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EFFECTS OF HYPEROXIC EXPOSURE ON THE PULMONARY EICOSANOID PROFILE AND THE RELATIONSHIP TO THE OXIDANT-INDUCED LUNG PATHOLOGY IN NEONATAL RATS

BY

KIM SHIGEKO KOYANAGI

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EFFECTS OF HYPEROXIC EXPOSURE ON THE PULMONARY EICOSANOID PROFILE AND THE RELATIONSHIP TO THE OXIDANT-INDUCED LUNG PATHOLOGY IN NEONATAL RATS

Submitted by **KIM SHIGEKO KOYANAGI**

In partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE.**

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To Mom and Dadbo,

Thank-you for giving me a life

that will now be happy!

ABSTRACT

The role of eicosanoids in the pathogenesis of Bronchopulmonary dysplasia was investigated by exposing neonatal rats to hyperoxia. A novel lung explant technique was developed to elucidate the *in vivo* pulmonary eicosanoid production during hyperoxic exposure.

The characteristic sequence of hyperoxic lung injury was produced as assessed by weight ratios and histological evaluation. After 7 days of acute hyperoxia, the DL/BW significantly decreased ($p<0.05$) indicating destructive tissue hypoplasia which was manifested in the acellular, attenuated appearance of the lung parenchyma. In the repair phase that followed, the DL/BW increased ($p<0.05$) reflecting reparative tissue hyperplasia which was due to an increased presence of interstitial cells and proliferation of Type II cells. After 7 and 17 days of exposure, the WL/DL was greater in the O_2 group ($p<0.05$) which was suggestive of pulmonary edema. An increased number of macrophages and PMNLs was seen in the interstitium and alveolar spaces.

After 1 day of hyperoxia, basal 6KF outputs and A.A. stimulated outputs of 6KF and TxB_2 were decreased ($p<0.05$). However, basal and A23187 stimulated LTB_4 levels were increased ($p<0.05$). Following 7 days of O_2, basal and stimulated outputs of the 3 eicosanoids measured were elevated ($p<0.05$). In the reduced hyperoxic environment on day 17, significantly lower outputs of basal 6KF, and stimulated TxB_2 were observed ($p<0.05$). At the same time, stimulated LTB_4 output was higher in the hyperoxic group compared to the normoxic group. O_2 exposure for 28 days, decreased basal and stimulated 6KF release but increased basal TxB_2 and LTB_4 outputs and stimulated LTB_4 output ($p<0.05$).

The lung injury produced by the hyperoxic exposure protocol reflected the characteristic exudative, hypoplastic phase followed by a hyperplastic, fibrotic repair phase. In conjunction with this sequence of pulmonary injury, hyperoxia caused a progressive alteration in the capacity of the lung to produce the individual eicosanoids. It is speculated that the relative balance between the eicosanoids is an important determinant in the pathogenesis of pulmonary oxygen toxicity.

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The journey that has brought me to this place in my life has been blessed by many people.

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To Jon: Thank-you for your words of wisdom and experience.

To Drew: Thank-you for sharing a piece of your imaginative side with me.

To Tamas: I have learned so many things from you about science but more importantly, about life.

To Fernando: I consider you my second brother!

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LIST OF ABBREVIATIONS AND SYMBOLS USED IN THE TEXT, FIGURES AND TABLES.

A.A.	:	Arachidonic acid
AB	:	Antibody
ANOVA	:	Analysis of variance
AOEs	:	Antioxidant enzymes
ARDS	:	Adult respiratory distress syndrome
AT	:	Ammonium Hydroxide-Triton X-100 Extraction Solution
A23187	:	Calcium ionophore
B	:	Bound
BALF	:	Bronchoalveolar lavage fluid
BPD	:	Bronchopulmonary dysplasia
B_o	:	Binding maximum
Ca^{2+}	:	Calcium ion
$CaCl_2$	:	Calcium chloride
cAMP	:	Cyclic adenosine 3',5'-monophosphate
CAT	:	Catalase
CO_2	:	Carbon dioxide
CPM	:	Counts per minute
DL/BW	:	Dry lung weight-to-body weight ratio
DNA	:	Deoxyribonucleic acid
DPM	:	Disintegrations per minute

ECF	:	Extracellular fluid
EDTA	:	Ethylenediaminetetraacetic acid
e.g.	:	For example
et al.	:	And others
	:	Fraction of inspired oxygen
	:	Gamma-glutamyl-transpeptidase
GP	:	Glutathione peroxidase
^3H	:	Tritium
HBSS	:	Hanks' balanced salt solution
HEPES	:	Hydroxyethyl-piperazine-2-ethanesulfonic acid
HMD	:	Hyaline membrane disease
HP	:	High pool
H_2O	:	Water
i.e.	:	Id est (Latin), that is to say
IP3	:	Inositol-1,4,5-trisphosphate
LDH	:	Lactate dehydrogenase
LIV/BW	:	Liver weight-to-body weight ratio
LTA4	:	Leukotriene A4
LTB4	:	Leukotriene B4
LTC4	:	Leukotriene C4
LTD4	:	Leukotriene D4
LTE4	:	Leukotriene E4
LTF4	:	Leukotriene F4
LTs	:	Leukotrienes
LP	:	Low pool

mRNA	:	Messenger ribonucleic acid
NADH	:	Nicotinamide adenine dinucleotide (reduced)
NADPH	:	Nicotinamide adenine dinucleotide phosphate (reduced)
NH_4OH	:	Ammonium hydroxide
NSB	:	Non-specific binding
O_2	:	Molecular oxygen
$PaCO_2$	:	Partial pressure carbon dioxide in arterial blood
PAF	:	Platelet activating factor
PaO_2	:	Partial pressure oxygen in arterial blood
PBS	:	Phosphate buffered saline
PBSG	:	Phosphate buffered saline with gelatin
PDGF	:	Platelet-derived growth factor
PGE_1	:	Prostaglandin E_1
PGE_2	:	Prostaglandin E_2
$PGF_{1\alpha}$	:	Prostaglandin $F_{1\alpha}$
$PGF_{2\alpha}$	:	Prostaglandin $F_{2\alpha}$
PGG_2	:	Prostaglandin G_2
PGH_2	:	Prostaglandin H_2
PGHS	:	Prostaglandin endoperoxide H synthase
PGI_2	:	Prostacyclin
PGs	:	Prostaglandins
pK_a	:	pH where acid half dissociated
PLA_2	:	Phospholipase A_2
PLC	:	Phospholipase C
PMA	:	Phorbol-12-myristate-13-acetate

PMNL	:	Polymorphonuclear leukocyte
PUFA	:	Polyunsaturated fatty acid
RBCs	:	Red blood cells
RDS	:	Respiratory distress syndrome
RIA	:	Radioimmunoassay
RPM	:	Revolutions per minute
SRS-A	:	Slow reacting substance of anaphylaxis
SD	:	Standard deviation
SOD	:	Superoxide dismutase
TxA_2	:	Thromboxane A_2
TxB_2	:	Thromboxane B_2
T	:	Total counts
v / v	:	Volume/volume
WL/BW	:	Wet lung weight-to-body weight ratio
WL/DL	:	Wet lung weight-to-dry lung weight ratio
5-HPETE	:	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
5-LO	:	5-lipoxygenase
6KF	:	6-keto-prostaglandin $F_{1\alpha}$
15-OH-PGDH	:	15-hydroxy-prostaglandin dehydrogenase
% intra-C.V.	:	Intra-assay coefficient of variation
% inter-C.V.	:	Inter-assay coefficient of variation
α	:	Alpha
γ	:	Gamma
μ	:	Micro

CHAPTER 1

INTRODUCTION

Oxygen is necessary for life. However, oxygen can also be toxic. This is of particular importance to preterm, newborn infants who are ventilated with high levels of oxygen in order to alleviate the symptoms of respiratory distress. Preterm infants are uniquely susceptible to the toxic effects of oxygen because their lungs are structurally and functionally immature (Frank, 1985).

What happens when lungs are exposed to hyperoxia?

There is a two phase pathogenic sequence of injury that occurs to the lung during exposure to elevated levels of oxygen (Clark and Lambertsen, 1971). The first phase is an acute injury that is initiated by necrosis of the resident lung cells with the subsequent development of pulmonary edema and inflammation. If healing is allowed to occur following this initial insult to the lung, a repair phase takes place. This second phase is characterized by hyperplasia of the resident lung cells with an alteration in the proportion of the different cell populations and pulmonary fibrosis. In preterm

infants, the end result is the development of a chronic lung disorder called Bronchopulmonary dysplasia (BPD) (Northway *et al.*, 1967).

How does hyperoxia cause pulmonary injury?

Oxygen free radicals are highly reactive forms of oxygen. They are produced by normal cellular proceses but are maintained at low levels by antioxidant defense systems. Under hyperoxic conditions, there is an over-production of oxygen free radicals within the resident lung cells. The antioxidant defenses are unable to effectively remove these highly reactive molecules before they interact with intracellular components such as DNA, enzymes and membrane lipids. This primary oxygen toxicity results in functional aberrations and ultimately in cell death.

What are eicosanoids?

Eicosanoids are 20-carbon derivatives of the unsaturated fatty acid, arachidonic acid (A.A.) which is stored esterified in membrane phospholipids. A.A. is liberated from membranes by the action of phospholipases A_2 and C. The free substrate may then be metabolized via 2 pathways: 1) the prostaglandin endoperoxide H synthase pathway (PGHS) giving rise to the prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) (Gryglewski *et al.*, 1976, Hamberg and Samuelsson, 1974), and 2) the 5-lipoxygenase (5-LO) pathway producing the leukotrienes (LTs) (Borgeat and Samuelsson, 1979).

Eicosanoids are potent biological mediators which are vasoactive, regulators of cell mitogenesis and differentiation and are important inflammatory mediators (Hall and Behrman, 1982). Each eicosanoid produces a characteristic array of physiological actions via interaction with specific receptors on the target cell. The final effect produced by the different eicosanoids are often antagonistic to one another. For example, PGI_2 is a vasodilator whereas TxA_2 and LTs are vasoconstrictors.

Why are eicosanoids important in the lung?

The lung is the major site of eicosanoid activity. This organ has the capacity to synthesize a wide array of eicosanoids (Nowak, 1984). The endothelial cells of the extensive pulmonary vascular bed have the potential to produce large quantities of PGI_2. Other resident lung cells such as the alveolar epithelial cells, fibroblasts, smooth muscle cells and alveolar macrophages are also capable of generating eicosanoids. Each cell type produces a characteristic array of eicosanoids, including PGI_2, PGE_2, TxA_2 and LTs. The lung is also important in the metabolism of eicosanoids. The predominate mechanism for the inactivation of circulating eicosanoids occurs by the clearance and metabolism upon passage through the pulmonary circulation (Gryglewski, 1980).

How does hyperoxia affect eicosanoids?

Altered levels of eicosanoids have been detected in lung lavage fluid following exposure to hyperoxia (Stenmark *et al.*, 1985, Taniguchi *et al.*, 1986, Hageman *et al.*, 1986, Smith *et al.*, 1986). Hyperoxia has been shown to affect many different aspects of the synthesis of eicosanoids. At the level of the cell membrane, lipid peroxidation caused by the interaction of O_2 free radicals with membrane lipids stimulates the release of A.A. (Warso and Lands, 1985). Hyperoxia causes alterations in various regulatory factors such as lipid peroxides and free calcium which control the various enzymatic steps resulting in altered activities of the enzymes (Egan *et al.*, 1976, Sun and McGuire, 1984).

Is there a link between hyperoxic lung injury and alterations in pulmonary eicosanoid production?

There is evidence that hyperoxia is capable of causing both lung injury, and altering the eicosanoid cascade in the lung. Therefore, it is hypothesized that an alteration in the normal homeostatic balance of eicosanoids in the lung may contribute to the pathogenesis of hyperoxic lung injury.

How was this hypothesis to be investigated?

Initially, two techniques were developed and characterized in order to examine eicosanoid production by the lung. Firstly, a fresh lung tissue explant technique was devised. An intact slice of lung

tissue was used because we wanted to examine the eicosanoid production as close as possible to the *in vivo* state. The second technique that was developed was radioimmunoassays (RIAs) for 6KF (stable metabolite of PGI_2), TxB_2 (stable metabolite of TxA_2) and LTB_4. The antisera were characterized for the optimal working titre and for their specificities. The RIAs were optimized and assessed for the reproducibility, accuracy and sensitivity in order to ensure confidence in the determinations made using this technique. Finally, experiments exposing neonatal rat pups to hyperoxia were undertaken to begin to address the hypothesis. Parameters of lung injury were assessed with simultaneous evaluation of the lung eicosanoid production. Through these parallel observations, perhaps a link between lung injury and eicosanoids can be established.

CHAPTER 2

LITERATURE REVIEW

2.1. BRONCHOPULMONARY DYSPLASIA

2.1.1. History and Definition of Bronchopulmonary Dysplasia

A previously unrecorded lesion in the lungs of newborn infants who required intensive oxygen therapy was first reported by Rosan *et al.*, in 1966. The lungs of these infants demonstrated abnormalities that differed from the known pathology of Respiratory Distress Syndrome (RDS) for which they were originally being treated. It was noticed that their lungs had irregular airspaces and hypertrophied connective tissue surrounding the bronchioles and interlobular regions. The term "bronchopulmonary dysplasia" was first introduced by Northway to describe this new clinical syndrome of chronic lung disease (Northway *et al.*, 1967). The name was chosen to emphasize the "dysplasia" of both the "bronchiolar" and the "pulmonary parenchymal" tissues of the lung in the pathological presentation.

BPD is a chronic pulmonary disorder and is observed specifically in newborn infants that have received elevated levels of oxygen in an attempt to alleviate an underlying respiratory problem. There are unique risk factors that predispose particular infants to develop BPD (Farell and Palta, 1986). In many cases these infants are preterm (<37 week gestation) and very-low-birth-weight (<1000 grams). They are susceptible because of the complications that arise from the structural and biochemical immaturity of their lungs. It is believed that BPD develops when O_2 toxicity is superimposed upon vulnerable newborn lungs. The etiology of BPD is thought to be multifactorial, with the most important factors being "oxygen + immaturity + pressure + time".

The diagnosis of BPD is made by assessment of specific clinical criteria. The infant will have shown signs of chronic respiratory distress with the presence of abnormal blood-gas values. Their treatment histories have included treatment with 80 to 100% O_2 via an intermittent positive-pressure respirator for at least 24 hours (Northway *et al.*, 1967). A chest radiograph will demonstrate changes in the lungs that are compatible with the etiology of BPD (Farrell and Palta, 1986).

2.1.2. Oxygen Therapy

Oxygen therapy is comprised of two components, firstly, an elevated fraction of inspired O_2 (FIO_2) and secondly, mechanically assisted ventilation. The main goal of O_2 therapy is to provide oxygen, i.e. maintaining the partial pressure of O_2 in arterial blood (PaO_2) at 50-70 mmHg (Oh *et al.*, 1983) and to remove carbon dioxide (CO_2), i.e. maintaining the partial pressure of CO_2 ($PaCO_2$) below 60-65 mmHg, in an attempt to correct abnormal blood gases and alleviate the ensuing acidosis (i.e. pH of less than 7.25). This treatment also serves to improve alveolar ventilation and reduce the work of breathing.

Pulmonary gas exchange in infants with respiratory distress is inefficient due to the structural immaturity of their lungs and widespread atelectasis caused by the lack of surfactant. This necessitates the use of O_2 therapy in order to overcome the vicious cycle of hypoventilation and underperfusion that is occurring in their lungs. The resultant hypoxia and hypercarbia stimulates pulmonary vasoconstriction causing hypoperfusion which further contributes to the uneven ventilation-perfusion ratio. The accumulating CO_2 shifts the acid-base equilibrium and increases the hydrogen ion concentration in the blood of the alveolar capillary microenvironment causing respiratory acidosis.

Implementation of O_2 therapy is beneficial to infants with compromised pulmonary function since it ensures adequate PaO_2

and $PaCO_2$. However, a paradox exists with the use of high levels of O_2 and mechanical ventilation. The unfortunate side-effect of the treatment is the development of pulmonary oxygen toxicity which then progresses to the chronic lung disease, BPD.

2.1.3. The Lung of the Newborn

The lungs of a newborn infant are much smaller in absolute size than an adult's however in the neonate this organ is proportionally much larger relative to the rest of the body. It is important to realize that the newborn's lungs are not merely a miniature version of adult lungs but are structurally quite different since they do continue to mature and develop postnatally (Weibel, 1967).

The bronchial tree is completely developed by the 16th week of intrauterine life during the glandular stage (Meyrick and Reid, 1977). From 16 to 24 weeks, the second stage called the canalicular stage occurs when capillaries begin to penetrate into the canalicular structures. During the alveolar stage which takes place between 24 to 40 weeks, single primitive alveoli begin to appear along the length of the straight airways to form respiratory bronchioles. Epithelial cells begin to differentiate and become mature alveolar-lining Type I and surfactant-synthesizing Type II cells (Weibel, 1967).

This maturational process of the lung does not halt at birth. A process called budding continues postnatally where complex clusters of new alveoli are formed by the partitioning of primitive air sacs thereby increasing the number of alveoli (Reid, 1967). This formation of new alveoli is most marked from 5 to 10 days postnatally and causes a sudden, rapid rate of growth of the surface area of the lung which is greater than the rest of the body (Weibel, 1967). Following this time until about 8 years of age, the lungs continue to grow in proportion to the body dimensions. The pulmonary vasculature also continues to develop such that the branching pattern of the pulmonary artery follows that of the proliferating airways. The number of capillaries increases in relation to the formation of new alveoli.

The alveolar fluid contained in the potential air spaces of the fetal lung must be removed upon commencement of air breathing at the time of parturition. Some of the fluid is passively emptied through the upper airways during the process of birth. The remaining fluid is cleared by movement from the airspaces into the interstitium as a result of the pressures caused during inspiration. This liquid is then removed from the interstitium via the lymphatic system.

At the time of birth, the air-blood barrier is relatively thick compared to its functional thickness of 0.5 μm and is composed of a relatively greater proportion of interstitial fibroblasts and connective tissue than blood carrying capillaries (Weibel, 1967). The proportion

of the components that make up the air-blood barrier changes during the maturation of the lung so that the majority of the interstitium becomes the capillary volume. The interstitium is also progressively thinned until the final functional respiratory membrane consists of an endothelial cell, Type I epithelial cell and their fused basement membranes.

2.1.4. The Lung of the Preterm Newborn

The normal term infant goes through 40 weeks of gestation. Preterm babies can be born as early as 28 weeks of gestation. They are introduced into the air breathing environment with a pulmonary architecture that is not yet fully equipped for the sudden adaptation that must occur. These infants born before the normal 40 weeks of gestation must still put in the time for their lungs to develop, i.e., they still go through the final stages of *in utero* lung development (Reid, 1979). A major difficulty arises because they must be actively using this immature system for extracting O_2 from the air.

The alveolar network is very under-developed. Portions of the lung in the preterm infant are just in the beginning of the alveolar stage where the first alveoli are being formed (Weibel, 1967). Airspaces that are lined by functionally immature and thicker epithelial cells translates into a gas exchange surface that compromises PaO_2 and $PaCO_2$. Overall, the preterm infant has less

compliant lungs with a decreased volume as a result of both atelectasis and fewer numbers of alveoli (Polgar, 1979).

The pulmonary vascular bed is not well-developed. There are fewer capillaries in immature lungs because the capillary proliferation that accompanies the increasing alveolar surface area has not had time to take place. The capillaries that are present in the immature lung have thicker endothelial cells and a narrower capillary lumen.

A relatively larger distance separates the airspaces and the pulmonary capillaries in the preterm lung. The alveolar capillaries remain deeply embedded in pulmonary interstitial tissue that is more cellular and has more connective tissue fibers and matrix. Also, the basement membranes of the capillary endothelium and alveolar epithelium have not yet fused. The Type I cells that line the alveoli and the endothelial cells of the capillaries are cuboidal in shape in the immature lung whereas in the mature lungs they are very thin and attenuated (Kotas, 1979). Together all of these factors contribute to the increased distance between the blood and the air.

2.1.5. Respiratory Distress Syndrome

Respiratory distress syndrome (RDS) is the most common respiratory disorder of the preterm newborn. It is the main reason that these babies are given O_2 therapy. The primary pulmonary injury that results from RDS, in addition to the secondary O_2 toxicity that results from the treatment, may lead to the development of BPD.

Babies who get RDS are almost always immature. They are prone to develop respiratory probiems because their lungs have not reached a differentiated stage in gestation where surfactant is produced in adequate amounts. Superimposed upon this biochemical deficiency is the structural immaturity of the pulmonary architecture. Immature lungs have an underlying surfactant deficiency and therefore the alveoli have high surface tension. The lungs of preterm infants are unstable during expiration and are prone to atelectasis. Also, they are poorly compliant and difficult to expand upon inspiration. The lack of surfactant promotes net forces that favours the leakage of vascular components into the alveoli which then compromises gas exchange. The acute pulmonary pathology resulting in RDS is characterized by pulmonary vasoconstriction, edema, hyaline membranes and necrosis of the epithelium.

2.1.6. Pulmonary Function Consequences

Bronchopulmonary dysplasia is a major clinical problem because the pathological changes sustained early in life have effects on efficient pulmonary function that persist into adulthood. This is a particular problem in the newborn because the injury occurs during a time when lung development is still taking place and therefore future lung structure may be altered.

Aberrations in pulmonary function may occur as early as 4 days from the initiation of treatment in infants with RDS requiring ventilation (Goldman *et al.*, 1983). The most marked finding was an elevation in pulmonary airway resistance. This alteration in pulmonary function was presumably due to the release of bronchoconstrictor agents (perhaps eicosanoids), in response to the acute injury caused by oxygen therapy.

Severe pulmonary functional abnormalities during advanced BPD have been documented in infants studied between 1 month and 2 years of age (Lindroth *et al.*, 1980). Following the healing phase of BPD, aberrations in lung mechanics result from the chronic pathological remodeling of the airways and the parenchymal architecture. Bronchi and distal airways have narrowed lumens due to epithelial cell, smooth muscle cell and fibroblast proliferation. The walls of the large airways demonstrate signs of peribronchiolar fibrosis and increased muscularity. Together, these structural modifications are responsible for the persistent high airway

resistance and bronchial hyper-reactivity. In the parenchyma of the lung, interstitial and intraalveolar fibrosis is present and the alveolar pattern depicts alternating regions of emphysema and atelectasis. Fibrosis results from an increased deposition of collagen causing the alveolar walls to become more rigid thereby decreasing the dynamic compliance of the lung (Benoist *et al.*, 1976). The abnormal arrangement of the alveolar regions results in the maldistribution of air which impairs the gas exchange function of the lung.

2.1.7. Pathology of the Acute Phase

The first phase of pathological alterations in the lung following hyperoxic exposure is considered to be an acute injury. The lesions to the lung commence due to oxygen toxicity within the resident lung cells. This initiates a characteristic sequence of progressive pulmonary injury which then evokes an inflammatory response (Kapanci *et al.*, 1969, Kaplan *et al.*, 1969, Clark and Lambertsen, 1971, Crapo *et al.*, 1978).

