoxia-exposed rats. By using real-time PCR, we demonstrated the differential expression of all the ionotropic GABA receptor subunits during fetal lung development and after hyperoxia exposure. We further performed the functional study on fresh or cultured type II cells and rats, by using real-time PCR, western blot, immunostaining, biotinylation, immunproecipitation, isotope, RNA intereference, and lung fluid clearance assays.

Findings and Conclusions:

1. The GABRP expression was regulated by culture conditions and closely associated with the type II cell-phenotype.
2. Among 19 known GABA receptor subunits, 17 were expressed in rat lungs and type II cells throughout the fetal and adult stages. Those subunits were differentially regulated during lung development and most of them were decreased by hyperoxia.
3. We found that adult rat type I cells and type II cells both expressed multiple ionotropic GABA receptor subunits. GABA and GAD were synthesized in type II cells. We identified one $GABA_A$ receptor complex, $\alpha1\alpha3\beta2\gamma2\pi$ in the type II cells. This receptor was localized on the apical but not basolateral plasma membranes. GABA increased Cl^- efflux dose-dependently, which was not due to CFTR, NKCC and CaCC. When type II cells were cultured on the air-liquid interface, GABA inhibited the basal and isoproterenol-stimulated Cl^- absorptions. Knock-down of the π-subunit eliminated the GABA-dependent Cl^- efflux. In anesthetized rats, GABA inhibited both basal and isoproterenol-stimulated alveolar fluid clearance. Therefore, we have identified a novel fluid transport pathway in adult rat alveolar epithelial cells, contributing to the ionotropic GABA receptors.

ADVISER's APPROVAL: Dr. Lin Liu