Endothelial Cell Injury

Pulmonary capillary endothelial cells are the most susceptible to the toxic effects of oxygen and are the first cells to show injury (Bowden and Adamson, 1974). The initial response of these cells to oxygen toxicity is demonstrated microscopically by signs of cellular swelling and edema (Clark and Lambertsen, 1971). Ultrastructural examination of endothelial cells at this stage shows pyknotic nuclei due to margination of chromatin, swollen perinuclear cisternae, rough endoplasmic reticuli and mitochondria. If the pathogenic sequence progresses, the cells die due to rupture of the plasma membrane and nuclear envelope (Crapo *et al.*, 1980). After dying, the broken segments of the endothelial cells lift off of the basement membrane (Crapo *et al.*, 1980, Barry and Crapo, 1985).

The toxic effects of hyperoxia on endothelial cells occur because of an over-production of toxic oxygen species at intracellular sites (Crapo *et al.*, 1983). A decrease in the number of capillary endothelial cells translates into a corresponding decrease in the capillary surface area available for gas exchange and a decrease in the capillary lumen volume thereby limiting the carrying capacity of blood (Crapo *et al.*, 1978, 1980). There are large segments of the alveolar septal walls that are completely devoid of capillaries which then promotes the collapse of the associated alveoli.

Vascular Permeability

As a result of endothelial cell injury, there is an increase in the transudation of fluid across the vascular endothelium. This may occur by an increase in the permeability of the vasculature and by a complete breakdown of the endothelial barrier, depending upon the severity of the injury to the capillary barrier (Bressack *et al.*, 1979).

During hyperoxia, an increase in the transudation of fluid into the interstitial compartment is produced, in part, by alterations in the Starling Forces. The pulmonary capillary hydrostatic pressure increases due to pulmonary hypertension and the interstitial oncotic pressure is elevated as a result of protein accumulation in the interstitium (Bressack *et al.*, 1979). There is also an increase in the permeability caused by injury to both the tight junctions between endothelial cells and the transport mechanisms through the cells (Kapanci *et al.*, 1969). These permeability changes precede the substantial fluid transudation that occurs following severe injury to the capillary endothelium which causes the complete breakdown of the barrier.

Platelets

In response to damage to the endothelium, inflammatory cells called platelets rapidly aggregate and adhere at local sites of injury in an attempt to mediate repair. This inflammatory reaction serves to promote repair of the endothelium when injury has occurred. However, when the injury is extensive and prolonged, the actions of

platelets can be detrimental. Extensive platelet aggregation can lead to thrombus formation that occludes the capillary lumen. Vasoconstriction, increased vascular permeability and the action of lysosomal enzymes creates a situation that promotes the production of interstitial edema. The extensive release of chemotactic agents may cause a prolonged and excessive leukocyte influx.

The platelet reaction has been implicated in playing a role in the acute phase of hyperoxic lung injury. The first quantitative change in the pulmonary capillary bed found during exposure to hyperoxia was an increase in platelet volume and the endothelial surface that they cover (Barry and Crapo, 1985).

Edema

One of the earliest consequences of an excessive inflammatory reaction during oxygen toxicity is the development of pulmonary edema (Crapo *et al.*, 1980). The first stages of interstitial edema are the result of mild pulmonary microvascular injury. This is caused by intracellular oxygen toxicity which increases the permeability of the endothelium (Matalon *et al.*, 1982). The severe damage to the endothelium is caused by both excessive intracellular generation of oxygen radicals and by the destructive actions of PMNLs (Flick *et al.*, 1931). The next step in progressive pulmonary edema is intraalveolar edema. This results when epithelial cell injury occurs due to the oxygen toxicity within these cells and is compounded by the destructive actions of the accumulating PMNLs. When there is

mild injury to the epithelial barrier, fluid transudation may be enhanced by an increase in transport of fluid and ions across an intact epithelium (Al-Bazza *et al.*, 1981, Eling *et al.*, 1988). The transudation of fluid into the alveoli may also be promoted by hydrostatic and oncotic pressure gradients which favour movement in this direction. When there is total compromise of the epithelium, fluid flows freely from the interstitium into the alveoli thereby interfering with gas exchange.

Inflammation

Inflammation occurs as a secondary reaction following the tissue damage that was originally caused by primary oxygen toxicity (Belch, 1989). The purpose of the inflammatory process is to repair the tissue injury. The inflammatory reaction characteristically includes an increase in blood flow and an influx of inflammatory cells to the site of injury. Biologically active substances (e.g. eicosanoids) that mediate and/or modulate these processes are released in elevated levels. Inflammation becomes a pathological process when there is excessive formation or inadequate inactivation of these mediators (Konig *et al.*, 1990). Instead of the injury being repaired, there is edema and compounded tissue damage by an over-exaggerated inflammatory cell response.

One component of the inflammatory reaction is a cell-mediated response which involves the emigration of cells from the blood into the tissues (Bainton, 1988). The normal function of inflammatory

cells such as PMNLs is to phagocytose the debris from necrotic cells and other damaged tissue components (Belch, 1989).

If there is a chronic stimulus such as prolonged hyperoxia or excessive eicosanoids, then PMNLs persist in an activated state (Hunninghake *et al.*, 1979). During excessive activation, potentially destructive oxygen radicals and lysosomal enzymes may be secreted by PMNLs in aberrant amounts and cause damage to surrounding tissue. The compounded tissue injury caused by the action of PMNLs and the secretion of chemotactic stimuli (e.g. LTB4) from PMNLs creates a vicious cycle that leads to prolonged and chronic inflammation (Belch, 1989). Alveolar macrophages also increase in numbers following hyperoxic exposure and compound hyperoxic lung injury by being a source of PMNL chemoattractants (LTB4) and by releasing collagenase and elastase (Reynolds, 1983).

Epithelial Cells

The lung epithelium forms a tight barrier between the pulmonary interstitium and the air spaces which is an effective boundary to macromolecules and to small solutes (Normand *et al.*, 1970, 1971). The epithelium consists of Type I and Type II pneumocytes that are connected by tight junctions over a continuous basement membrane.

Exposure to hyperoxia produces injury to the Type I cells that is apparent by an abnormal ruffled appearance of their surface (Crapo *et al.*, 1980). When the Type I cells become necrotic, there is

a resultant loss in the functional integrity of the epithelial barrier (Kapanci *et al.*, 1969). The Type II cells exhibit injury during hyperoxia by the blunting of the microvilli on their airway surface (Crapo *et al.*, 1980). Ultrastructural examination of the Type II cells shows that there is an increase in free ribosomes, dilation of the endoplasmic reticulum and expansion in mitochondrial volume (Rosenbaum *et al.*, 1969, Crapo *et al.*, 1980).

Interstitium

During the acute stage of hyperoxia, there is an increase in the width of the interstitium due to both the increase in the total number of cells and to interstitial fluid accumulation (Kapanci *et al.*, 1969, Kaplan *et al.*, 1969, Crapo *et al.*, 1980). The resident interstitial cells i.e., fibroblasts and smooth muscle cells may actually decrease in numbers with a lethal dose of oxygen although they do exhibit more resistance to the toxic effects of hyperoxia than endothelial and epithelial cells. It is the influx of non-resident cells i.e., PMNLs, macrophages and mononuclear cells that is responsible for the elevated cellular population of the interstitium (Kapanci *et al.*, 1969, Kaplan *et al.*, 1969). The increase in the interstitial width is not due to more connective tissue deposition since exposure to hyperoxia causes a decrease in lung collagen during the acute stage (Curran *et al.*, 1984). This breakdown of connective tissue fibers occurs directly by oxygen radicals acting on the protein structure and by the

increased amounts of collagenase that are released from activated macrophages and PMNLs (Kimbel, 1984).

Alveoli

During the acute stage of hyperoxic lung injury, there is a marked decrease in the number of functional alveolar units available for gas exchange. Exposure to hyperoxia causes widespread atelectasis of alveoli due to decreased perfusion and blockage of airways by necrotic debris (Kapanci *et al.*, 1969). Also, the marked inhibition of the normal postnatal alveolarization serves to decrease the alveolar surface area (Meyrick and Reid, 1982, Massaro *et al.*, 1985). This is caused by the inhibitory effects of hyperoxia on lung protein and DNA synthesis (Clark and Lambertsen, 1971, Northway *et al.*, 1976, Frank and Massaro, 1980). The reduction in alveolar formation at this critical stage of lung development may have long term consequences that leads to a permanent reduction in the surface area for respiratory function (Sobonya *et al.*, 1983).

2.1.8. Pathology of the Repair Phase

The second phase of the pathological sequence in pulmonary oxygen toxicity is a proliferative reaction. It is the transition from the acute phase to this repair phase that leads to permanent changes in the lung that culminate to produce the chronic lung disease.

Endothelial Cell Regeneration

If conditions are favourable for healing, endothelial cells are capable of regenerating following the initial oxidant insult. The surviving endothelial cells begin to repopulate the denuded endothelium (Bowden and Adamson, 1974). The proliferation is a normal reaction to the initial erosion of the blood vessel wall (Clark and Lambertsen, 1971). However, when the endothelial injury is extensive, cells that have endured become hypertrophied (Crapo *et al.*, 1980). The expansion of the size of the cells is an attempt to balance the loss in the total cell number. The ultrastructural changes reflect an adaptive transformation that augments their resistance to hyperoxia. These oxygen-adapted cells possess enhanced metabolic machinery such as extensive mitochondria and polyribosomes for the purpose of manufacturing increased levels of antioxidant enzymes.

Blood vessels

The pulmonary vascular bed becomes morphologically abnormal as pathological repair occurs. Although blood vessels exhibit hyperoxia-induced angiogenesis, the recovered vasculature has altered features (Roberts *et al.*, 1983). There is thickening and hyalinization of the walls of pulmonary vessels due to muscularization and fibrosis (Bennett and Smith, 1934). The result is a marked narrowing of the lumen which then encroaches on blood flow and contributes to pulmonary hypertension. Also, there is extension of the muscular layer of the blood vessel closer to the peripheral areas of the lung where smooth muscle cells are not normally found (Reid, 1979, Jones *et al.*, 1984, Hu and Jones, 1989).

Epithelial Cells

The epithelium of the lung shows one of the most striking changes following oxygen toxicity. The alveolar lining becomes a continuous layer of granular Type II pneumocytes (Kapanci *et al.*, 1969, Kaplan *et al.*, 1969). This is due to the greater capacity for division of this cell type over Type I cells. Type I cells are terminally differentiated epithelial cells whereas Type II cells represent the reserve cell of the alveolar lining which are capable of cell division. The oxygen-adapted Type II cells have unique ultrastructural changes that reflect their increased ability to synthesize antioxidant enzymes (AOEs) and surfactant (Crapo *et al.*, 1980).

Alveoli

There is a decrease in the overall number and concentration of functional alveoli (Crapo *et al.*, 1980). The alveoli that are present are either atelectic or emphysematous (Reid, 1979). The decrease in alveolar number may be the combined result of the destruction of alveolar walls and atelectasis and impaired alveolar multiplication by inhibition of the normal postnatal septal growth (Butcher and Roberts, 1981). The emphysematous alveoli may arise from an over-inflation of the air spaces that are open, in an attempt to compensate for the neighbouring scarred and collapsed alveoli (Reid, 1979).

Interstitium

The interstitial compartment also demonstrates marked changes during the proliferative phase. The interstitial space between the alveolar air space and the pulmonary capillaries doubles in width following hyperoxia (Crapo *et al.*, 1980). The increased thickness of the interstitial space during the proliferative phase, is attributed to augmented cellularity and volume of collagen fibers while the contribution of edema fluid is reduced (Clark and Lambertsen, 1971). The thickening of the overall gas exchange membrane is compounded by the increase of Type II cells which are cuboidal as opposed to the normal respiratory lining cell Type I cells which are flat and thin (Kapanci *et al.*, 1969).

Fibrosis

Fibrosis is a hallmark of lung repair following pulmonary oxygen toxicity. Fibrosis is characterized by a proliferation of fibroblasts in conjunction with a pathological increase in connective tissue deposition, particularly collagen. The damage to collagen that occurred during the acute phase is followed by altered deposition and cross-linking of newly formed collagen.

Fibrosis may occur in various locations in the lung. Peribronchiolar fibrosis is noted following damage to the airways (Northway *et al.*, 1967). Fibrosis occurs in the interstitium following the resolution of edema (Reid, 1979). Interstitial edema may stimulate the proliferation of fibroblasts by transporting growth promoting substances to this location (Hesterberg and Last, 1981). Exudate within the alveolar spaces may become organized *in situ* thereby causing intraalveolar fibrosis which will obliterate these gas exchange units. Parts of the vascular bed may be compromised by becoming condensed within fibrotic scars hence are not able to participate in gas exchange. The large blood vessels may also become fibrosed making the walls of the arteries and veins thicker and contributing to pulmonary hypertension (Bennett and Smith, 1934, Hu and Jones, 1989).

Inflammation

A prolonged and excessive inflammatory response produces a vicious cycle of injury. This initial defense mechanism served the purpose of mediating tissue protection and repair. However, when inflammatory processes are chronically activated, they contribute to and compound the original injury (Belch, 1989). The failure to recover from pulmonary oxygen toxicity may be due to a delayed resolution of the inflammatory response which then contributes to the development of the chronic proliferative phase. During this aberrant repair phase, there is a persistent presence of phagocytes possibly due to inappropriately controlled levels of chemoattractants i.e., the leukotrienes. The chronic presence of inflammatory cells suggests that pathological fibrosis will be the long-term sequela of the injury.

2.2. OXYGEN TOXICITY

2.2.1. Generation of Oxygen Free Radicals

Oxygen free radicals are compounds which contain an oxygen atom with an unpaired electron (Halliwell, 1987). This is a thermodynamically unfavourable state, therefore, these species are highly reactive. Oxygen free radicals are found in various forms: superoxide anion, hydrogen peroxide, singlet oxygen, and hydroxyl radical. Each has a different degree of reactivity and these oxygen species may be interconverted. The hydroxyl radical is more reactive than superoxide and hydrogen peroxide and is the agent that is primarily responsible for direct cellular damage.

Oxygen free radicals are formed when some of the electrons passing down the mitochondrial electron transport chain are transferred to O_2 before reaching cytochrome c oxidase (Halliwell, 1987). Under normal conditions, this accounts for 95% of the oxygen consumed by most mammalian cells (Freeman and Tanswell, 1985). There are many enzymes present in cells that produce oxygen radicals as normal or inadvertant products of their catalytic reactions. Oxygen radicals may also be generated during the auto-oxidation of soluble low molecular weight molecules. These cytosolic molecules include thiols, hydroquinones, catecholamines, flavins and tetrahydropterins (Freeman and Tanswell, 1985). Oxygen radicals

are also formed by the Haber-Weiss reaction involving the direct reduction of hydrogen peroxide by superoxide in the presence of a metal catalyst which results in the formation of hydroxyl radical (Haber and Weiss, 1934). Another reaction that forms reactive oxygen species is the Fenton reaction where superoxide first reacts with an oxidized metal such as iron, causing reduction of the metal and generating O_2 (Cross *et al.*, 1987).

2.2.2. O_2 Radicals During Normoxia

Reactive oxygen species are generated in the mitochondria and account for the major oxygen consumption in cells. During normal cellular respiration, superoxide and hydrogen peroxide are generated by the electron transport chain (Freeman and Tanswell, 1985). Free radicals may also be generated by the plasma membrane, nuclear membrane and by microsomes which contain membrane-bound enzymes that can generate oxygen radicals as byproducts of catalytic cycling. Intracellular reactive oxygen species can also be produced in the cytosol by the auto-oxidation of soluble low molecular weight molecules (Freeman and Tanswell, 1985).

2.2.3. O_2 Radicals During Hyperoxia

The oxidative stress caused by hyperoxia affects primarily the lung, since it is directly exposed to the inspired gases (Freeman and Crapo, 1981). During oxidative stress, there is an increase rate of reactive oxygen species generated by the electron transport components of the inner mitochondrial membrane. Particularly, the production of superoxide by cyanide-resistant submitochondrial particles increases directly as a function of oxygen tension (Turrens *et al.*, 1982).

The intramitochondrial antioxidant enzymes that usually maintain homeostatic levels of reactive oxygen species are overwhelmed by the excessive amounts that are produced (Matalon and Egan, 1984). Therefore, the reactive oxygen species, mainly hydrogen peroxide, may escape from the mitochondria into the cytoplasm to react with other cellular components (Freeman and Tanswell, 1985). The cellular constituents that are susceptible to damage by the oxygen species include carbohydrates, membrane lipids, proteins both structural and functional, and DNA (Cross *et al.*, 1987).

The reactive oxygen species that are membrane-derived diffuse directly into the cytosol and damage intracellular components. Lung microsomes can generate superoxide at a 3 to 5-fold increased rate during hyperoxia which in turn react with microsomal membrane components (Turrens *et al.*, 1982).

Injurious reactive oxygen species may also come from an extracellular source. The lung injury that occurs due to intracellular oxygen toxicity initiates an inflammatory response which involves the influx of phagocytic cells. When these phagocytes are stimulated, they respond with respiratory bursts generating even more oxidizing agents (Cross *et al.*, 1987).

2.2.4. Antioxidant Defenses

The main component of the antioxidant defense system is the antioxidant enzymes (AOEs) but also included are compounds that react with oxygen free radicals with the subsequent formation of an inert compound. Antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GP) are capable of inactivating reactive oxygen species by converting them to inert compounds such as H_2O and O_2. Under normal circumstances the activity of these enzymes is sufficient to remove oxygen free radicals as they are formed. Thus, a homeostatic balance is established so that the free radicals are inactivated before they can react with cellular components. The mitochondrial production of oxygen species is contained within the organelle by the AOEs within the matrix.

An alternative antioxidant mechanism to AOEs has been proposed and involves the scavenging of oxygen radicals by compounds such as polyunsaturated fatty acids (PUFA) (Sosenko *et*

al., 1988). This hypothesis proposes that PUFA that are found in non-critical, non-membrane sites (i.e. triglycerides) could scavenge excess oxygen free radicals and prevent their interaction with critical membrane PUFA (i.e. phospholipids).

Albumin and soluble molecules such as uric acid and glucose can offer protection to cellular components by removing the oxygen free radicals from the extracellular compartment (Halliwell and Gutteridge, 1985). Vitamin E and uric acid can stop the chain reaction of lipid peroxidation (Cross *et al.*, 1987).

2.2.5. Development of AOEs in the Newborn

Neonatal animals are more tolerant to hyperoxia than are adult animals (Holtzman *et al.*, 1989). There is a developmental decrement in AOE levels that occurs with maturation. The reason for the higher levels of AOEs in the neonate is that their lung cells are able to induce an increased rate of AOE synthesis in response to the hyperoxic stimulus (Frank and Sosenko, 1987). This is necessary for successful fetal adaptation to extrauterine life where they must be prepared for transition to the relatively hyperoxic environment of 21% O_2. It has been shown that SOD and CAT activity doubles during the final 10 to 15% of gestation in parallel with the development of the surfactant system (Walther *et al.*, 1989, Tanswell *et al.*, 1986). Prematurity is associated with reduced pulmonary AOE activities

(Tanswell and Freeman, 1984) and the preterm neonate will not be prepared to handle the relative hyperoxia of 21% O_2. Therefore, the neonate with immature lungs is even more susceptible and likely to develop oxygen toxicity.

2.2.6. Induction of AOEs During Hyperoxia

Hyperoxia is a stimulus that causes an increase in the activity of AOEs. The phenomenon of tolerance and adaptation to the lethal effects of oxygen is associated with an induction of AOEs (Crapo *et al.*, 1980). This induction has been shown to occur at the level of increased mRNA expression for SOD and CAT (Shinwell *et al.*, 1989). The different sensitivities of lung cells to hyperoxia are due to differences in the ability of the particular cell to increase its AOEs (Crapo and Teirney, 1974).

2.2.7. O_2 Free Radicals on Sub-cellular Components

When highly reactive oxygen free radicals are not inactivated by the antioxidant defense systems, they are free to react with various cellular components. This can range from causing fragmentation of DNA, damage to structural proteins such as collagen and elastin, alterations to and inactivation of enzymes, and

interaction with membrane components causing lipid peroxidation (Cross *et al.*, 1987). Lipid peroxidation diminishes membrane fluidity, increases non-specific permeability to ions and may inactivate membrane-bound enzymes. When this chain reaction is uncontrolled, the result may be cell necrosis (Halliwell, 1987).

The end result of these reactions may be cell death if the damage is extensive. However, if the cell remains viable, there may be oxidant-induced modifications in gene expression thus altering the functional status of the cell or the proliferative state of the cell. Alterations or inactivation of existing enzymes leads to aberrant metabolic functioning of the cell, i.e. increased or decreased eicosanoid output.

Lipid peroxidation causes alterations in the phospholipids of the membranes that results in the release of fatty acids from the membrane lipids i.e., A.A. (Petruska *et al.*, 1990). This aberrant release of fatty acids may thus result in pathological synthesis of lipid mediators. In addition, lipid hydroperoxides and other peroxides such as hydrogen peroxide are activators of A.A. metabolic enzymes (Warso and Lands, 1985).

2.2.8. O_2 Free Radicals on Cellular Populations

The initial injury caused by hyperoxia occurs to the capillary endothelium. The endothelial cells are the most susceptible to the direct effects of hyperoxia because they contain less intracellular AOE activity (Freeman and Tanswell, 1985). Also, the intracellular generation of oxygen radicals causes inactivation of antiproteases within endothelial cells, thus leaving them with decreased defenses against the secondary effects of hyperoxia mediated by PMNL proteases (Cheronis *et al.*, 1987).

Type II cells are more resistant to the direct toxic effects of hyperoxia than are the Type I cells and endothelial cells (Crapo *et al.*, 1978). Enhanced resistance of the alveolar Type II cells is the result of the specific hyperoxic-induced increase in [illegible] AOEs (Jongkind *et al.*, 1989). Injury to the Type I membrane is repaired by a selective proliferation of the more resilient Type II cells (Crapo *et al.*, 1980). The epithelium is better equipped to handle increased levels of oxygen radicals due to the increased proportion of oxidant-adapted Type II cells that cover the surface (Coalson, 1986).

Hyperoxia causes alterations in the functional status of alveolar macrophages and induces the release of factors that recruit and activate damaging PMNLs (Shasby *et al.*, 1982). Excessive, intracellular generation of oxygen radicals can lead to cell death as evident by the diminished numbers of pulmonary alveolar macrophages following acute hyperoxic exposure (Ogden *et al.*, 1984).

Also, hyperoxia inhibits mitosis and prevents proliferation of these cells (Sherman *et al.*, 1988).

Lung fibroblasts found in the interstitium respond biphasically (both hypo- and hyperplasia) depending upon the degree of hyperoxic challenge. If at lethal levels, these cells are irreversibly damaged and have a decreased ability for normal multiplication. However, at sub-lethal levels, these cells may respond by becoming hyperplastic in response to oxidant-induced stimuli. Fibroblasts are relatively more resistant to hyperoxia than are endothelial cells because the rate of induction of their AOEs (glutathione peroxidase) is twice that of endothelial cells (Jongkind *et al.*, 1989).

2.2.9. Oxygen Toxicity in the Newborn

The preterm neonate is particularly susceptible to hyperoxic injury because it is ill-adapted to protect itself (Frank, 1985). In particular, the preterm lung has an incompletely developed AOE defense system and therefore is at increased risk (Yam *et al.*, 1978).

The unique, age-related problem of oxygen toxicity in the preterm newborn is related to the fact that the newborn lung is in a stage of rapid growth and maturation (Avery *et al.*, 1985). With the introduction of the hyperoxic stress on the growing lung, this normal development of the alveoli is interrupted. Hyperoxia inhibits DNA synthesis (Northway *et al.*, 1976) and inhibits protein synthesis

(Frank and Massaro, 1980). This anti-mitogenic effect prevents septal growth and therefore does not allow for the sub-division of large air spaces into smaller alveoli (Roberts *et al.*, 1983).

2.3. EICOSANOIDS

2.3.1. History and Definition of Eicosanoids

Eicosanoids are biologically active compounds that are derived from 20-carbon unsaturated fatty acids, the most important being arachidonic acid (A.A.). They are formed by the actions of two main enzymatic pathways: prostaglandin endoperoxide H synthase (PGHS) forming the prostaglandins (PG), prostacyclin (PGI_2) and thromboxane A_2 (TxA_2) and lipoxygenases forming the leukotrienes (LTs). The term prostaglandin was introduced by von Euler and applies to compounds based on prostanoic acid. Leukotrienes were named to describe their three conjugated double bonds and their leukocyte sources.

Eicosanoids were first noticed in human seminal plasma by Kurzrok and Lieb (Von Euler, 1967). This fluid contained biologically active compounds that gave it the ability to cause contraction of uterine smooth muscle (Von Euler, 1934). Similar observations were made by Goldblatt in 1933 (Goldblatt, 1933). The first eicosanoids to be isolated were PGE_1 and $PGF_{1\alpha}$ by Bergstrom and colleagues in 1960 (Bergstrom and Sjovall, 1960). Prostacyclin was discovered by Vane and colleagues in 1976 as a factor that was produced by vascular microsomes which inhibited platelet aggregation (Gryglewski *et al.*, 1976). Thromboxane A_2 was first described as

being a new eicosanoid that was formed in platelets and was a potent platelet aggregating agent (Hamberg and Samuelsson, 1974).

Lipoxygenases were known to exist in plants and were responsible for the insertion of oxygen into polyunsaturated fatty acids (Hamberg and Samuelsson, 1967). This enzymatic activity was then found to be present in human cells such as platelets, which possesses 12-lipoxygenase activity (Hamberg and Samuelsson, 1974) and in leukocytes, which possesses 5-lipoxygenase (Borgeat and Samuelsson, 1979). Initially there was interest in the similarities between the cysteinyl-containing leukotrienes, LTC4, LTD4 and LTE4 and the inflammatory products, the slow-reacting substance of anaphylaxis (SRS-A). Later, it was discovered that they were the same compounds (Murphy *et al.*, 1979).

2.3.2. Eicosanoid Synthesis

2.3.2.1. *Arachidonic Acid Release*

Arachidonic acid (A.A.) is stored in membrane phospholipids esterified at the sn-2 position (Irvine, 1982). It must be liberated from these stores since only the free form of the fatty acid can be metabolized into eicosanoids (Lands and Samuelsson, 1968). This is accomplished mainly by the hydrolytic action of phospholipase A2

(PLA_2) (Flower and Blackwell, 1976). Liberation of A.A. also occurs by the sequential actions of phospholipase C (PLC) (Hong and Deykin, 1982) producing diacylglycerol then 1-diacylglycerol lipase followed by 2-monoacylglycerol lipase which ultimately releases A.A. (Balsinde *et al.*, 1991). Arachidonic acid release is relatively rapid following stimulation, i.e., in 5 to 60 seconds (Smith, 1989). The rate-limiting step in the eicosanoid pathway appears to be the availability of the precursor since addition of exogenous A.A. leads to a dose-dependent increase in eicosanoid synthesis (Irvine, 1982).

2.3.2.2. *Prostaglandin Endoperoxide H Synthase Metabolites*

Free A.A. is acted upon by prostaglandin endoperoxide H synthase (PGHS) to form prostaglandins, prostacyclin and thromboxane. PGHS exhibits two distinct catalytic activities. First, its cyclooxygenase action adds two molecules of oxygen to form the hydroperoxide, PGG_2. Second, is the hydroperoxidase activity mediating a net two-electron reduction of the 15-hydroperoxyl group of PGG_2 to form PGH_2 (Ohki *et al.*, 1979). PGH_2 is then converted to the various prostaglandins, prostacyclin or thromboxane by the subsequent actions of the particular enzymes based upon the availability of the particular enzymes within a given cell. PGE_2 is formed by the action of an isomerase. PGI_2 is synthesized from

PGH_2 following the action of PGI_2 synthetase and TxA_2 is formed by the TxA_2 synthetase.

2.3.2.3. *5-Lipoxygenase Metabolites*

Free A.A. may also be acted upon by 5-lipoxygenase which catalyzes the specific di-oxygenation of A.A. at the C-5 position to form 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). This unstable intermediate is enzymatically converted to the allylic epoxide, LTA4, by the dehydrase activity which causes the stereospecific removal of the 10D(R) hydrogen atom and loss of water (Maas *et al.*, 1982). LTA4 is highly unstable and is rapidly converted to various more polar compounds both enzymatically and non-enzymatically (Borgeat and Samuelsson, 1979). The enzymatic conversion of LTA4 to the biologically active compounds may follow two pathways.

The enzymatic hydrolysis of LTA4 by LTA4 hydrolase produces 5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid (LTB4). The second pathway produces 5-hydroxy-6-S-glutathionyl-7,9,11,14-eicosatetraenoic acid (LTC4) by the action of LTC4 synthase which adds the tripeptide, glutathione, to LTA4 at the C-6 position (Hammarstrom *et al.*, 1979). LTC4 is transformed into the cysteinylglycinyl derivative LTD4 by the cleavage of the glutamic acid residue by the enzyme γ-glutamyl-transpeptidase (γ-GTP).

LTD4 is metabolized to the cysteinyl derivative, LTE4 by the removal of a glycine residue by a dipeptidase action (Parker *et al.*, 1980). It has been shown that incubation of LTE4 can form LTF4 by the reincorporation of glutamic acid by the action of γ-GTP (Anderson *et al.*, 1982).

2.3.3. Characteristics of Eicosanoid Enzymes

2.3.3.1. *Phospholipases*

The phospholipases that release A.A. from membrane glycerolphospholipids are phospholipase A2 (PLA2) (Flower and Blackwell, 1976) and phospholipase C (PLC) (Hong and Deykin, 1982). PLA2 prefers phosphatidylcholine as a substrate and PLC uses phosphatidylinositol (Holtzman *et al.*, 1988). Evidence suggests that it is the arachidonyl-specific PLA2 which is primarily responsible for substrate release that leads to eicosanoid formation (Gao and Serrero, 1990).

PLA2 must be translocated from the cytosol to the membrane in order to be activated. This translocation is calcium-dependent (Channon and Leslie, 1990). The enzyme is activated by stimulation of cell surface receptors (Neufield and Majerus, 1983) that are coupled to G-proteins (Murayama *et al.*, 1990). The activation of the

G-proteins in turn control the activities of PLA_2 (Silk *et al.*, 1989) and PLC (Jeremy *et al.*, 1988). Ligands that bind to these receptors include hormones, growth factors and the eicosanoids themselves (Duval and Freyss-Beguin, 1992).

2.3.3.2. *Prostaglandin Endoperoxide H Synthase*

Prostaglandin endoperoxide H synthase (PGHS) is a membrane bound enzyme (Pagels *et al.*, 1983) that is located mainly in the microsomes and nuclear membrane and is not found in the plasma or mitochondrial membranes (Rollins and Smith, 1980). Its two enzymatic activities reside in a single protein (Samuelsson *et al.*, 1978).

This enzyme requires low levels of activator hydroperoxides in order to be continuously activated (Hemler and Lands, 1980). It is proposed that the enzyme has a saturable activator site that binds the peroxides (Lands *et al.*, 1976). The product of this enzyme, PGG_2, is itself a hydroperoxide and thus it forms a positive feedback loop to enhance its own production since the reaction starts slowly and then accelerates (Hemler and Lands, 1980). In turn, PGG_2 can also serve as a negative feedback when levels of the hydroperoxide product become too high such as when the cyclooxygenase activity exceeds the ability of the peroxidase activity to remove PGG_2. The cyclooxygenase activity is inhibited by excessive levels of oxidants

(i.e. peroxides) generated during catalysis (Chen *et al.*, 1987) and therefore this negative feedback loop may be the mechanism of self-inactivation of the enzyme that occurs (Kent *et al.*, 1983). Normally, this does not occur since it is the cyclooxygenase step that is usually rate limiting (Murota *et al.*, 1983).

Cyclooxygenase activity is stimulated by growth factors such as transforming growth factors (Tashjian *et al.*, 1985), epidermal growth factor (Yokota *et al.*, 1986), platelet-derived growth factor (Goerig *et al.*, 1987) and also by cytokines such as interleukin-1 and interleukin-2 (Frasier-Scott *et al.*, 1988). This stimulation may occur at the level of enhanced gene transcription or increased mRNA stabiliy since these factors increase cyclooxygenase mRNA levels (Rosen *et al.*, 1989).

2.3.3.3. *Prostacyclin Synthetase and Thromboxane A_2 Synthetase*

Prostacyclin synthetase catalyzes the conversion of its substrate PGH_2 to prostacyclin (PGI_2), an enol-ether (Moncada, 1982). PGH_2 may be derived from intracellular A.A. metabolism or from transcellular substrate shunting (Marcus *et al.*, 1980). This enzyme has been localized to the plasma and nuclear membranes and may be a cytochrome P-450 (DeWitt and Smith, 1983). PGI_2 synthetase is a constitutive enzyme and is present in excessive amounts in cells capable of synthesizing prostacyclin (Murota *et al.*,

1983). The reaction speed of PGI_2 synthetase is 40 times greater than that of cyclooxygenase, therefore it is the cyclooxygenase step that is rate-limiting (Murota *et al.*, 1983). This enzyme may undergo self-inactivation during catalysis (DeWitt and Smith, 1983) by the inhibitory actions of lipid hydroperoxides (Salmon *et al.*, 1978).

Prostacyclin synthesis is stimulated by thrombin (Weksler *et al.*, 1978), adenosine diphosphate (Boeynaems and Galand, 1983), histamine (Baenziger *et al.*, 1981), bradykinin (Hong and Deykin, 1982). After endothelial cells have been stimulated to produce prostacyclin, they become unresponsive to a second stimulus, due to inactivation of PGI_2 synthetase and/or PGHS (Kent *et al.*, 1983, Egan *et al.*, 1976).

Thromboxane synthetase catalyzes the metabolism of PGH_2 into thromboxane A_2 (Moncada, 1982). TxA_2 synthetase is located predominantly in platelets but may be found in other cell types (see section 2.3.4.). This enzyme may be a cytochrome P-450 - type hemoprotein. Platelet thromboxane synthetase activity is associated with dense tubular membranes (Needleman *et al.*, 1986). The appearance of TxA_2 in platelets is accompanied by the appearance of malondialdehyde which has been used as an alternative measure of thromboxane production (Cohen, 1985). It has been suggested that this enzyme may require calcium ions for synthetic activity (Knapp *et al.*, 1977).

2.3.1.4. *5-Lipoxygenase*

The 5-lipoxygenase enzyme is stored as a cytosolic enzyme where it is thought to be in an inactivated state. In order to become activated it must be translocated to the membrane, a process which is calcium dependent (Rouzer and Samuelsson, 1987). The two activities of this enzyme, i.e., 5-lipoxygenase and dehydrase activities are associated with the same protein (Matsumoto *et al.*, 1988). This enzyme complex requires calcium, adenosine triphosphate, a fatty acid hydroperoxide, the presence of two high molecular weight cytosolic factors and one membrane-bound factor for maximum activity (Rouzer *et al.*, 1986). It undergoes self-inactivation following covalent binding of the substrate, A.A., at the active site (Rouzer *et al.*, 1986). 5-lipoxygenase is sensitive to inhibition by peroxides as is PGHS (Sun and McGuire, 1984).

The 5-lipoxygenase enzyme may be activated by chemotactic peptides, platelet-activating factor, and immune complexes, through interaction with surface receptors (Borgeat and Naccache, 1990). The calcium dependence of the enzyme suggests that alterations of calcium metabolism (calcium influx or release from intracellular stores) may be involved in the regulation of the 5-lipoxygenase (Borgeat and Naccache, 1990). In contrast to the PGHS pathway where the first step (i.e., cyclooxygenase) is rate limiting, the rate limiting step in LT biosynthesis occurs at the levels of the LTA4 hydrolase and LTC_4 synthase (Jakschik and Kuo, 1983).

2.3.3.5. *LTA4 Hydrolase & LTC4 Synthase*

LTA4 hydrolase catalyzes the conversion of LTA4 to form LTB4. This enzyme appears to be expressed in a variety of cell types including those that lack 5-lipoxygenase activity (Mendina *et al.*, 1989). LTA4 hydrolase is a soluble enzyme since it is found dissolved within the cytosolic fraction (Ziboh *et al.*, 1984). The 5-lipoxygenase and LTA4 hydrolase activities reside in the same protein (Rouzer *et al.*, 1986). In neutrophils, the LTA4 hydrolase limits the synthesis of LTB4 (Fruteau de Laclos *et al.*, 1984). When cells are maximally stimulated with A23187, the 5-lipoxygenase will produce amounts of LTA4 that exceeds the catalytic capacity of LTA4 hydrolase, consequently, the excess LTA4 undergoes non-enzymatic hydrolysis to 6-trans-LTB4 and 6-trans-12-epi-LTB4 (Borgeat and Naccache, 1990). LTB4 synthesis is triggered by platelet-activating factor (PAF), chemotactic peptides and phagocytic particles which are inflammatory stimuli (Dahinden *et al.*, 1988).

The LTC4 synthase or glutathione transferase, γ-GTP and dipeptidase are particulate enzymes that have been localized in the plasma membrane (Jakschik *et al.*, 1982). It has been speculated that LTC4 is converted to LTD4 as it passes through the plasma membrane to the outside of the cell since the LTD4-forming enzyme is in the plasma membrane (Needleman *et al.*, 1986).

2.3.4. Eicosanoid Synthesis in the Lung

The cellular sites of eicosanoid synthesis in the lungs include fibroblasts, alveolar macrophages, Type II alveolar epithelial cells, mast cells, smooth muscle cells and endothelial cells (Nowak, 1984). Individual cell types respond to A.A. liberation by producing a characteristic array of metabolites. This is a function of both the intracellular enzymatic network and the receptors present on cell membranes (Garcia *et al.*, 1987). It has been proposed that there is a cellular compartmentalization for the generation of eicosanoids such that prostacyclin is from the pulmonary endothelium, TxA_2 is from platelets and interstitial cells of the parenchyma, and LTs are mainly generated from inflammatory cells (Gryglewski *et al.*, 1978).

Almost all mammalian cells contain the phospholipases and the PGHS enzyme, with the exception of erythrocytes (Moncada, 1982). 5-lipoxygenase is mostly found in phagocytic cells (such as leukocytes and macrophages) and lymphocytes (Garcia *et al.*, 1987). Endothelial cells do not contain 5-lipoxygenase, but can synthesize leukotrienes by cell-to-cell transfer of the intercellular leukotriene precursor, LTA_4 (Claesson and Haeggstrom, 1988).

Prostacyclin synthetase is found in the greatest abundance in endothelial cells (Spector, 1988). Endothelial cells make 10 times more PGI_2 than other eicosanoids (Olson and Tanswell, 1987). Other cells such as alveolar epithelial cells, platelets, inflammatory cells

(Scott *et al.*, 1980), smooth muscle cells (Hla and Bailey, 1989) and fibroblasts are capable of synthesizing prostacyclin.

Thromboxane synthetase is predominantly localized in platelets (Moncada *et al.*, 1976). However endothelial cells, leukocytes, macrophages, smooth muscle cells (Gryglewski *et al.*, 1978), epithelial cells (Chauncey *et al.*, 1988) and lung fibroblasts (Hopkins *et al.*, 1978) also contain this enzyme (Cohen, 1985). TxA_2 synthetase is not found in Type I or Type II pneumocytes (Nusing *et al.*, 1990).

LTA4 hydrolase is found mainly in phagocytic cells such as macrophages (Scott *et al.*, 1980), neutrophils and other granulocytes. LTB4 is the main leukotriene produced by neutrophils and alveolar macrophages (Freeland *et al.*, 1988, Kumlin and Dahlen, 1989, Borgeat and Naccache, 1990) and is produced in amounts that are 10 times greater than LTC4 (Lee *et al.*, 1985). Blood monocytes produce LTB4 as well as LTC4 (Borgeat and Naccache, 1990). Epithelial cells are capable of generating LTB4 (Chauncey *et al.*, 1988).

LTC4 synthase is located in inflammatory cells such as PMNLs and macrophages (Scott *et al.*, 1980). Among PMNLs, eosinophils are the major source of LTC4 (Borgeat and Naccache, 1990, Lee *et al.*, 1985) but basophils may also synthesize LTC4 (Fox *et al.*, 1985). Endothelial cells have been shown to contain this enzyme but not 5-lipoxygenase since these cells can metabolize LTA4 into LTC4 but cannot convert exogenous A.A. into LTC4 (Claesson and Haeggstrom, 1988). Platelets have been shown to be able to convert LTA4

derived from granulocytes into LTC4 which indicates that they contain the LTC4 synthase enzyme (Edenius *et al.*, 1988).

2.3.5. Eicosanoid Metabolism

Eicosanoids are autocrine and paracrine hormones and not the classical circulating hormones. They are synthesized, act and are metabolized within a localized site (O'Flaherty, 1982). The rapid inactivation of these mediators is important because they are very potent biological compounds and it is desirable to contain their actions to a localized area. The lung is the major site of prostaglandin metabolism (Gryglewski, 1980). The selective elimination of eicosanoids by the lung is not due to sequestration following uptake and storage in the lung tissue but to their active enzymatic metabolism (Piper *et al.*, 1970). There is a rapid access of eicosanoids to intracellular catabolizing enzymes which is effected by a carrier-mediated, energy requiring transport mechanism (Bito *et al.*, 1977). The lung tissue contains an abundance of a cytosolic enzyme, 15-hydroxy-prostaglandin dehydrogenase (15-OH-PGDH) which catalyzes the initial step of PG metabolism by the oxidation of the 15-hydroxy group.

Prostacyclin has been implicated as playing the role of a circulating vasoregulatory hormone (Gryglewski *et al.*, 1978). This hypothesis was put forth since PGI_2 is continuously released from

the endothelial surface of the lungs into the systemic circulation and since it is not metabolized to a great extent by the lung. The slow metabolism of PGI_2 is due to the poor affinity of prostacyclin with the transport system of the lungs (Armstrong *et al.*, 1977). Prostacyclin has a half-life of 10 minutes before spontaneous hydrolysis to 6-keto $PGF_{1\alpha}$, which is non-enzymatic (Pace-Asciak and Wolfe, 1971).

Thromboxane A_2 is the biologically active form of the compound. It is highly unstable and is non-enzymatically decomposed to the hemiacetal, TxB_2, within 30 seconds (Hamberg *et al.*, 1974). The more slowly formed enzymatic metabolites are 15-dihydro-thromboxane B_2 and 2,3-dinor thromboxane B_2 via the lung metabolic processes.

LTB_4 is metabolized mainly in neutrophils which is initiated by a receptor-mediated uptake mechanism (Goldman and Goetzl, 1982). Within these cells the leukotriene is catabolized by ω-oxidation to 20-OH-LTB_4 and subsequently to 20-COOH-LTB_4. These metabolites have diminished biological activity compared with LTB_4 (Hansson *et al.*, 1981). LTB_4 is not metabolized by eosinophils, blood monocytes or alveolar macrophages to a great extent (Borgeat and Naccache, 1990).

LTC_4 is rapidly degraded to LTD_4 and LTE_4 by sequential enzymatic steps and represents the main inactivation pathway (Hammarstrom, 1982). Although the two metabolites do possess some biological activity, they are 50% less potent than the parent

compound LTC4 following one passage through the lungs (Piper *et al.*, 1981). Another degradative pathway for LTC4, as well as for LTD4 and LTE4, is their conversion to diastereoisomeric sulfoxides which possess very little or no biologic acitivity (Lee *et al.*, 1982).

2.3.6. Cellular Mechanisms of Action

Eicosanoids are mediators and/or modulators of cellular actions. It had been proposed that due to the intrinsic lipid solubility and membrane permeability of eicosanoids, that they may alter membrane fluidity and hence affect membrane-associated enzymes (Johnson and Ramwell, 1973). Due to distinctive effects of the individual eicosanoid on certain cell types it is more likely that they communicate with cells via specific cell surface receptors that have now been identified. These plasma membrane receptors are coupled to G-proteins (Rodbell, 1980) that control second messenger cascade systems such as cyclic adenosine monophosphate (cAMP) and inositol trisphosphate (IP3) (Smith, 1989).

Upon binding to the receptor, G-proteins are activated and stimulate the activity of the adenylate cyclase which increases intracellular cAMP formation (Gorman *et al.*, 1977). Cyclic-AMP then activates protein kinase C which in turn phosphorylates the proteins that will produce the final cellular action (Hall and Behrman, 1982). The IP3 cascade is activated when the receptor linked G-protein

stimulates a membrane-associated phosphodiesterase to hydrolyse phosphatidyl-inositol-4,5-bisphosphate, resulting in the formation of IP3 and 1,2,-diacylglycerol. IP3 then mobilizes intracellular calcium stores. The increase in free calcium synergizes with 1,2-diacylglycerol to activate protein kinase C which catalyzes the phosphorylation of various proteins which mediates the final cellular event (Berridge, 1984).

Specific prostacyclin receptors are found on platelets, endothelial cells, neutrophils, smooth muscle cells and fibroblasts (Leigh *et al.*, 1984). Prostacyclin stimulates adenylate cyclase causing an increase in cAMP levels within these cells (Goff *et al.*, 1978).

Thromboxane A2 receptors are found on platelets, endothelial cells, neutrophils, smooth muscle cells and fibroblasts (Lord *et al.*, 1976). When TxA2 binds to the surface receptor, it functions to cause inhibition of the adenylate cyclase thereby decreasing the intracellular levels of cAMP (Gorman *et al.*, 1979). Thromboxane regulates the intracellular levels of calcium perhaps via activation of the inositol trisphosphate system since it has been shown to cause mobilization of intracellular calcium (Gerrard *et al.*, 1977).

Leukotriene B4 receptors are mainly found on phagocytic cells. The binding of the ligand to the receptor activates the IP3 cascade and affects plasma membrane ion channels (Crooke *et al.*, 1989). Interaction of LTB4 with the specific receptor activates G-proteins and turns on the IP3 cascade resulting in the mobilization of

intracellular calcium stores and stimulation of protein kinase C. LTB4 stimulation of the G-protein also enhances influx of extracellular calcium by opening a receptor-operated calcium channel (Crooke *et al.*, 1989).

Leukotriene C4 receptors are found on endothelial cells (Chau *et al.*, 1986), epithelial cells, phagocytes, smooth muscle cells and fibroblasts (Nicosia *et al.*, 1985). The activation of LTC4, D4, E4 receptors also utilizes the IP3 cascade system causing the release of intracellular calcium stores and activation of protein kinase C (Crooke *et al.*, 1989).

2.3.7. Biological Actions of Eicosanoids

Eicosanoids are potent biological compounds that have been ascribed a plethora of roles such as the control of airway diameter, vascular resistance, inflammatory responses, ion transport and more recently, influencing cell growth and differentiation.

2.3.7.1. *Prostacyclin*

Prostacyclin is the major A.A. metabolite that is produced by the lungs which is mainly attributed to the rich vascularization of the lung (Zijlstra *et al.*, 1987). Prostacyclin is a potent systemic and

pulmonary vasodilator (Armstrong *et al.*, 1977) and bronchodilator (Gardiner and Collier, 1980). It possesses these actions through the ability to cause relaxation of smooth muscle cells in the vasculature and the airways. PGI_2 is a powerful inhibitor of platelet aggregation (Moncada *et al.*, 1976). This prostaglandin inhibits the proliferation of fibroblasts and smooth muscle cells by inhibiting DNA synthesis (Owen, 1985). In addition to modulating cell proliferation, PGI_2 may have profound effects on the differentiated state of cells (Needleman *et al.*, 1986).

Increases in cAMP have been shown to: increase surfactant release from Type II epithelial cells, i.e., biochemical lung differentiation (Davis *et al.*, 1990); induce new PGHS synthesis (Kusaka *et al.*, 1988); and inhibit the capacity of fibroblasts to deposit connective tissue components such as collagen (Baum *et al.*, 1978). Since PGI_2 causes an increase in intracellular cAMP levels, it may also play a regulatory role in these processes through this second messenger system.

2.3.7.2. *Prostaglandin E_2*

PGE_2 stimulates chloride ion secretion by epithelial cells, and therefore is involved in lung fluid fluxes (Eling *et al.*, 1988). PGE_2 induces mild vasoconstriction in the pulmonary vascular bed (Kadowitz *et al.*, 1975). However, it has the opposite effect on airway

smooth muscle, since it is a bronchodilator (Rosenhale *et al.*, 1968). PGE_2 inhibits cell proliferation of fibroblasts and the collagen synthetic capacity of these cells (Goldstein *et al.*, 1985).

2.3.7.3. *Thromboxane A2*

Thromboxane A_2 is a powerful platelet aggregator (Hamberg *et al.*, 1974). It is a vasoconstrictive agent of both veins and arteries and has the ability to contract bronchial smooth muscle (Svennson *et al.*, 1977). TxA_2 induces cell differentiation (Xie *et al.*, 1992).

2.3.7.4. *Leukotriene B4*

Leukotriene B_4 plays an important role in inflammation and host defense mechanisms. LTB_4 causes contraction of bronchial smooth muscle, however this effect is indirect since it is mediated by increased levels TxA_2 that are induced by LTB_4 (Piper and Tippins, 1982). LTB_4 has been shown to produce an increase in vascular permeability in the presence of a vasodilatory agent (Bray *et al.*, 1981). LTB_4 is a potent chemotactic, chemokinetic and proaggregatory compound for PMNLs (Ford-Hutchinson *et al.*, 1980). Specifically, it causes the specific adherence of PMNLs to vascular endothelial cells and then stimulates their diapedesis into

extravascular tissues (Bray *et al.*, 1981). Once they are in the extravascular tissues, PMNLs are activated by LTB4 to degranulate (Showell *et al.*, 1982) and increase the production of oxygen free radicals (Serhan *et al.*, 1982). This leukotriene may stimulate DNA synthesis and enhance cell proliferation (Palmberg *et al.*, 1989). The intracellular events caused by LTB4 are in part mediated through alterations in levels of intracellular calcium. Calcium binds to intracellular myofibrils (Leff, 1988) to cause chemotaxis and may act on lysosomes to cause them to fuse with the plasma membrane and degranulate (Rae and Smith, 1981).

2.3.7.5. *Leukotriene C4*

LTC4 has the ability to contract the trachea, bronchi, small airways and small blood vessels by acting directly on smooth muscle cells (Piper *et al.*, 1982). The action of peptidoleukotrienes on the vasculature, however, differs depending upon which vessels they are acting upon. They cause arteriolar constriction but dilation of venules (Needleman *et al.*, 1986). LTD4 and LTE4 exhibit similar effects on the smooth muscle of the airways and the vasculature but are less potent than the parent compound, LTC4 (Piper *et al.*, 1982). LTC4 causes an increase in vascular permeability (Dahlen *et al.*, 1981) which is thought to be due to a direct action on the endothelial lining in the blood vessels (Piper *et al.*, 1982).

LTC_4 induces proliferation of fibroblasts (Phan *et al.*, 1988), smooth muscle cells (Palmberg *et al.*, 1989) and epithelial cells by inducing DNA synthesis (Leikauf *et al.*, 1990). This leukotriene stimulates collagen synthesis in fibroblasts (Phan *et al.*, 1988).

2.3.8. Effects of Oxygen on Eicosanoids

The eicosanoid cascade is oxidant-sensitive. Most of the evidence implicates reactive oxygen species as playing an important role in the control of eicosanoid synthesis (Sedor, 1986). Elevated levels of oxygen radicals have been shown to both stimulate and inhibit eicosanoid production (Adler *et al.*, 1987). The oxygen free radicals that are produced during hyperoxia may affect eicosanoid production at various levels of the cascade.

Eicosanoid synthesis is activated by reactive oxygen species in part due to peroxidation and perturbations of membrane phospholipids that cause an increase in the release of arachidonic acid (Warso and Lands, 1985). Lipid peroxidation also activates phospholipase A_2 perhaps by increasing calcium ion influx due to alterations in membrane permeability characteristics (Lebedev *et al.*, 1982). Also, the elevated levels of lipid peroxides induced by hyperoxia causes a reduction in the re-uptake of calcium into microsomes therefore causing a sustained level of free calcium (Iwata *et al.*, 1986).

Oxygen stimulation has been implicated to have an effect on PGHS since oxidant-induced PG synthesis was found to occur before membrane perturbations causing A.A. release (Lee *et al.*, 1989). The increase in the peroxide tone during hyperoxia serves to activate the cyclooxygenase activity of PGHS when present at low levels (Lands, 1985). However, at high peroxide tone, the enzyme may be self-inactivated by oxidizing equivalents (Egan *et al.*, 1976). When high concentrations of A.A. are present, it has been shown that there is a preferential synthesis of TxA_2 over PGI_2 (James and Walsh, 1988). They suggest that PGI_2 synthetase is saturated at lower concentrations of substrate than is TxA_2 synthetase. Perhaps, prostacyclin synthetase may be inactivated at high peroxide levels whereas thromboxane synthetase is preferentially activated (Wang *et al.*, 1988).

5-lipoxygenase also demonstrates the biphasic reaction to lipid hydroperoxide levels, where it is activated by low concentrations and inactivated by higher levels (Sun and McGuire, 1984).

The enzymes that metabolize eicosanoids such as 15-OH-PGDH, are inactivated by hyperoxia which would cause increased levels of eicosanoids to be present and may prolong their actions (Chaudhari *et al.*, 1979).

Hyperoxia may have an indirect effect on the levels and the relative profile of eicosanoids produced by the lung because of alterations in the cellular populations present. A decrease in the endothelial cell population caused by the initial cellular destruction

may cause a decrease in the prostacyclin produced by the lung. The influx of platelets may contribute to an increase in TxA_2 production in the pulmonary tissue. The increase in the neutrophil and macrophage pools may thereby cause an increase in the amount of leukotriene output by the lung. Type II cell and fibroblast hyperplasia during the chronic phase may also contribute to an alteration in the normal profile of eicosanoids produced by the lung.

2.3.9. Role of Eicosanoids in BPD

Stenmark *et al.* (1985) measured an increase in the levels of all lipid mediators in bronchoalveolar lavage fluid of infants with BPD. Lipoxygenase products and TxB_2 were elevated to a greater extent than was 6KF. Increased levels of LTB_4 in lavage fluid of infants with BPD shows temporal correlation with changes in airway reactivity (Motoyama *et al.*, 1986). Increased LTC_4 could participate in the bronchoconstriction, edema and pulmonary vasoconstriction that is present in the lungs of infants with BPD (Lewis and Austen, 1984). Both PGHS metabolites, 6KF and TxB_2 levels have been detected in elevated levels in the plasma of neonates that have been diagnosed with lung disease (Hutchison *et al.*, 1985) but the concentration of thromboxane greatly exceeded that of 6KF (Stenmark *et al.*, 1985). The imbalance favouring TxB_2 could be

responsible in part for the bronchoconstriction and pulmonary vasoconstriction observed (Lewis and Austen, 1984).

Indirect evidence for the role of eicosanoids in BPD was implicated by studies showing clinical and physiological improvement in lung function in infants with BPD following steroid administration which inhibited eicosanoid synthesis (Avery *et al.*, 1985).

The role of lipid mediators has been strongly implicated since they are detected in concentrations capable of having pathophysiological effects. Lipid mediators play a part in a complex mediator network that also involves oxygen radicals, proteases, and lymphokines which act in concert to initiate and promote lung injury (Stenmark *et al.*, 1987). The exact mechanisms that lead to the morphological and functional aberrations of the lungs in neonates with BPD have not yet been established and therefore require further investigation.

CHAPTER 3

RESEARCH PLAN

3.1. OVERALL OBJECTIVE AND HYPOTHESIS

Overall Objective

The overall objective is to examine the alterations in eicosanoid output associated with the development of lung injury during pulmonary oxygen toxicity in the neonatal rat.

Hypothesis

Prolonged exposure to hyperoxia causes lung injury which is in part mediated by oxidant-induced aberrations in the balance of the pulmonary eicosanoid profile.

3.2. SPECIFIC AIMS

1) To examine the pathological effects of *in vivo* hyperoxic exposure on the lungs of neonatal rat pups.

2) To elucidate the eicosanoid profile of the neonatal rat lung in response to *in vivo* hyperoxic exposure.

3) To relate changes in the eicosanoid profile to the observed pathological changes in the lung following *in vivo* hyperoxic exposure.

3.3. RATIONALE

3.3.1. Effects of Hyperoxia on the Lung

Evidence leading to the belief that hyperoxia affects the lung.

Direct exposure of the lung to hyperoxia is known to cause pulmonary oxygen toxicity (Frank and Massaro, 1980). Pulmonary oxygen toxicity constitutes an initial acute insult to the lung which is followed by a healing phase if the lung adapts to a reduced level of oxygen or hyperoxia is removed. There is a characteristic etiology of

the effects of hyperoxia on the lung. During the acute phase, resident cells of the lung's airways and parenchyma are damaged causing lung hypoplasia. This initiates an inflammatory response with an influx of platelets and phagocytes in conjunction with interstitial and intraalveolar edema. If the inflammatory processes are prolonged or excessive, the subsequent repair response of the lung is abnormal. There is a hyperplastic response of different cellular populations in the lung, i.e. fibroblasts, Type II pneumocytes, macrophages and endothelial cells (Crapo *et al.*, 1980). Collagen synthesis is excessive and results in intraalveolar and interstitial fibrosis. The increase in cell numbers and connective tissue contributes to a thickening of the interstitium.

The rationale of the methods employed in the examination of the effects of hyperoxia on the lung.

In order to mimic this known progression of lung injury, a specific hyperoxic exposure regime was employed. It consisted of exposure to >95% oxygen for 7 days, to induce an excessive, acute lung injury, followed by 21 days at 60% oxygen, to prolong the inflammatory reaction yet allow for adaptive repair. Animals were examined for injury after 1 and 7 days of exposure, during what was believed to be the acute phase and then after 17 and 28 days as representative times of the repair phase. The markers of lung pathology were chosen to test for the injury that was expected to be occurring during the progression of the lung damage. Therefore, lung

edema, pulmonary tissue weight changes in relation to the body weight and histology of the lung were examined.

3.3.2. Effects of Hyperoxia on Eicosanoids

Evidence that the eicosanoid pathways are affected by hyperoxia.

The eicosanoid pathways are oxidant-sensitive. The production of PGHS products may demonstrate a biphasic response with an initial stimulation by peroxides followed by an inhibition due to self-inactivation of the enzyme (Hemler and Lands, 1980). An increase in the levels of PGHS products during hyperoxia may be due to a decrease in the activity of the metabolic enzymes (Chaudhari *et al.*, 1979). Leukotrienes have been found to increase following hyperoxic exposure (Taniguchi *et al.*, 1986).

The novel aspects of how this study elucidates the effects of hyperoxia on eicosanoids.

Previous hyperoxic exposure models examined mainly adult animals where eicosanoids were measured in bronchoalveolar lavage fluid or from vascular perfusate. In order to examine the eicosanoid profile of whole lung tissue with the *in vivo* architecture intact, this study employed a newly developed tissue explant model. The eicosanoids, 6KF, TxB_2, LTB_4 were measured since they constitute the major metabolites produced by the lung (Zijlstra *et al.*, 1987).

The profile of eicosanoids was examined at the different stages to determine if there was a differential effect of hyperoxia during the progression of lung injury.

3.3.3. Eicosanoids and Hyperoxic Lung Injury

Evidence that eicosanoids possess the ability to mediate pathological actions similar to those observed in hyperoxic lung injury.

Eicosanoids may be mediators that cause the observed lung pathology during hyperoxic injury. Eicosanoids are capable of eliciting a wide array of physiological actions, some of which are important during the pathogenesis of hyperoxic lung damage. PGI_2 is postulated to serve a protective role since when its production is inhibited, lung injury is amplified (Hageman *et al.*, 1986). PGI_2 may oppose lung damage through the ability to cause vaso- and bronchodilation, inhibition of platelet and PMNL aggregation (Moncada *et al.*, 1976), inhibition of cell proliferation (Rabinovitch, 1987) and decrease in collagen synthesis (Goldstein *et al.*, 1986). TxA_2 may mediate some of the pathological effects because it can cause platelet aggregation and vaso- and bronchoconstriction. LTs may also promote the development of lung injury since these metabolites are found in elevated amounts in correlation with lung damage and blockade of their synthesis results in reduced injury (Taniguchi *et al.*, 1986, Hageman *et al.*, 1986). Leukotriene B_4 may

contribute to lung injury through its ability to enhance neutrophil chemotaxis and activation. This LT induces fibroblast proliferation thereby contributing to fibrosis (Phan *et al.*, 1988). LTC4 can cause vaso- and bronchoconstriction and increases vascular permeability (Dahlen *et al.*, 1981). It can also promote collagen synthesis from fibroblasts (Phan *et al.*, 1988).

Why is this study different from previous examinations of hyperoxia and eicosanoids?

Previous studies have examined only the acute phase of hyperoxic exposure and have focused on the role of individual eicosanoids. However, it is speculated that the balance of eicosanoid levels relative to each other is the determining factor in the pathogenesis of lung injury. Therefore, in this study, the effect of hyperoxia on the levels of 3 eicosanoids were determined throughout the progression from the acute to the repair phase and related to the changes in the pathological markers of lung damage.

CHAPTER 4

EXPLANT TECHNIQUE

4.1. INTRODUCTION OF THE EXPLANT TECHNIQUE

The lung is a complex organ that is composed of various functional compartments and has approximately 40 different cell types. Many different models have been used in order to study biochemical processes that occur within the lung. These include bronchoalveolar lavage, vascular perfusion, cell culture of isolated lung cells and organ culture. Each method has advantages and disadvantages that must be evaluated in accordance with what is of importance to the particular study. Bronchoalveolar lavage allows the assessment of the environment of the airways and alveoli. However, the information obtained is a static picture and does not give an accurate reflection of what is taking place in the other compartments of the lung. Vascular perfusion assesses only the blood side of the lung and again does not give information about the environment of the rest of the lung. Cell culture allows the study of

individual cell types but the *in vivo* cell-to-cell interactions are lost. The normal functional responses of the cells depends on the communication between cells, therefore it is desirable to retain the normal lung architecture. For example, it has been shown that growth and differentiation of lung epithelium is dependent on the presence of mesenchymal cells (Masters, 1976). Therefore maintaining the normal architectural relationships permits biochemical assessments that truly reflect the *in vivo* environment.

The purpose of using the explant technique in the present study was to be able to quantitate eicosanoid release as it may occur *in vivo*. This technique has the advantage of being able to observe the functional synthetic capacity of the lung in a controlled environment while retaining the *in vivo* architecture of the cell-to-cell interactions and the connective tissue connections. The dynamics of these interactions on eicosanoid output is of greater interest in the present studies than the behaviour of the individual cells in isolation. The disadvantage of the explant system is that the heterogeneity of the cell types complicates the interpretation of biochemical data. Tissue slices have been used previously to study the respiration of lung tissue (Black *et al.*, 1951) and its hormonal sensitivity (Adamson *et al.*, 1975). A system of lung tissue explants has been well-characterized and was found to be valid in the examination of fetal lung development (Gross *et al.*, 1978). However, these explants were cultured over several days and morphological and biochemical maturation of the lung was found to occur. This was not desirable in

the present studies and therefore a short-term, fresh tissue explant technique was developed.

4.2. EXPLANT PREPARATION

Pups where euthanized by an intraperitoneal injection of 3 parts Ketamine (100 mg/ml) and 1 part Rompun (20 mg/ml) at a dosage of 10 mg Ketamine:0.6 mg Rompun/g body weight using a 1 ml syringe and a 30 gauge needle. A mid-line incision was made through the abdomen and thorax. The lungs were removed and the left lobe was carefully dissected free of visible blood vessels and airways. The tissue was placed in ice cold Hanks' balanced salt solution (HBSS) in a petri dish and placed on ice (for details see section 6.2.3.).

After the lungs had been removed from all the pups, the left lobe was sliced longitudinally to a thickness of 500 µm using a McIllwain tissue chopper. This thickness of tissue was used since a larger slice would require that the incubation atmosphere be supplemented with oxygen in order to adequately oxygenate the central portions of the explant. A smaller slice was not used because the cut edges of the slice would occupy a proportionally larger portion of the explanted tissue (Gross *et al.*, 1978). Tissue slices were separated with paint brushes while in ice cold HBSS. The explants

from different animals were pooled and mixed together in order to be a representative sample of the entire litter. Approximately 40 to 50 mg of tissue (8 to 10 slices) was placed on nylon mesh grids (original design KSK) which were placed in 12-well culture plates. Slices were carefully placed so that they did not overlap and the full surface of the explant was flattened on the grid. After lung slices were placed on the grids, the tissue was washed three times by pipetting cold HBSS over the explant. Then pre-warmed media was added to the wells so that the liquid level just reached the bottom surface of the c[illegible] and the optimal volume was determined to be 700 µl. The plates were covered to maintain the humidity of the ambient air and incubated in a shaking water bath at 37°C.

To determine the optimal conditions of this short-term explant system, several preliminary experiments were performed.

4.3. CHARACTERIZATION OF EXPLANT TECHNIQUE

4.3.1. Explant Incubation Medium

The basic function of a salt solution is to maintain the pH and the osmotic balance in the medium and to provide the tissue with water and essential inorganic ions. The media used was Hanks' balanced salt solution (HBSS) (Hanks, J., 1976). This was found to be sufficient because the incubation was short-term and not a tissue culture experiment where supplemental nutrition and antibiotics would be necessary. The media was effectively buffered with 15 mM hydroxyethyl-piperazine-2-ethanesulfonic acid (HEPES, pKa=7.3 at 37°C), a zwitterion (Lee *et al.*, 1989). The pH was assessed throughout the duration of the incubation and was found to remain stable at 7.38. A bicarbonate buffer was not used since it was not desirable to manipulate the gaseous atmosphere i.e., the CO_2 environment which would add another variable to the experiment. Calcium chloride ($CaCl_2$) was added at a concentration of 1.67 mM (as found in HBSS containing $CaCl_2$, Sigma) to ensure an adequate supply of Ca^{2+} for the calcium-dependent enzymes, phospholipase A_2 and 5-lipoxygenase. The volume of buffer added to each well to bring the liquid level to the bottom of the explant was determined to be 700 µl/well. Then, by capillary action and by the gentle rocking of the shaking water bath, the top surface of the explant was kept

moist. The exposure of tissue to the ambient air allowed the explant to be adequately oxygenated via direct gas diffusion (Gonzales *et al.*, 1985).

4.3.2. Basal Eicosanoid Release

In initial studies, it was found that the handling and chopping of tissue caused a large release of eicosanoids. This artificially induced eicosanoid generation which produced an artifact. This would not be representative of the actual *in vivo* eicosanoid synthetic activity. It was necessary to determine conditions that would return the lung tissue eicosanoid output to resting levels.

The explants were prepared as described above. After 10, 30 or 60 minute intervals of incubation, the media were aspirated from the bottom of the separate wells, placed into chilled glass test tubes and frozen at -70°C until they were assayed. Fresh, pre-warmed media were added to the wells, the plates were covered and again incubated for 10, 30 or 60 minute intervals. This media changing procedure was repeated for a total period of 210 or 240 minutes. The media samples were assayed directly by radioimmunoassay (RIA) for 6KF, TxB_2 and LTB_4. Following the incubation, the tissue from each well was blotted and weighed. Eicosanoid release was expressed per milligram wet tissue weight per minutes of incubation interval and plotted against duration of incubation.

Eicosanoid output was found to be greatest during the first time interval for each eicosanoid measured (6KF, TxB_2 and LTB_4) for all 3 durations of incubation (Figures 4.1., 4.2. and 4.3.). The eicosanoid release declined in a linear fashion with increasing incubation time. When the media were changed every 10 minutes, the levels of all 3 eicosanoids declined 10 fold from the maximum levels that were released during the first interval to the lowest levels when the basal plateau was reached (Figure 4.1.). When media changes were every 30 minutes, absolute levels declined on average 5 fold (Figure 4.2.). This suggests that frequent changes may have a washing-out effect. When the media were changed every 10 minutes or 30 minutes, the basal output plateau was reached after 120 minutes of incubation. However, if the media were changed every 60 minutes, the eicosanoid output did not plateau, even after 240 minutes of incubation and absolute levels were higher (Figure 4.3.).

It was desirable to choose the shortest possible incubation time in order to minimize and prevent tissue alterations from *in vivo*. Therefore, a 150 minute incubation period was used in order to achieve basal release. When the lowest eicosanoid levels were reached, the plateau remained at a stable, steady-state pattern of release. Media changes were made every 30 minutes since more frequent changes did not facilitate reaching basal levels of eicosanoid output.

4.3.3. Recovery of Eicosanoids

Eicosanoids are not stored within cells but are synthesized and released from cells into the media upon demand (Lands, 1976). Explants were to be used to assess the eicosanoid production from the lung. Therefore, in order to assess the accuracy of the eicosanoid determinations, the recovery of exogenously added eicosanoid standards following incubation with the explants was assessed. This was done to ensure confidence that the values obtained using this technique accurately reflect the eicosanoids being released from the explants into the media.

Lung explants were incubated to basal eicosanoid release for 150 minutes with 30 minute interval media changes. In the following 30 minute period, 6KF, TxB_2 or LTB_4 standards at concentrations of 500 pg/0.1 ml, 125 pg/0.1 ml and 31.25 pg/0.1 ml were added to separate wells that contained lung explants. The media were aspirated and frozen until RIA. Six replicates of each of the added standard concentrations were also assayed and then averaged to determine the actual amount of each eicosanoid added. Basal eicosanoid release (120 min to 150 min) was subtracted from the time period where the standards were added (150 min to 180 min) to account for the tissue eicosanoid contribution. The percent recovery was calculated as follows in Equation 4.I.:

EQUATION 4.I.

$$\frac{\text{total measured - tissue production}}{\text{eicosanoid added}} \times 100 = \% \text{ Recovery}$$

Similar percent recoveries were found for the 3 concentrations (500 pg/0.1 ml, 125 pg/0.1 ml, 31.25 pg/0.1 ml) of unlabelled eicosanoid added (Table 4.A.). The average percent recoveries of the 3 concentrations used, were 100%, 99%, and 102% for 6KF, TxB_2 and LTB_4, respectively.

It was concluded that once eicosanoids were released by the lung tissue into the media, they were not metabolized by the tissue during the incubation time interval. Eicosanoids were recoverable and were not lost due to binding to tissue, components in the media, inserts or plates. The levels of eicosanoids that were measured were an accurate reflection of the amounts being released by the explants.

4.3.4. Arachidonic Acid Dose-Response

Arachidonic acid (A.A.) is the major substrate of the PGHS and 5-lipoxygenase enzymes for the synthesis of eicosanoids. To find an enzyme saturating dose of A.A. for the explant system, the tissues were stimulated with increasing concentrations of substrate. Excess

exogenous A.A. eliminates the limiting factor of the availability of substrate in affecting the activity rates of PGHS and 5-lipoxygenase. From this, the overall effect of hyperoxia on these enzymes can be assessed. The stimulation of the explant was to be maximal since it was desirable to observe the greatest total capacity of the lung tissue to synthesize eicosanoids.

Lung explants were incubated to basal eicosanoid release for 150 minutes with 30 minute interval media changes. In the following 30 minute period, A.A. in 700 µl of pre-warmed HBSS was added in concentrations of 0.1, 1, 10, 100 and 1000 µM to separate wells in replicates of 6. The media were aspirated and frozen until RIA. The lung tissue was blotted and weighed for eicosanoid standardization. RIAs were performed on A.A. media samples in the absence of tissue to subtract as background. Basal eicosanoid release (120 min to 150 min) and A.A. media backgrounds were subtracted from the total stimulated release (150 min to 180 min) to represent the eicosanoid synthesized from exogenous A.A. stimulation only. Stimulated eicosanoid synthesis was standardized to explant tissue wet weight and expressed per minute of incubation. Three replicate experiments were performed.

The dose-response curves for 6KF and TxB_2 are shown in Figure 4.4.. There was a dose-response relationship since eicosanoid synthesis increased with increasing doses of A.A.. At concentrations of 0.1 to 10 µM, A.A. was not present at enzyme saturating doses since stimulation was not maximal. Perhaps these doses are too low

to cause adequate diffusion of A.A. through the tissue, into the cells and to the eicosanoid synthetic enzymes. From 10 to 100 μM A.A., there was significant stimulation of release of both 6KF and TxB_2. Levels of 6KF increased 7 fold and TxB_2 increased 4 fold with 100 μM A.A. over the levels stimulated by the 0.1 μM dose. The greater stimulation of 6KF may be a reflection of the greater number of endothelial cells which predominantly synthesize prostacyclin. At 1000 μM A.A., eicosanoid production significantly decreased as compared to 100 μM A.A.. This may be due to the enzyme inactivation that is known to occur by the negative feedback of the product on the PGHS enzyme (Egan *et al.*, 1976).

The optimal dose of A.A. used in subsequent explant studies was 100 μM since this concentration of exogenous A.A. stimulated the greatest output of both 6KF and TxB_2. Surprisingly, LTB_4 synthesis was not stimulated at any dose of A.A.. However, it was found later (see 4.3.6) that calcium ionophore, A23187 stimulated LTB_4 output.

4.3.5. Arachidonic Acid Time-Course

The use of intact tissue presents an obstacle since exogenously added A.A. must diffuse through the tissue, into the cells and to the sites of the eicosanoid synthetic enzymes. Therefore, it was

necessary to define the time needed for A.A. to be incubated with the explants in order to achieve maximal stimulation.

Explants were incubated to basal eicosanoid release for 150 minutes with 30 minute interval media changes. Then 100 μM A.A. in 700 μl of HBSS was added to the wells. Media samples from separate wells were taken after 5, 15, 30, 45, 60 and 75 minutes of incubation, aspirated and frozen until assay. Six replicates were assessed at each time point. Eicosanoid release stimulated by A.A. during the various times was calculated by subtracting the endogenous basal tissue contribution for the same amount of time (i.e., basal release for 5, 15, 30, 45, 60 and 75 min) and standardized to explant tissue wet weight per minute of incubation. Three replicate experiments were performed.

Figure 4.5. shows the time-dependent pattern of eicosanoid release when explants were incubated with 100 μM A.A.. Eicosanoid release for both 6KF and TxB_2 increased linearly from 0 to 30 minutes of A.A. stimulated incubation. After 5 minutes, levels of 6KF were greater than TxB_2 for the remaining durations of the incubation suggesting preferential metabolism of A.A. through the PGI_2 pathway which is the characteristic profile for the lung. For incubation times longer than 30 minutes, eicosanoid release reached a steady-state plateau as no further increase was observed.

In subsequent explant studies, maximal A.A. stimulation was performed for a duration of 30 minutes since it was demonstrated that this was sufficient time for the A.A. to travel to the cellular sites

of synthesis. After 30 minutes of incubation, the eicosanoid synthetic machinery was functioning at maximum capacity and longer durations would therefore be of no further benefit.

4.3.6. A23187 Dose-Response

A23187 is a calcium ionophore that causes an increase in the levels of free intracellular calcium mainly by increasing the transport of extracellular calcium into the cell. The availability of maximal levels of calcium allows for the optimal activation of the phospholipase A_2 (PLA_2) and 5-lipoxygenase enzymes which are calcium-dependent (Rouzer and Samuelsson, 1987). Use of A23187 would stimulate the optimal release of the endogenous stores of A.A. by maximally activating PLA_2. For LTB_4 synthesis to be stimulated, intracellular calcium levels must be increased in order to activate the 5-lipoxygenase enzyme.

The A23187 dose-response protocol was the same as for A.A. dose-response experiments except that doses of A23187 were 1.25, 12.5, 25, 50 and 75 μM.

Figure 4.6. shows the response of explants to increasing doses of A23187 and this reflects the ability of the tissue to synthesize eicosanoids from stimulated release of endogenous substrate. 6KF output with 50 μM A23187 increased by 15 fold over levels stimulated by the 1.25 μM dose and was the greatest stimulated

response of the 3 eicosanoids. TxB_2 and LTB_4 outputs increased 5 to 6 fold. All 3 curves were found to increase linearly in the concentration range of 1.25 to 50 μM A23187. Eicosanoid output reached a maximum at 50 μM and then plateaued since no further increase was seen with 75 μM. The levels of stimulated eicosanoid synthesis were less with A23187 than measured following incubation with the optimal concentration of A.A., 100 μM. For example, maximum 6KF release was 10.0 pg/0.1 ml with 50 μM A23187 but was 15.0 pg/0.1 ml with 100 μM A.A. Perhaps A23187 stimulation was not releasing sufficient amounts of endogenous A.A. that would maximally saturate PGHS.

The availability of calcium was important in the synthesis of eicosanoids from the explants since a dose-response relationship exists. The effect was similar for all three eicosanoids which may indicate that the predominant stimulatory effect of A23187 was in the activation of PLA_2. The calcium dependence of the 5-lipoxygenase enzyme was demonstrated since LTB_4 synthesis was stimulated and a dose-response relationship did exist in the presence of A23187. In the subsequent explant experiments, 50 μM A23187 was used.

4.3.7. A23187 Time-Course

As for A.A., the optimal time for the ionophore to diffuse to the site of action was determined for maximal stimulation to be obtained. The protocol used to examine the time course of stimulation with 50 μM A23187 was the same as used for the A.A. time course study.

6KF and LTB4 release increased from 0 to 15 minutes of incubation and then plateaued at a maximum for longer incubation periods as shown in Figure 4.7.. TxB2 output rose sharply in the first 5 minutes but did not stabilize until 30 minutes. At 5 minutes of incubation TxB2 levels were greater than 6KF and LTB4. After 15 minutes of incubation, 6KF levels surpassed TxB2 and LTB4 levels and remained higher throughout the duration of the experiment.

A23187 stimulated the explants to maximum eicosanoid release by 15 to 30 minutes whereas exogenous A.A. required 30 minutes. Perhaps the metabolism of endogenous A.A. is more rapid since it would not have to traverse the plasma membrane in order to reach the metabolic enzymes. In subsequent experiments, 30 minute incubations with A23187 were used so that all 3 eicosanoids were produced at maximum levels and to coincide with the A.A. stimulation experiments.

4.3.8. Viability of Explants

The enzyme lactate dehydrogenase (LDH) is a macromolecule that is contained in all cells. If a cell is undergoing toxicity, there is an increase in the permeability of the plasma membrane which promotes the release of intracellular constituents such as LDH. An increased release of LDH from cells is a conventional index of cytotoxicity (Bergmyer, 1978). This was used in order to assess the viability of the explants throughout the incubation.

Tissue explants were incubated in HBSS for a total of 210 minutes, with media changes every 30 minutes. Samples of media were taken after 0, 30, 60, 90, 120, 150, 180 and 210 minutes and frozen until LDH assay. To determine total LDH, tissue was homogenized in 1.0 ml of ice cold HBSS containing 0.1% Triton X-100 (in order to solubilize the lipid components of the membranes) and then frozen. LDH was measured in the media and in the tissue sample.

The LDH assay measures the metabolic activity of the enzyme. Therefore assessments determine whether the enzyme is localized within the cells or whether it is detectable in the media following release from the cell. The LDH assay was performed using a kit from SIGMA. Briefly, 1.0 ml pyruvate substrate was added to a vial containing 1.0 mg/ml NADH and incubated in a water bath at 37°C for 5 minutes. Then 0.1 ml of standard or sample was added and incubated for 30 minutes. 1.0 ml of Sigma colour reagent was added

and mixed well for 20 minutes at room temperature. Then 10.0 ml of 0.40 N sodium hydroxide was added. Samples were read against a water reference at 450 nm on a spectrophotometer. A calibration curve was constructed by using serial dilutions of the pyruvate substrate and the amount of pyruvate is inversely proportional to the amount of LDH activity. The amount of LDH was determined from the linear regression equation. Percent LDH release from tissue was calculated as follows in Equation 4.II.:

EQUATION 4.II.

$$\frac{\text{LDH in media}}{\text{LDH in media + LDH in tissue}} \times 100 = \%\ \text{LDH Release}$$

The greatest percent LDH release was 10 to 12% which occurred between 1 and 30 minutes of incubation as seen in Figure 4.8.. Then, from 30 to 120 minutes, the percent LDH release declined, and from 120 to 210 minutes, it leveled to negligible values.

Therefore, LDH was released from the explants at the beginning of the incubation, perhaps due to chopping and manipulation of the tissue. There was injury to the outermost cells of the explant but the remaining cells were not injured or dying since they released low levels of LDH (Gross *et al.*, 1978). In addition, the fact that the explants were able to synthesize eicosanoids upon stimulation demonstrated that they were still metabolically functional. Therefore, within the experimental time frame of 180 minutes, the

cells that were not injured by chopping remained viable and metabolically active.

4.4. SUMMARY OF EXPLANT TECHNIQUE

The development of a short-term lung explant technique was undertaken because a method to assess the eicosanoid production by the intact lung had not been developed. Other methods that had been used previously to assess the eicosanoid profile of the lung were limited in the airway or vascular compartments. The lung has many different compartments such as the airspaces, vascular lumens and the interstitial spaces. Eicosanoids are produced by the various cells in each of these areas of the lung. The interaction of eicosanoids between all components of the lung tissue taken together is an important determinant of the functional and biochemical status of the lung. The lung explant technique was chosen because the interaction of all the cell types in the lung was desirable and a close relationship to the *in vivo* condition was of greater importance than the behaviour of the individual compartments or the individual cell populations. The use of explants was found to be a viable method permitting a dynamic study of the eicosanoid production from lung tissue that had been pre-exposed to hyperoxia.

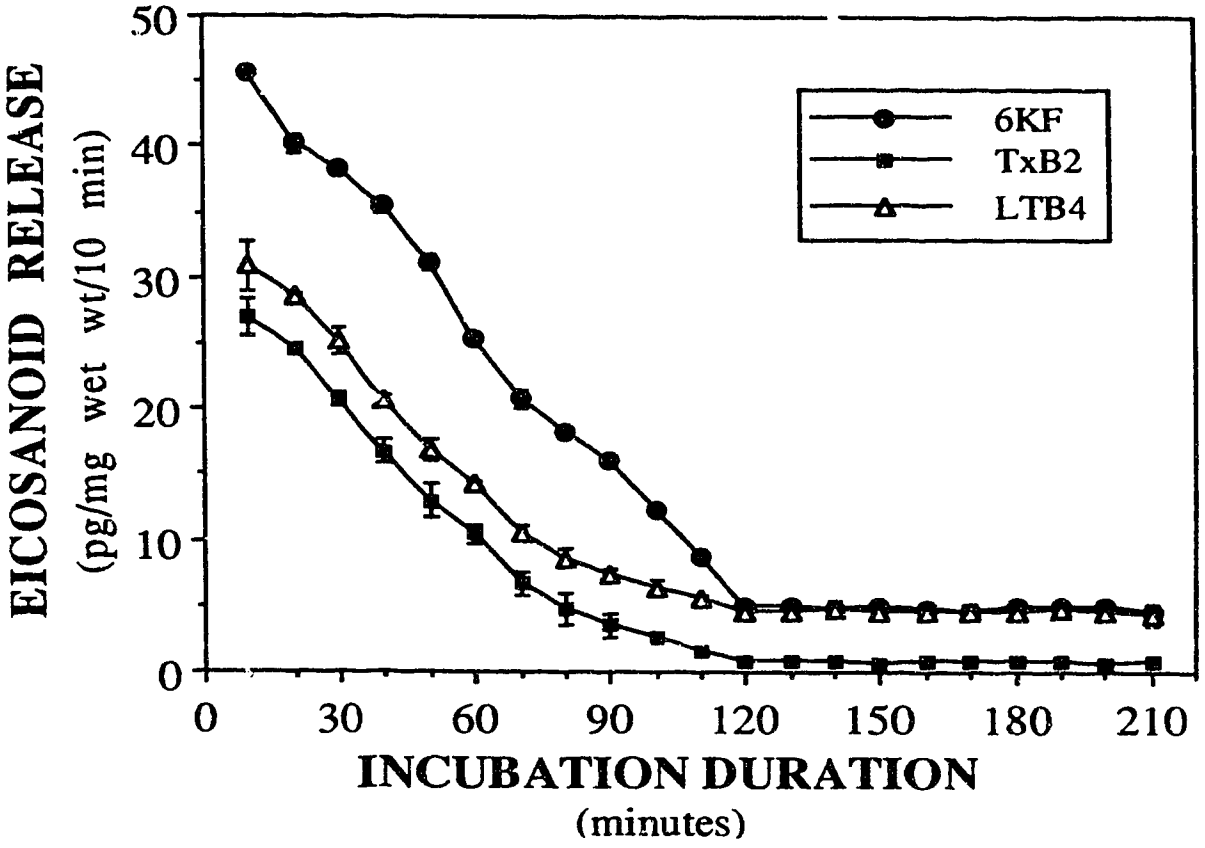


FIGURE 4.1. TIME COURSE OF EICOSANOID RELEASE WITH 10 MINUTE MEDIA CHANGES.
Lung tissue explants were incubated for 210 minutes and the media were changed at 10 minute intervals. Results are expressed as the amount of eicosanoid (6KF, TxB_2, LTB_4) (pg/mg wet wt/10 min) that was released into the medium over the 10 minute interval. Eicosanoid release declined linearly with time of incubation and plateaued to basal output after 120 minutes. Values represent MEAN ± SD of 6 replicate wells. This is a representative graph from one experiment (n=3).

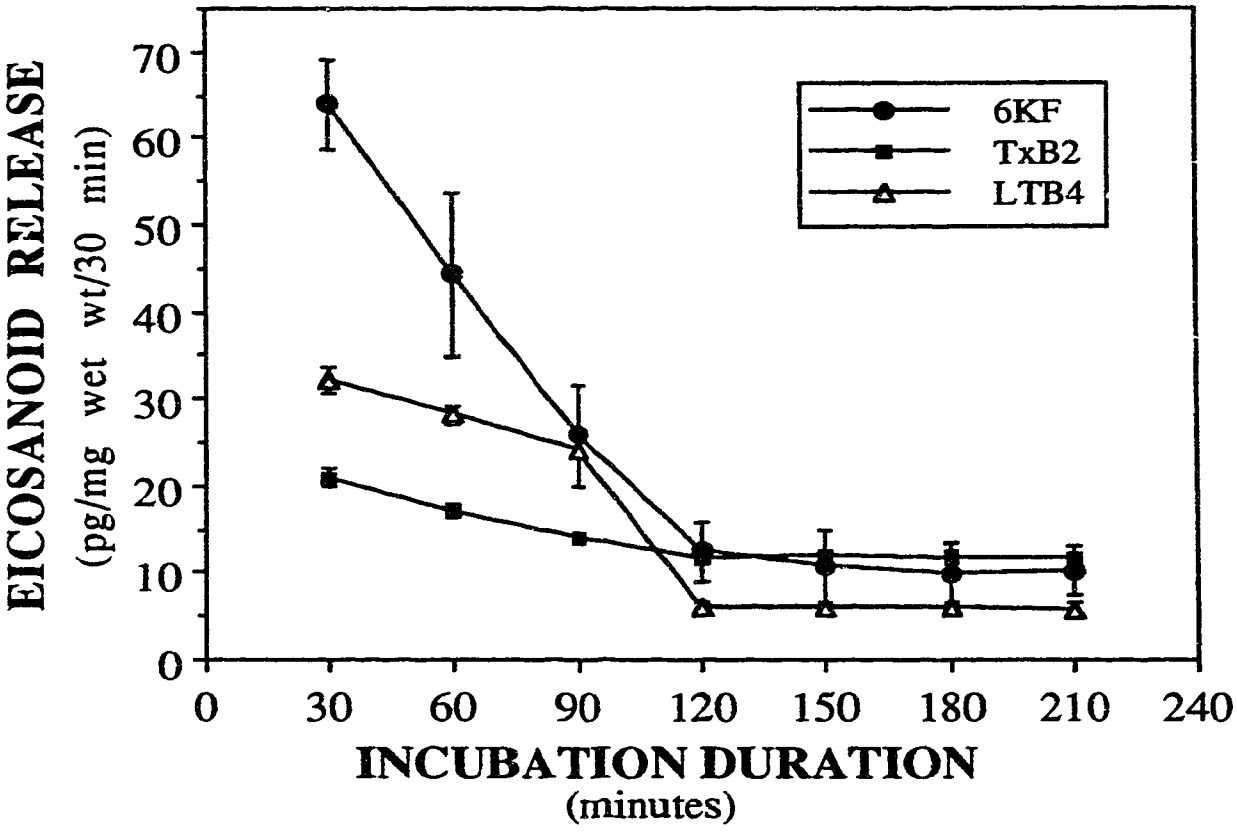


FIGURE 4.2. TIME COURSE OF EICOSANOID RELEASE WITH 30 MINUTE MEDIA CHANGES.
Lung tissue explants were incubated for 210 minutes and the media were changed at 30 minute intervals. Results are expressed as the amount of eicosanoid (6KF, TxB_2, LTB_4) (pg/mg wet wt/30 min) that was released into the medium over the 30 minute interval. Eicosanoid release declined linearly with time of incubation and plateaued to basal output after 120 minutes. Values represent MEAN ± SD of 6 replicate wells. This is a representative graph from one experiment (n=3).

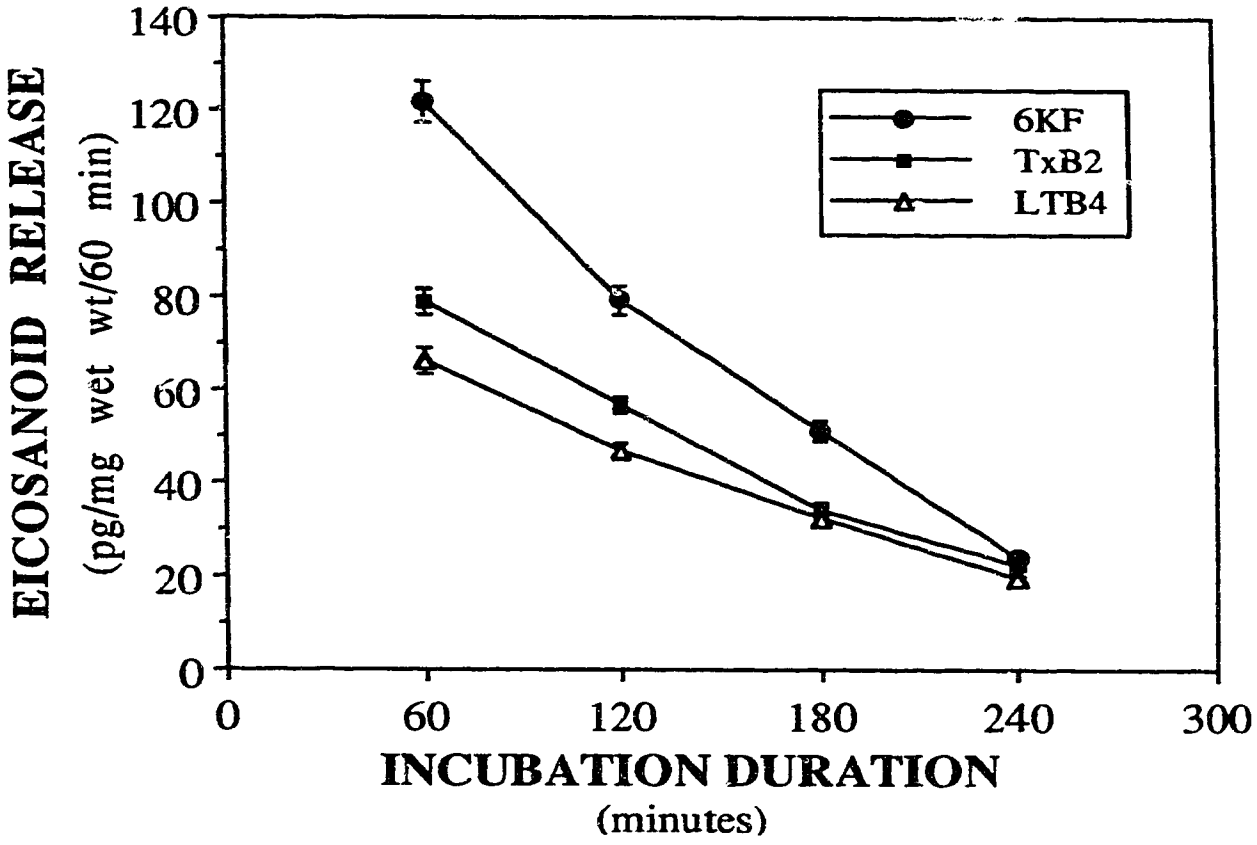


FIGURE 4.3. TIME COURSE OF EICOSANOID RELEASE WITH 60 MINUTE MEDIA CHANGES.

Lung tissue explants were incubated for 240 minutes and the media were changed at 60 minute intervals. Results are expressed as the amount of eicosanoid (6KF, TxB_2, LTB_4) (pg/mg wet wt/60 min) that was released into the medium over the 60 minute interval. Eicosanoid release declined linearly with time of incubation but did not plateau even after 240 minutes. Values represent MEAN ± SD of 6 replicate wells. This is a representative graph from one experiment (n=3).

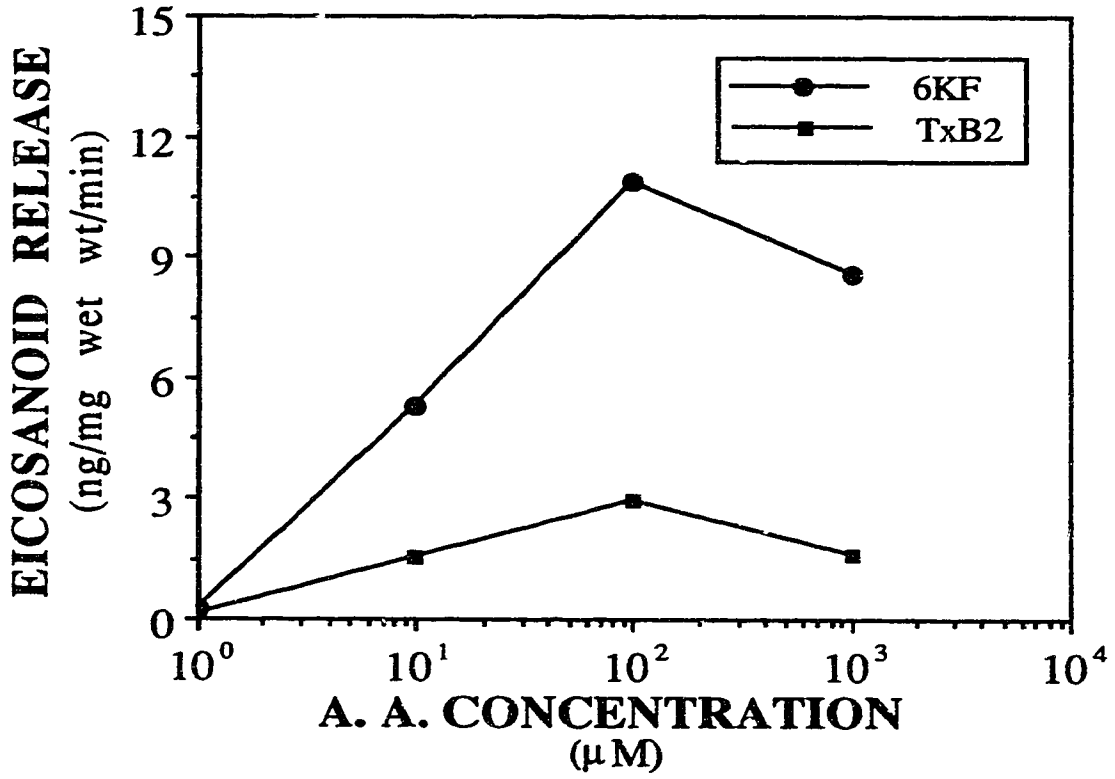


FIGURE 4.4. DOSE-RESPONSE CURVE FOR 6KF AND TxB_2 PRODUCTION BY LUNG EXPLANTS WITH ARACHIDONIC ACID (A.A.) STIMULATION.

Explants were incubated with 0.1, 1, 10, 100 and 1000 μM A.A. in replicates of 6, for 30 minutes. 6KF and TxB_2 production from exogenous A.A. was determined after correction for eicosanoid production from endogenous A.A. For maximum eicosanoid release, the optimal dose of A.A. was 100 μM. Values represent MEAN ± SD of 3 replicate experiments.

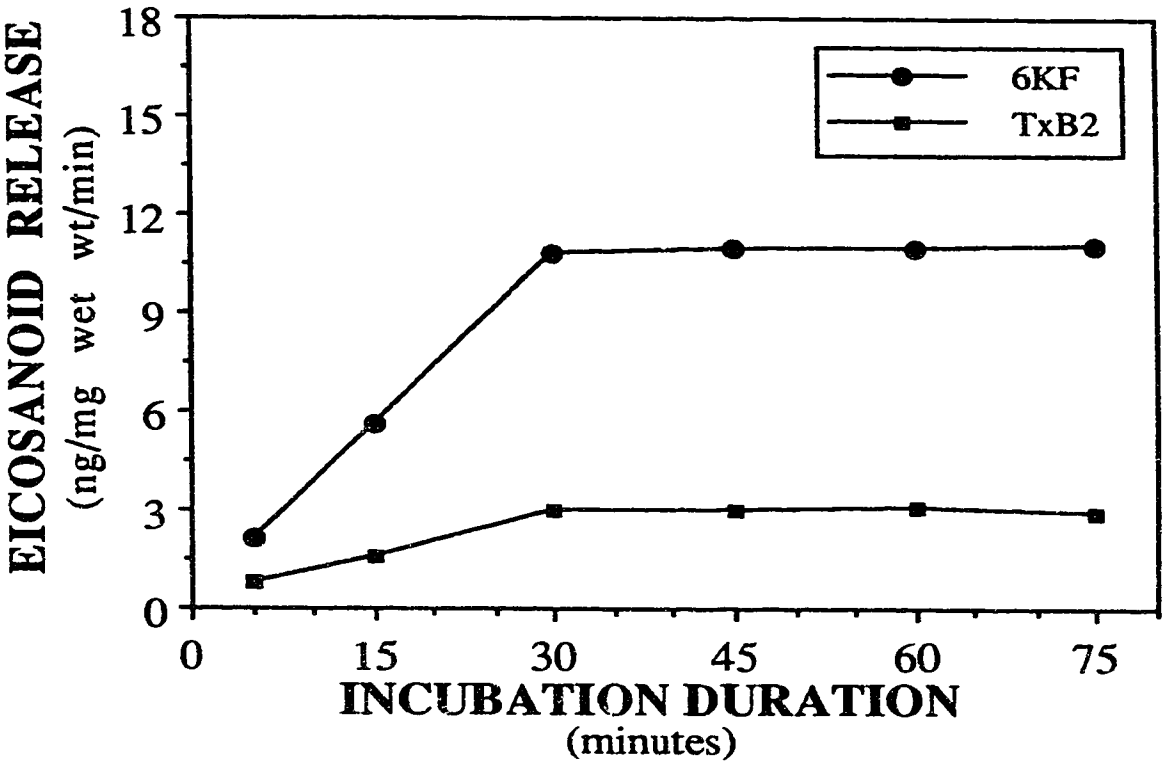


FIGURE 4.5. TIME-COURSE OF 6KF AND TxB_2 RELEASE FROM LUNG EXPLANTS WITH 100 μM A.A. STIMULATION.

Explants were incubated with 100 μM A.A. for 5, 15, 30, 45, 60 and 75 minutes in replicates of 6. 6KF and TxB_2 production from exogenous A.A. was determined after correction for eicosanoid production from endogenous A.A.. Eicosanoid release increased linearly from 1 to 30 minutes and then plateaued. Values represent MEAN ± SD of 3 replicate experiments.

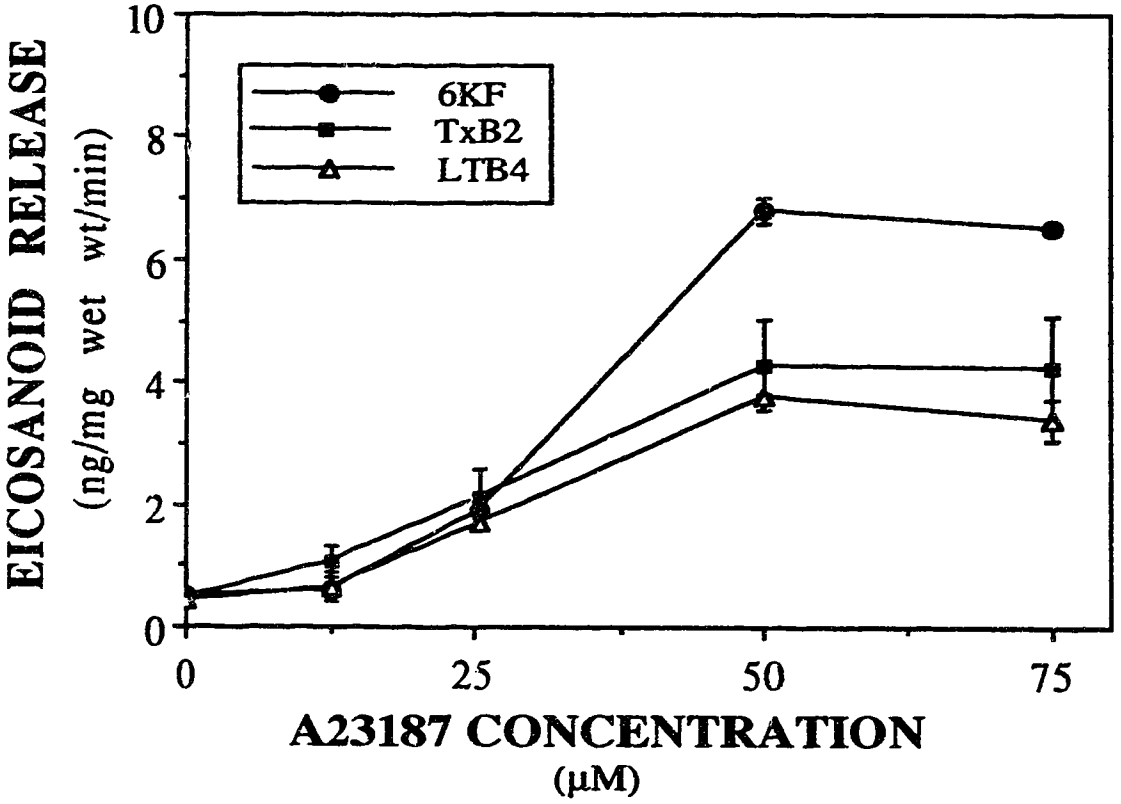


FIGURE 4.6. DOSE-RESPONSE CURVE FOR 6KF, TxB_2 AND LTB_4 PRODUCTION BY LUNG EXPLANTS WITH CALCIUM IONOPHORE (A23187) STIMULATION.

Explants were incubated with 1.25, 12.5, 25, 50 and 75 μM A23187 in replicates of 6, for 30 minutes. 6KF, TxB_2 and LTB_4 production following A23187 stimulation was determined after correction for basal eicosanoid production. For maximum eicosanoid release, the optimal dose of A23187 was 50 μM. Values represent MEAN ± SD of 3 replicate experiments.

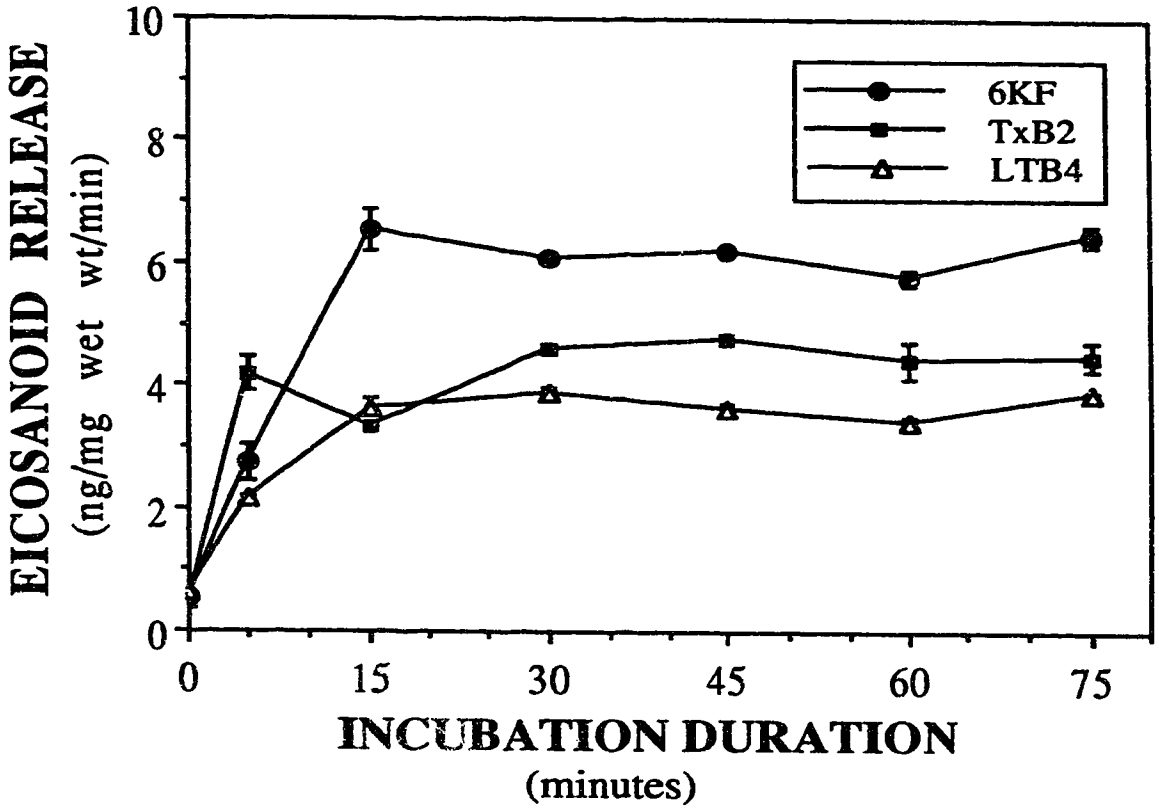


FIGURE 4.7. TIME-COURSE OF 6KF, TxB_2 AND LTB_4 RELEASE FROM LUNG EXPLANTS WITH 50 μM A23187 STIMULATION.
Explants were incubated with 50 μM A23187 for 5, 15, 30, 45, 60 and 75 minutes in replicates of 6. 6KF, TxB_2 and LTB_4 production following A23187 stimulation was determined after correction for basal eicosanoid production. Eicosanoid release increased linearly and then plateaued after 15 to 30 minutes. Values represent MEAN ± SD of 3 replicate experiments.

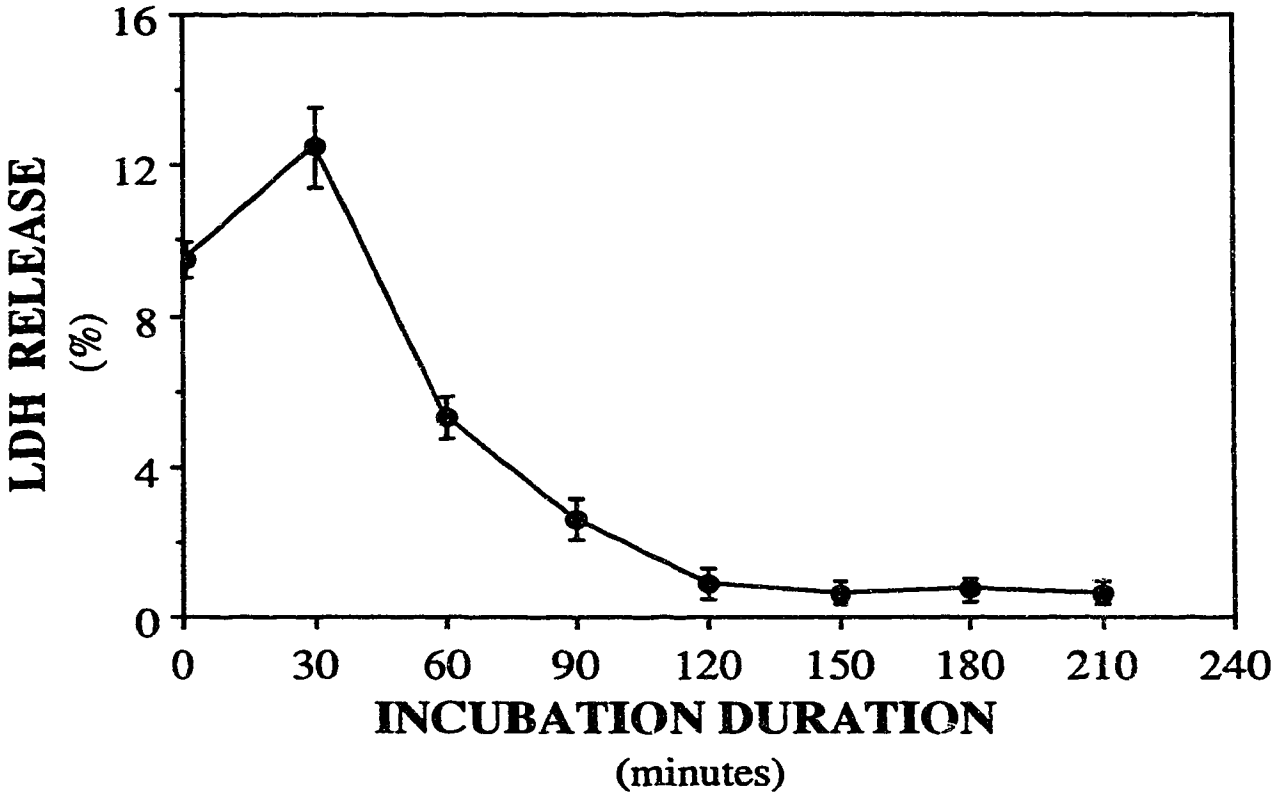


FIGURE 4.8. LACTATE DEHYDROGENASE (LDH) RELEASE FROM LUNG EXPLANTS.
LDH released into the incubation media was determined at 30 minute intervals for 210 minutes. LDH release was expressed as a percent of total cellular LDH (see Equation 4.II.). Levels of LDH detected in the media were highest in the first 30 minute interval and then declined to negligible levels after 120 minutes of incubation. Values represent MEAN ± SD of 12 replicate wells. This is a representative graph from one experiment (n=3).

TABLE 4.A.

RECOVERY OF KNOWN AMOUNTS OF EICOSANOIDS ADDED DURING EXPLANT INCUBATIONS.

Known amounts of 6KF, TxB_2 and LTB_4 (500 pg, 125 pg and 31.25 pg/0.1 ml) were added to separate wells (replicates of 6) of the incubation system for 30 minutes in order to assess the ability to recover eicosanoids following release into the media. Percent recovery of the amount added was calculated by Equation 4.I. (see text). There was a good correlation between added amounts of eicosanoid and the amount recovered. Values represent MEAN ± SD of 3 replicate experiments.

DOSE ADDED (pg/0.1 ml)	DOSE MEASURED (pg/0.1 ml)	PERCENTAGE RECOVERY (%)
6KF		
500	504.1 ± 7.6	100.8
125	122.3 ± 3.5	97.8
31.25	31.8 ± 0.4	101.8
TxB_2		
500	499.7 ± 5.7	99.9
125	123.7 ± 2.5	99.0
31.25	30.9 ± 0.2	98.9
LTB_4		
500	505.7 ± 4.5	101.1
125	127.3 ± 3.1	101.8
31.25	32.2 ± 0.3	103.0

CHAPTER 5

RADIOIMMUNOASSAY

5.1. INTRODUCTION OF RIA

Radioimmunoassay (RIA) is a technique that is used to quantitate minute quantities of biological substances. The procedure was first discovered by Dr. Solomon Berson (1956) as an unexpected consequence of studies undertaken to examine the metabolism of radiolabelled insulin in non-diabetic and diabetic subjects (Berson *et al.*, 1956). It was observed that ^{131}I-labelled insulin disappeared at a slower rate in patients who had previously been administered insulin. They hypothesized that the retarded clearance rate of the radiolabelled insulin was caused by its binding to antibodies that were formed in response to the administered, exogenous insulin. This original observation was then translated into the theoretical concepts of RIA which utilizes the binding of labelled antigen to a fixed concentration of antibody as a quantitative method.

The theoretical basis of this assay makes use of the competitive binding of radiolabelled ligand and unlabelled ligand to a specific antibody. After an equilibrium is established, the amount of unlabelled ligand is obtained by determining its inhibitory effect on the binding of radioactively labelled antigen to the specific antibody. The amount of unlabelled ligand that is bound to the antibody is inversely proportional to the amount of radioactivity of the labelled ligand that is measured. Comparison is made to the inhibitory effect of known concentrations of unlabelled ligand, called standards. An assumption of the RIA technique is that the antibody reaction with the standards and the ligand in the unknown samples is identical.

The antibody-antigen reaction is highly specific due to the absolute requirements of complementary atomic groups, 3-dimensional shape and the charge between the binding site and the antigen. When these criteria are met, there is a good fit between the antigenic determinant and the binding site on the antibody which lends to the high specificity of the reaction. The optimal intermolecular attractive forces are formed producing a high affinity binding of the true antigen which is responsible for the sensitivity of RIA.

In order to evaluate the potential physiological and pathophysiological roles of eicosanoids, it is necessary to be able to measure their concentrations in biological samples. RIA is a useful method to quantitate eicosanoids because it is very specific, sensitive and practical for a large number of samples (Yalow, 1978). In order

to ensure confidence in the measurements, the antisera and the assay itself require characterization. Characterization of the antisera is necessary because the antibody titre within the serum, and affinity or avidity of the antibody molecule varies in every new batch. Therefore, the optimal antibody dilution and the specificity of the new antisera for 6KF (stable hydrolysis metabolite of prostacyclin, PGI_2), TxB_2 (stable metabolite of TxA_2) and LTB_4 were determined. To characterize the assay procedure, parameters evaluating the precision, accuracy and sensitivity of the technique were assessed.

5.2. MATERIALS AND METHODS

5.2.1. Materials

The radiolabelled eicosanoids were obtained from Amersham Canada Limited (Oakville, Ontario).

6KF	6-keto[5,8,9,11,12,14,15(n)-^{3}H] prostaglandin $F_{1\alpha}$ Specific Activity: 4.4-6.6 TBq/mmol, 120-180 Ci/mmol catalogue #TRK.618
TxB_2	[5,6,8,9,11,12,14,15(n)-^{3}H] thromboxane B_2 Specific Activity: 4.4 TBq/mmol, >120 Ci/mmol catalogue #TRK.620
LTB_4	[5,6,8,9,11,12,14,15(n)-^{3}H] leukotriene B4 Specific Activity: 5.6 TBq/mmol, >150 Ci/mmol catalogue #TRK.692

The standard eicosanoids were obtained from Cayman Chemical Company (Ann Arbor, Michigan).

6KF Prost-13-en-1-oic acid,9,11,15-trihydroxy-6-oxo-(9α,11α,13E,15S)
Molecular Formula: $C_{20}H_{34}O_6$ MW=370.5
catalogue #15210

TxB_2 Thromboxa-5,13-dien-1-oic acid,9,11,15-trihydroxy-(5Z,9α,13E,15S)
Molecular Formula: $C_{20}H_{34}O_6$ MW=370.5
catalogue #19030

LTB_4 6,8,10,14-Eicosatetraenoic acid,5,12-dihydroxy-[S[R*S*-(E,Z,E,Z)]]
Molecular Formula: $C_{20}H_{34}O_4$ MW=336.5
catalogue #20110

The 6KF antibody was obtained from Cayman Chemical Company (Ann Arbor, Michigan).

Polyclonal sheep antiserum
Approximate titre for RIA (final dilution)=1:15 000
catalogue #115212

The TxB_2 antibody was a kind gift from Jean-Marie Moutquin (Laval University, Quebec City, Quebec).
Polyclonal sheep antiserum
Approximate titre for RIA (final dilution)=1:50 000

The LTB_4 antibody was a generous donation from Merck-Frosst Canada (Mississauga, ON).
Polyclonal sheep antiserum
Approximate titre for RIA (final dilution)=1:15 000

Solutions

Phosphate Buffered Saline with Gelatin (PBSG)

0.04 M	$NaH_2PO_4 \cdot H_2O$ (monobasic)(Sigma Chem. Co.-St. Louis, MO)
0.06 M	$Na_2HPO_4 \cdot 7\ H_2O$ (dibasic)(Sigma Chem. Co.-St. Louis, MO)
0.15 M	NaCl (BDH Inc.-Toronto, ON)
0.015 M	Na Azide (J.T. Baker Chemical Co.-Phillipsburg, NJ)
1.00 g	Gelatin (Fisher Scientific Co.-Fair Lawn, NJ)
1 litre	Distilled deionized H_2O

pH=7.1

(For PBS omit gelatin)

Charcoal Solution

100 ml	PBSG
100 ml	PBS (no gelatin)
1.0 g	Carbon decolourizing alkaline Norit-A (Fisher Scientific Co.-Fair Lawn, NJ)
0.1 g	Dextran T70 (Pharmacia-Uppsala Sweden)

Scintillation Fluid

CytoScint (ICN Biomedicals Inc.-Irvine, CA)

5.2.2. Assay Procedure

Antisera were diluted in PBSG to the appropriate working dilutions for RIA. Tritiated (^{3}H) eicosanoids were diluted in PBSG to give approximately 10 000 disintegrations per minute (DPM)/0.1 ml. A standard curve was constructed by serial dilutions at eicosanoid concentrations of 1000 pg, 500 pg, 250 pg, 125 pg, 62.5 pg, 31.25 pg, 15.6 pg, 7.8 pg and 3.9 pg/0.1 ml. Standards were diluted in the same buffer as the unknown samples (HBSS). Aliquots (0.1 ml) of the standard eicosanoids or appropriately diluted unknown samples were added to 12 x 75 mm glass test tubes in duplicate. ^{3}H-eicosanoids were added to the tubes in 0.1 ml aliquots followed by 0.1 ml of the antibody solution to give a total incubation volume of 0.3 ml. The mixture was incubated at 4°C for 16 to 20 hours. Unbound, labelled and unlabelled eicosanoid molecules were absorbed onto dextran-coated charcoal by adding 0.5 ml of charcoal solution and incubating for 10 minutes at 4°C. The mixture was centrifuged at 2500 revolutions/minute (rpm) for 10 minutes at 4°C and the supernatant, containing the antibody-antigen complexes was decanted into vials containing 4 ml of scintillation fluid. The level of radioactivity was determined in counts per minute (CPM) and disintegrations per minute (DPM) by a liquid scintillation system (LS 5000TD, Beckman Instruments, Fullerton, CA).

Total (T) and non-specific binding (NSB) tubes containing 0.1 ml of sample buffer (HBSS), 0.1 ml of PBSG and 0.1 ml of

radiolabelled eicosanoid and B_o tubes containing 0.1 ml of sample buffer, 0.1 ml ^{3}H-eicosanoid and 0.1 ml of antibody were included in duplicate. After 16 to 20 hours of incubation at 4°C, 0.5 ml of a mixture of 1 part PBSG to 1 part PBS was added to the T tubes and 0.5 ml charcoal solution was added to NSB and B_o tubes and processed as above. T tubes determine the total amount of radioactivity actually contained within test tubes after processing through the entire assay procedure. NSB tubes assess the background binding of the radiolabelled eicosanoid to substances other than the antibody. B_o tubes determine the maximum amount of radiolabelled eicosanoid that is able to bind to the antibody at equilibrium. The standard curve is constructed by plotting the percentage bound (B) of maximum bound (B_o) on a linear scale versus the eicosanoid concentration on a log scale. Percent B/B_o is calculated from the DPM values as follows in Equation 5.I.:

Equation 5.I.

$$\frac{\text{Bound - NSB}}{\text{Maximum Bound - NSB}} \times 100 = \%B/Bo$$

5.3. CHARACTERIZATION OF THE ANTISERA

The antisera for 6KF, TxB_2 and LTB_4 were newly obtained batches of sera. Although approximate titres were supplied by the sources, it was necessary to determine the exact working titre to be used in our RIA procedure because every batch of serum possesses individual characteristics. The specificity of the antisera for the parent eicosanoid must be assessed since other structurally related eicosanoids may potentially cross-react by binding to the antibody.

5.3.1. Determination of Antibody Titre

In order to determine the working concentration of the antisera to be used in the RIA procedure, serial dilutions of the plasma containing the antibody were made where the mid-range included the dilution suggested by the source of the antisera. The 6KF and LTB_4 antisera were serially diluted and tested at concentrations of 1 part serum to 500 parts PBSG (1:500), 1:1000, 1:5000, 1:10 000, 1:15 000, 1:20 000, 35 000, 50 000 and 1:100 000. The TxB_2 antiserum was evaluated at dilutions in the range of 1:500, 1:1000, 1:5000, 1:10 000, 25 000, 1:50 000, 60 000, 1:75 000 and 1:100 000. T and NSB tubes were included in replicates of 6. B_0 tubes were made using each of the antibody dilutions indicated

above, in replicates of 6 each. The tubes were incubated for 16 to 20 hours at 4°C. One part PBSG to one part PBS (0.5 ml) was added to T tubes. Charcoal solution was added (0.5 ml) to NSB and B_o tubes and processed as above (see 5.2.2.). From the radioactivity in DPM, the amount of radiolabelled eicosanoid bound to the antibody, in the absence of any unlabelled eicosanoid (B_o) was expressed as a percentage of the total amount of radiolabel added (Equation 5.II.).

Equation 5.II.

$$\frac{Bo - NSB}{T - NSB} \times 100 = \% B_o/T$$

The % B_o/T values were plotted against the dilutions of the antibody used. By interpolation from this curve, the dilutions of the antibody were determined for values that gave 30%, 40%, 50% and 70% B_o/T. Then, 3 replicate standard urves were run at each of these dilutions in order to examine the characteristics of the standard curves produced. The entire antiserum dilution tests were run 3 times for each eicosanoid.

The 6KF, TxB_2 and LTB_4 antibody dilution curves are shown in Figures 5.1.a., 5.2.a., and 5.3.a., respectively. For the 6KF antiserum, antibody dilutions of 1:3000, 11 500, 16 000 and 21 500 were found for 70%, 50%, 40% and 30% B_o/T, respectively. The standard curve produced using an antiserum dilution of 1:21 500 (i.e., 30% B_o/T) had the greatest slope in the linear portion of the curve and a narrow

concentration range from 9 pg/0.1 ml to 65 pg/0.1 ml (Figure 5.1.b.). This curve was shifted to the left indicating that its useful range was limited to relatively lower eicosanoid concentrations. At antiserum dilutions for 50% and 70% B_o/T, the standard curves were flat at the lower concentration range, between 3.9 pg/0.1 ml to 31.25 pg/0.1 ml and therefore had decreased sensitivity. These curves were right-shifted with a usable concentration range from 61.5 pg/0.1 ml to 1000 pg/0.1 ml (Figure 5.1.b.). The 40% B_o/T standard curves were linear for the greatest range of the standard concentrations, from 10 pg/0.1 ml to 800 pg/0.1 ml and showed sensitivity (see 5.4.3.) at a concentration lower than 3.9 pg/0.1 ml (Figure 5.1.b.).

For the TxB_2 antibody, dilutions of 1:5000, 31 500, 52 000 and 78 000 were determined by interpolation for 70%, 50%, 40% and 30% B_o/T, respectively. Similar findings resulted from the standard curves constructed at these 4 antibody dilutions as for the 6KF antibody. Again, the antibody dilution for 40% B_o/T gave the best standard curve although the range was less than that for 6KF. The curve was linear in the range of 10 pg/0.1 ml to 600 pg/0.1 ml and the sensitivity was less than 3.9 pg/0.1 ml (Figure 5.2.b.).

The LTB_4 antibody dilutions of 1:700, 7500, 15 000 and 20 000 were found for 70%, 50%, 40% and 30% B_o/T, respectively. The standard curves generated by the different antibody dilutions demonstrated similar characteristics as with the 6KF antiserum. The optimal standard curve was generated by the 40% B_o/T antibody

dilution where the range was 7.8 pg/0.1 ml to 800 pg/0.1 ml and the sensitivity was less than 3.9 pg/0.1 ml (Figure 5.3.b.).

For effective competition, the number of antibody binding sites must be less than the amounts of labelled and unlabelled ligand present. In order to achieve this, the antibody is diluted so that 40% to 70% of the labelled ligand binds in the absence of unlabelled ligand (Pegg, 1976). If the antibody is too concentrated, as at 50% and 70% B_o/T, there is a loss of the sensitivity of the assay as evidenced by the flat portion of the curves at low concentrations. This is due to the increased number of antibody binding sites present. Therefore the ^{3}H-eicosanoid that is displaced by small amounts of standard eicosanoid would be masked by the larger pool of ^{3}H-eicosanoid-antibody complexes. If the antibody is too dilute, as at 30% B_o/T, there is a loss in the range of concentrations of standard that can be measured as evidenced by the steeper slope of the curve. This results from too few antibody molecules present. Therefore, excess amounts of unlabelled eicosanoid will have already displaced the maximum amount of ^{3}H-eicosanoid and saturated the antibody binding sites. Any further increases in the amount of unlabelled eicosanoids will not be detected since there are no binding sites available.

The antibody dilutions that gave 40% B_o/T produced standard curves optimized for both sensitivity and range. Therefore, for the subsequent RIA procedures, the antibody dilutions used for 6KF, TxB_2 and LTB_4 were 1:16 000, 1:52 000 and 1:15 000, respectively.

These values agreed with the quotations by the suppliers of the antisera (see 5.2.1.).

5.3.2. Cross-reactivity of the Antisera

A competitive binding assay such as RIA depends on the specificity of the reaction between the antibody binding site and the ligand. Antisera obtained at different times will show variation in the degree of specificity. Therefore, this must be assessed with each new batch of antiserum. Cross-reaction may occur when molecules other than the specific ligand react with the binding site on the antibody of the parent eicosanoid. Eicosanoids are a family of related compounds derived from the same substrate, arachidonic acid, and therefore are structurally similar. It is necessary to assess the ability of the antibody molecules for 6KF, TxB_2 and LTB_4 to distinguish between the different eicosanoids.

The specificity of the antisera is assessed by measuring the ability of related eicosanoids and A.A. to displace the radiolabelled parent eicosanoid that is bound to the antibody. The antisera tested were 6KF, TxB_2 and LTB_4. The related eicosanoids tested were 6KF, TxB_2, LTB_4, LTC_4, LTD_4, LTE_4, PGE_2 and $PGF_{2\alpha}$. Triplicate 0.1 ml aliquots of each related eicosanoid in concentrations of 100 ng, 50 ng, 10 ng, 5 ng, 1 ng, 0.5 ng and 0.1 ng/0.1 ml were incubated with 0.1 ml of the parent eicosanoid's antibody and 0.1 ml of radiolabelled

parent eicosanoid. A.A. was tested at concentrations of 3045 ng (100 μM), 1522.5 ng, 304.5 ng, 152.25 ng, 30.45 ng, 15.22 ng, 3.04 ng and 1.52 ng/0.1 ml. A standard curve using the parent eicosanoid was run simultaneously. The RIA procedure was followed as above (5.2.2.). The $\%B/B_o$ was plotted on a linear scale against the mass of eicosanoid added (plotted on a log scale) for 6KF, TxB_2 and LTB_4 (Figure 5.4., 5.5., and 5.6., respectively). The mass of each tested eicosanoid that displaced 50% of the bound radiolabelled parent eicosanoid was determined by interpolation and used to calculate the % cross-reactivity (Equation 5.III.).

Equation 5.III.

$$\frac{\text{mass of parent eicosanoid at 50\% Bo}}{\text{mass of test eicosanoid at 50\% Bo}} \times 100 = \%\ \text{cross-reactivity}$$

The percent cross-reactivities for 6KF, TxB_2 and LTB_4 are shown in Table 5.A.. For the 6KF antibody, two other compounds containing the prostanoid structure, TxB_2 and PGE_2 demonstrated low cross-reactions of 0.16% and 0.13%, respectively. The leukotrienes and A.A. exhibited insignificant cross-reactivities of 0.04%. PGE_2 showed a slight cross-reaction of 4.2% with the TxB_2 antibody. The cross-reaction of PGE_2 is probably not a major problem in the lung explant system since quantities of PGE_2 were measured and found to be present only in very low levels. In turn, a

mass of PGE_2 would need to be 22 times greater than TxB_2 to cause 50% displacement of ^3H-TxB_2. The other two PGHS products, 6KF and $PGF_{2\alpha}$, exhibited minor cross-reactions with the TxB_2 antibody of 0.21% and 0.80%, respectively. The 5-lipoxygenase products and A.A. demonstrated insignificant cross-reactions. The LTB4 antibody cross-reacted with the three other LTs on average by 0.15%. Any cross-reaction would be attributed to the shared conjugated triene structure. The other compounds tested showed insignificant cross-reactivities of 0.05%.

The eicosanoids that were tested for cross-reactivity were chosen because they have been shown to be present to some extent within the lung (Zijlstra *et al.*, 1987a). A.A. was tested since it was added to the *in vitro* explant system that was used and it was necessary to ensure that the levels used would not interfere with the RIA determinations. The curves for the tested compounds that were parallel to the standard curve indicated that reaction was occurring at the specific binding site. This did not pose a problem because the amounts of the cross-reacting substances needed to displace 50% of the radiolabelled eicosanoid were several orders of magnitude greater than for the parent eicosanoid. Also, these cross-reacting eicosanoids are present in the lung at lower concentrations than 6KF, TxB_2 and LTB4 (Zijlstra *et al.*, 1987a). It was concluded that the antisera for 6KF, TxB_2 and LTB4 were specific and therefore ensured confidence that the measurements truly reflect the specific eicosanoid levels.

For eicosanoid measurements made from the explant system, media samples were quantitated by direct RIA. Due to the high specificity of the antisera, prior separation or purification of the individual eicosanoids was unnecessary. Extraction procedures to separate the eicosanoids from proteins was apt to be an unnecessary step because eicosanoids are released directly into a serum free medium in the explant system. To confirm this assumption, media samples from explants were divided in half. Then, one half were subjected to SEP-PAK extraction prior to RIA (Taniguchi *et al.*, 1986) and the other half were assessed by direct RIA. There were no differences in the values obtained. Therefore, there were no interfering substances in the samples that would give falsely high values and the values were not inaccurately low due to losses caused by binding to proteins.

5.4. CHARACTERIZATION OF RIA PROCEDURE

The characterization of the RIA procedure involved assessment of the parameters which affirm the confidence in values that were obtained. The precision, accuracy and sensitivity of the technique were determined. To accomplish this, 3 replicate standard curves were run for 6KF, TxB_2 and LTB4. Each trial included at least 4 B_o tubes and 6 replicate high pools (HP) at a concentration of 250 pg/0.1 ml and 6 low pools (LP) at 25 pg/0.1 ml.

5.4.1. Precision of RIA

The precision of the assay must be assessed and optimized in order to ensure reproducibility of the measurements made. Precision is defined as the closeness of repeated measurements of the same quantity (Sokal and Rohlf, 1981). The variability within the procedure of the technique and the variability between assays performed at different times must be examined in order to have knowledge of the inherent variation that was present.

Intra-assay Coefficient of Variation

The intra-assay coefficient of variation (%intra-assay C.V.) was calculated to assess the variability within a given assay. This value was calculated for both the HP and LP to determine the variability in different regions of the range of the standard curve (Equation 5.IV.).

Equation 5.IV.

$$\frac{\text{SD of HP or LP replicates}}{\text{mean of HP or LP replicates}} \times 100 = \%\text{intra-assay C.V.}$$

The values obtained for the 3 replicate trials were averaged together (Table 5.B.). The intra-assay variation was greater in the low range of the standard curve than in the higher range for 6KF and LTB_4. Therefore, there would be a greater degree of confidence in values that were interpolated from the 250 pg/0.1 ml region of the curve. For TxB_2, the variation within the assay was constant throughout the range of the standard curve. The possible sources of variation that were reflected in the %intra-assay C.V. may be caused by the several pipetting steps, the decanting step following the charcoal separation, the liquid scintillation counting, or any differences that occurred between the individual test tubes in a group of replicates. The intra-assay variation for these RIAs was in agreement with other laboratories (Salmon *et al.*, 1982). Coefficients

of variation of 10% are acceptable and indicated satisfactory reproducibility (Pegg, 1976).

Inter-assay Coefficient of Variation

The %inter-assay C.V. was calculated to determine the variability between assays when performed at different times. This value was determined for the HP and LP to assess the contribution of the sources of this variation at different concentrations of the curve (Equation 5.V.).

Equation 5.V.

$$\frac{\text{SD of 3 replicate standard curve averages of HP or LP}}{\text{mean of 3 replicate standard curves averages of HP or LP}} \times 100 = \%\text{inter-assay C.V.}$$

The inter-assay coefficients of variation are presented in Table 5.B.. For 6KF, the variation between assays was the same along the range of the standard curve. For TxB_2 and LTB_4, the inter-assay variation was greater at the low end of the standard curve. This meant that from assay to assay, there was more confidence in determining values that fell in the higher concentration range of the curve. In general, the 10% variation found was acceptable and in

agreement with values found by others utilizing the same assays (Salmon *et al.*, 1982). This indicated that the measurements made from assays performed at different times can be related to one another with confidence. The sources of variation between assays reflect differences that occur when the assays were performed at different times, by different people, differences in the solutions being used, changes occurring in the standards or antibody with storage over time. The %intra-assay C.V. values were found to be lower than the %inter-assay C.V. values which is the usual finding (Pegg, 1976). This means that differences that occur when assays are performed at different times contributes more to variability than differences from test tube to test tube within a given assay.

5.4.2. Accuracy of RIA

Accuracy is defined as the closeness of a measured or computed value to its true value (Sokal and Rohlf, 1981). It is difficult to actually assess this and there is no established method that is universally accepted. Dose interpolation from the standard curve of known amounts of standard should show a correlation to the calculated amount added, and is indicative of accuracy.

To investigate the accuracy to the RIAs, 3 replicate standard curves were run for 6KF, TxB_2 and LTB_4. Simultaneously, triplicates of standards at calculated concentrations of 25 pg, 50 pg, 100 pg, 200

pg and 400 pg/0.1 ml were subjected to RIA and quantitated by dose interpolation from the standard curve.

Table 5.C. lists the calculated concentration of the known standard that was added and the value that was determined by dose interpolation following RIA. There was no significant difference between the calculated and measured values as assessed using Student's *t* -test at $p<0.05$. Therefore, the levels measured by RIA were highly reflective of the calculated amounts of eicosanoid added, indicating the accuracy of this technique.

5.4.3. Sensitivity

The sensitivity of the assay is represented by the lower limit of detectability. This is defined as being the dose of eicosanoid which is significantly different from zero (Pegg, 1976). To find the smallest detectable amount of eicosanoid, it is necessary to determine the variability around the zero standard, i.e., B_o. The sensitivity is given by Equation 5.VI..

Equation 5.VI.

$$\text{mean of Bo replicates} \pm (\text{SD} \times 0.05\ {}^{t}n) = \text{sensitivity}$$

The term $0.05^{t}n$ (Student's *t* for n degrees of freedom with $p<0.05$) approaches a value of 2 with a sufficiently large number of

determinations of B_o (Pegg, 1976). Therefore, it is usually acceptable to assess the limit of sensitivity as the concentration achieved at more than 2 SD units of difference from the B_o value of a number of B_o replicates (Pegg, 1976). This value was determined by first calculating the mean and SD of the DPM values for the B_o replicates. Then, using Equation 5.VII., the value of the DPM of the lower limit was obtained. The $\%B/B_o$ was calculated by Equation 5.I. and the assay sensitivities were obtained by interpolating from the standard curves for 6KF, TxB_2 and LTB_4 (Figure 5.7., 5.8. and 5.9., respectively).

Equation 5.VII.

mean of Bo replicates - 2 x SD = DPM of lower limit

The lower limit was determined for each of the 3 replicate standard curves and averaged together. The RIAs for 6KF, TxB_2 and LTB_4 were very sensitive since the lower limits of detectability were found to be lower than the lowest concentration, i.e. 3.9 pg/0.1 ml that was used in the standard curves. This was in agreement with the findings of others who found lower limits of detectability for 6KF of 0.1 pg/0.1 ml, TxB_2 of 0.05 pg/0.1 ml and LTB_4 of 0.125 pg/0.1 ml (Hageman *et al.*, 1986). It was of great importance to know the smallest concentrations of eicosanoid that could be quantitated because it was suspected that the levels that were to be measured in

the explant system may be low. Since the assays are sensitive, they would be useful in determining very low levels of eicosanoids and perhaps may make it unnecessary to concentrate the samples.

5.5. SUMMARY OF RIA

Radioimmunoassay was chosen to quantitate eicosanoids due to the specificity and sensitivity of the technique. Specificity is beneficial because although eicosanoids are related in structure, they have very different biological effects. Therefore, it was advantageous to use a technique that was capable of reliably distinguishing between the different members of this family of compounds. The sensitivity of the RIA technique was important since the levels of these mediators may be present in very small quantities in the system that it was to be employed. With the specificity, precision, accuracy and sensitivity of the RIA procedure well-characterized for 6KF, TxB_2 and LTB_4, this was a useful and reliable tool to be used in the experiments to follow.

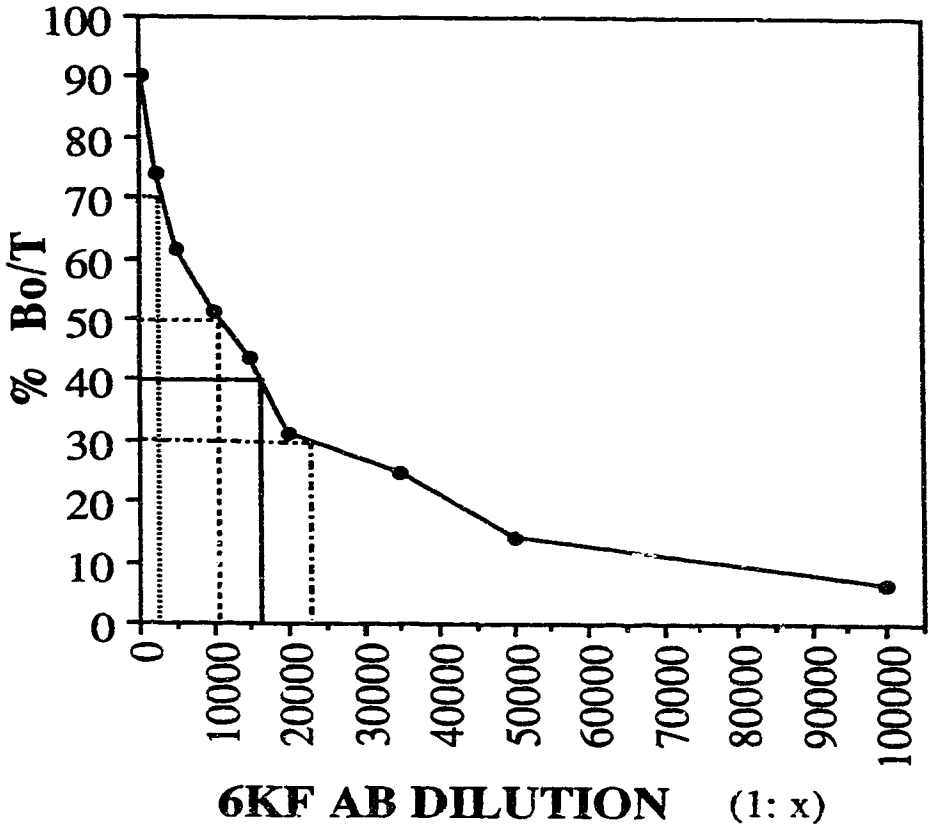


FIGURE 5.1.a. ANTISERUM DILUTION CURVE FOR 6-KETO-PROSTAGLANDIN $F_{1\alpha}$ (6KF).

Serial dilutions from 1:500 to 1:100 000 (v/v) of antiserum were incubated with ^{3}H-6KF (120-180 Ci/mmol). Percentages of total counts added bound by the antisera (see Equation 5.II.) were plotted against the antiserum dilution tested. Optimal antiserum dilution to be used in the final RIA procedure was determined to be 1:16 000 (v/v) by interpolation at 40% binding of total counts. Values represent MEAN ± SD of 6 replicates. This is a representative graph from one experiment (n=3 experiments).

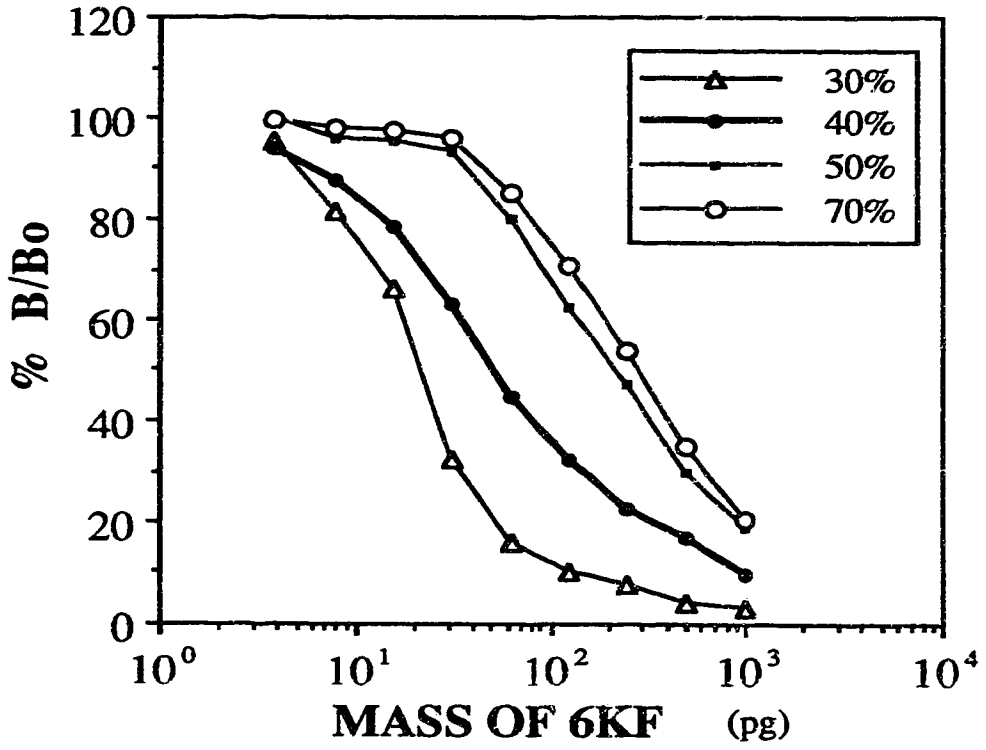


FIGURE 5.1.b. STANDARD CURVES FOR 6KF AT VARIOUS ANTISERUM DILUTIONS.
Standard curves in a concentration range from 3.9 pg to 1000 pg/0.1 ml were run using 6KF antiserum dilutions that gave 30%, 40%, 50% and 70% B_O/T as indicated by dose interpolation from Figure 5.1.a.. The 30% B_O/T curve was left shifted and had a narrow linear range. The 40% B_O/T curve optimized both range and sensitivity. The 50% and 70% B_O/T curves were less sensitive at low 6KF concentrations. This is a representative graph of 3 replicate standard curve trials.

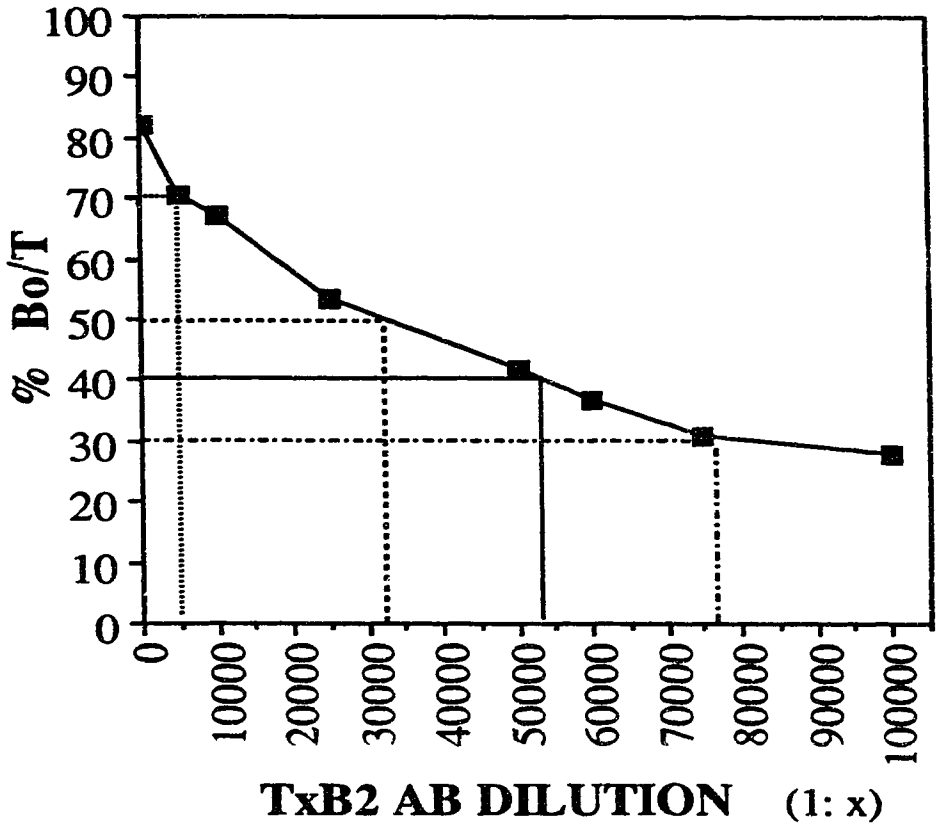


FIGURE 5.2.a. ANTISERUM DILUTION CURVE FOR THROMBOXANE B_2 (TxB_2).
Procedure was same as described in Figure 5.1.a.. Tracer used was ^{3}H-TxB_2 (>120 Ci/mmol). The optimal antiserum dilution for the final RIA procedure was determined to be 1:52 000 (v/v) by interpolation at 40% binding of total counts. Values represent MEAN ± SD of 6 replicates for one representative experiment (n=3 experiments).

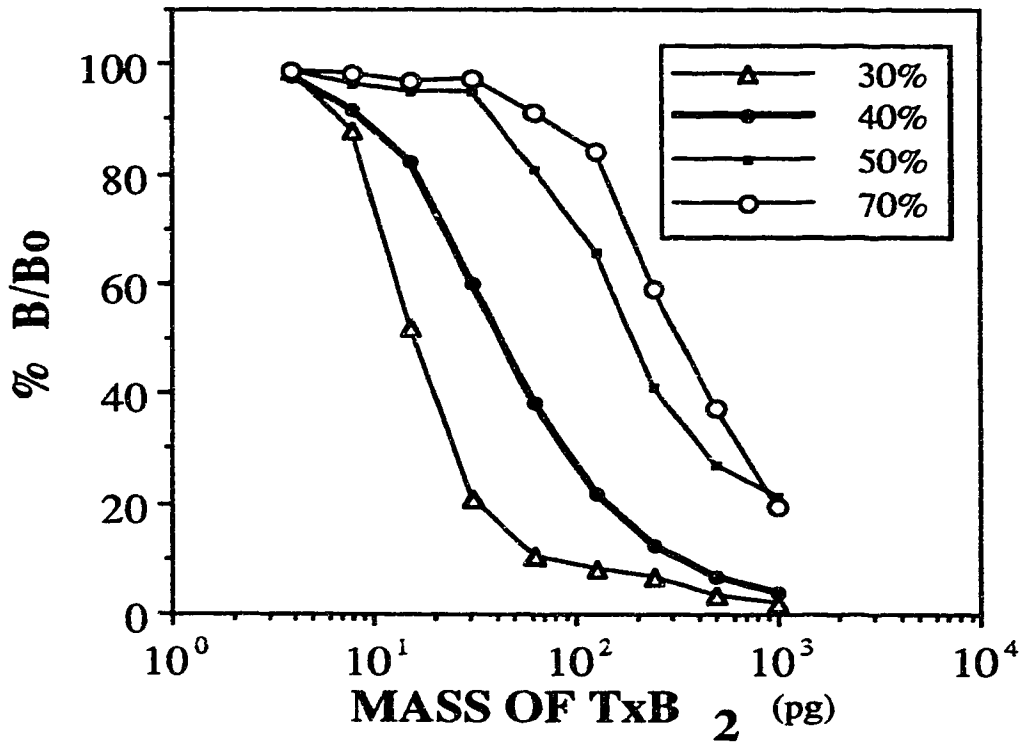


FIGURE 5.2.b. STANDARD CURVES FOR TxB_2 AT VARIOUS ANTISERUM DILUTIONS.
Standard curves in a concentration range from 3.9 pg to 1000 pg/0.1 ml were run using TxB_2 antiserum dilutions that gave 30%, 40%, 50% and 70% B_o/T as indicated by dose interpolation from Figure 5.2.a.. The 30% B_o/T curve was left shifted and had a narrow working range. The 40% B_o/T curve optimized both range and sensitivity. The 50% and 70% B_o/T curves were less sensitive at low TxB_2 concentrations and were right shifted. This is a representative graph of 3 replicate standard curve trials.

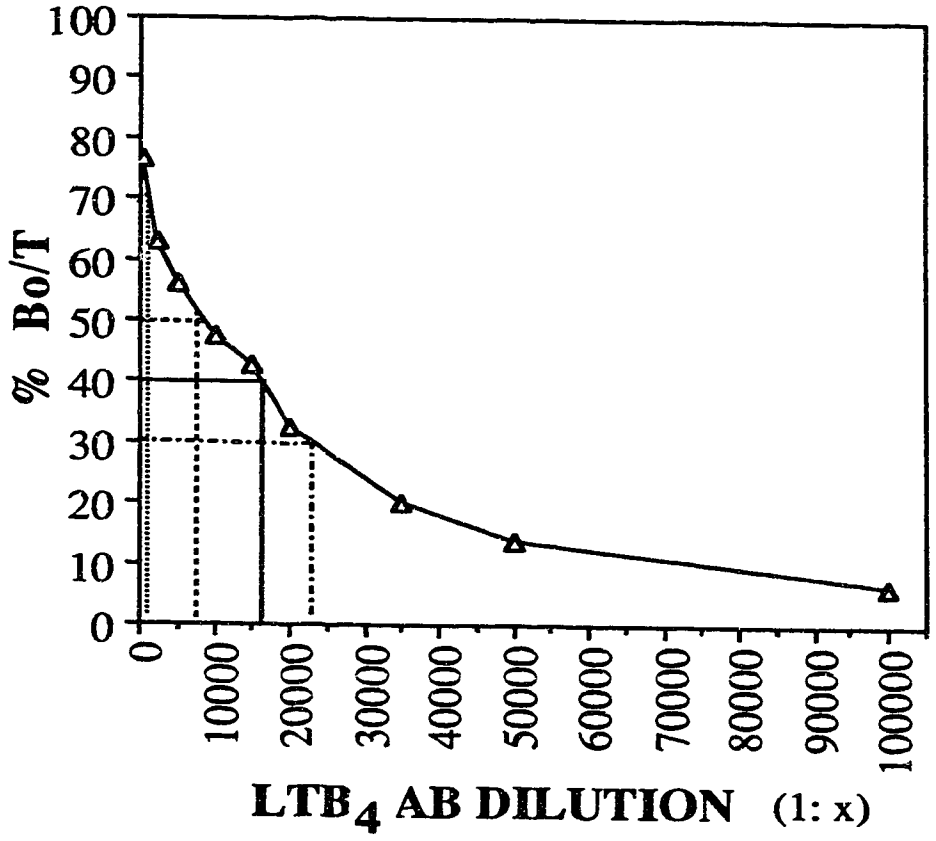


FIGURE 5.3.a. ANTISERUM DILUTION CURVE FOR LEUKOTRIENE B4 (LTB_4).
Procedure was same as described in Figure 5.1.a.. The radiolabelled eicosanoid used was ^3H-LTB_4 (>150 Ci/mmol). The optimal antiserum dilution for the final RIA procedure was determined to be 1:15 000 (v/v) by interpolation at 40% binding of total counts. Values represent MEAN ± SD of 6 replicates for one representative experiment (n=3 experiments).

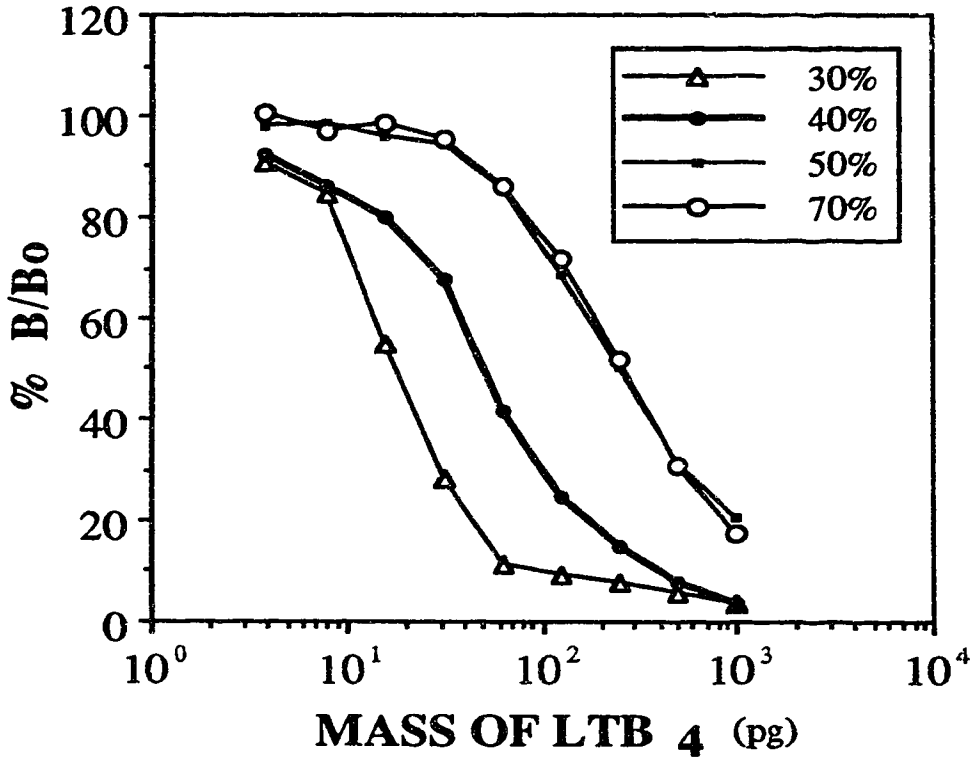


FIGURE 5.3.b. STANDARD CURVES FOR LTB4 AT VARIOUS ANTISERUM DILUTIONS.
Standard curves in a concentration range from 3.9 pg to 1000 pg/0.1 ml were run using LTB4 antiserum dilutions that gave 30%, 40%, 50% and 70% B_o/T as indicated by dose interpolation from Figure 5.3.a.. The 30% B_o/T curve was left shifted and had a compressed working range. The 40% B_o/T curve optimized both range and sensitivity. The 50% and 70% B_o/T curves were less sensitive at low LTB4 concentrations and were right shifted. This is a representative graph of 3 replicate standard curve trials.

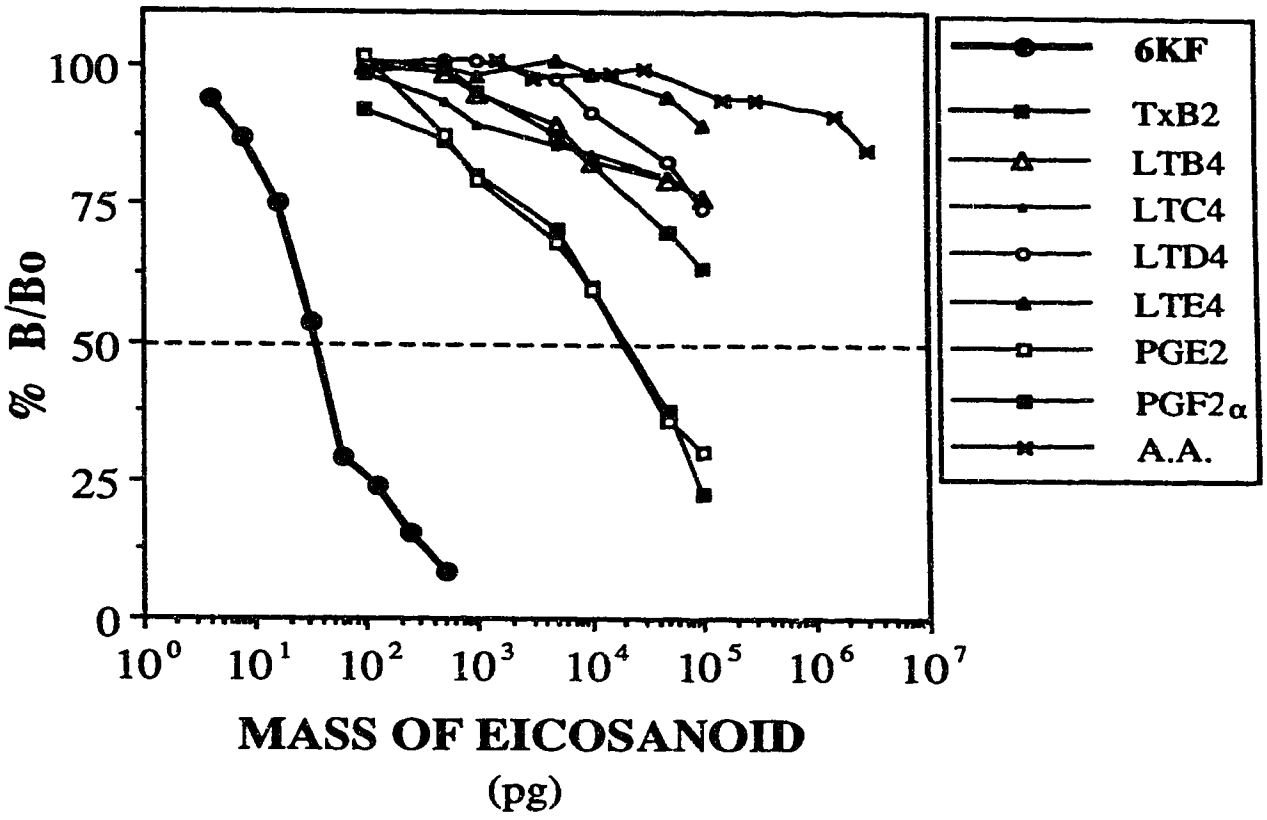


FIGURE 5.4. SPECIFICITY OF 6KF ANTISERUM.
Increasing concentrations of related compounds (TxB_2, LTB_4, LTC_4, LTD_4, LTE_4, PGE_2, $PGF_{2\alpha}$, from 0.1 ng to 100 ng/ 0.1 ml, A.A. from 1.52 to 3045 ng/0.1 ml) were incubated with ^{3}H-6KF (120-180 Ci/mmol) and 6KF antiserum (1:16 000 v/v). The percentages of ^{3}H-6KF displaced from the antigen binding site by related compounds (see Equation 5.I.) were plotted against the mass of the added compound. The mass of the cross-reacting substance that produced 50% displacement of ^{3}H-6KF was determined by interpolation and used for calculation of the percent cross-reactivity (see Equation 5.III.). See Table 5.A. for a summary of cross-reactivities. Values represent MEAN ± SD of triplicates. This is a representative graph from one experiment (n=3 experiments).

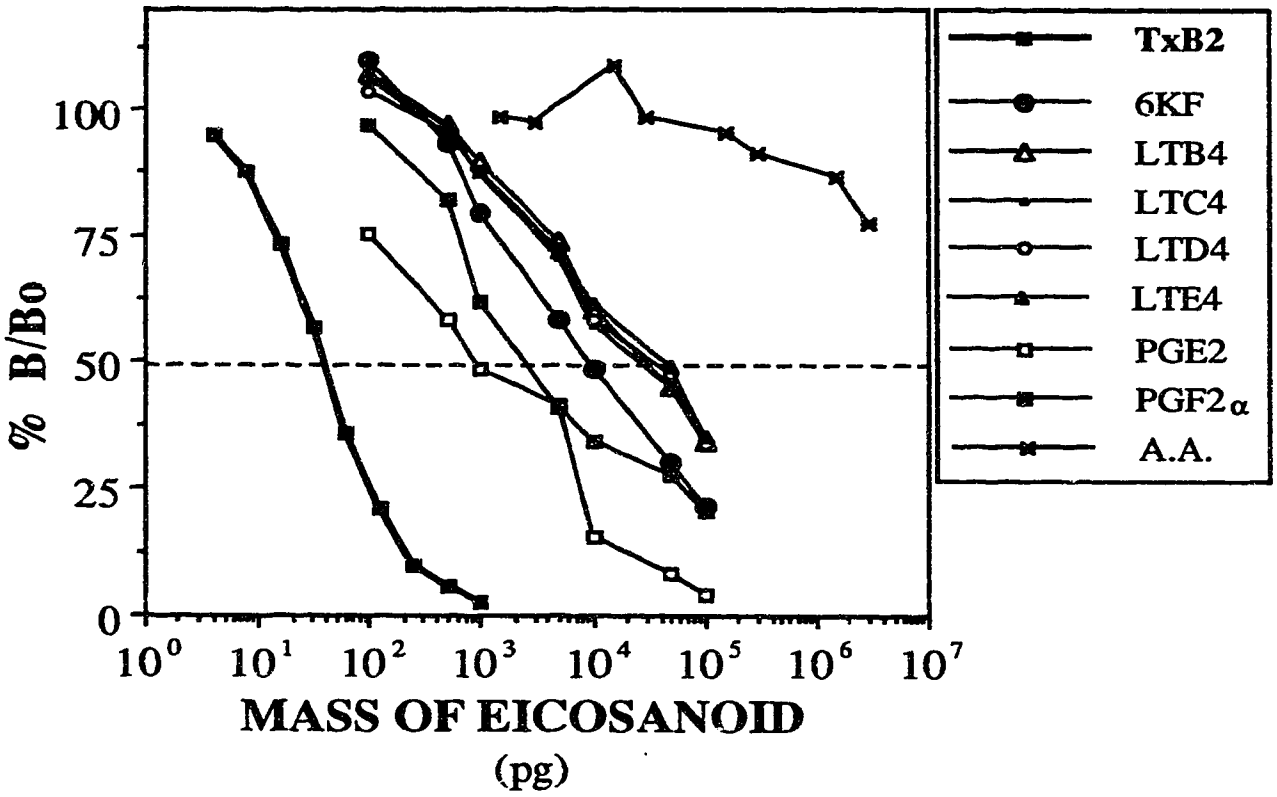


FIGURE 5.5. SPECIFICITY OF TxB_2 ANTISERUM.
The procedure used was as described in Figure 5.4.. The radiolabelled eicosanoid used was ^3H-TxB_2 (>120 Ci/mmol) and the TxB_2 antiserum was diluted to a working titre of 1:52 000 (v/v). See Table 5.A. for summary of cross-reactivities. Values represent MEAN ± SD of triplicates for one representative experiment (n=3 experiments).

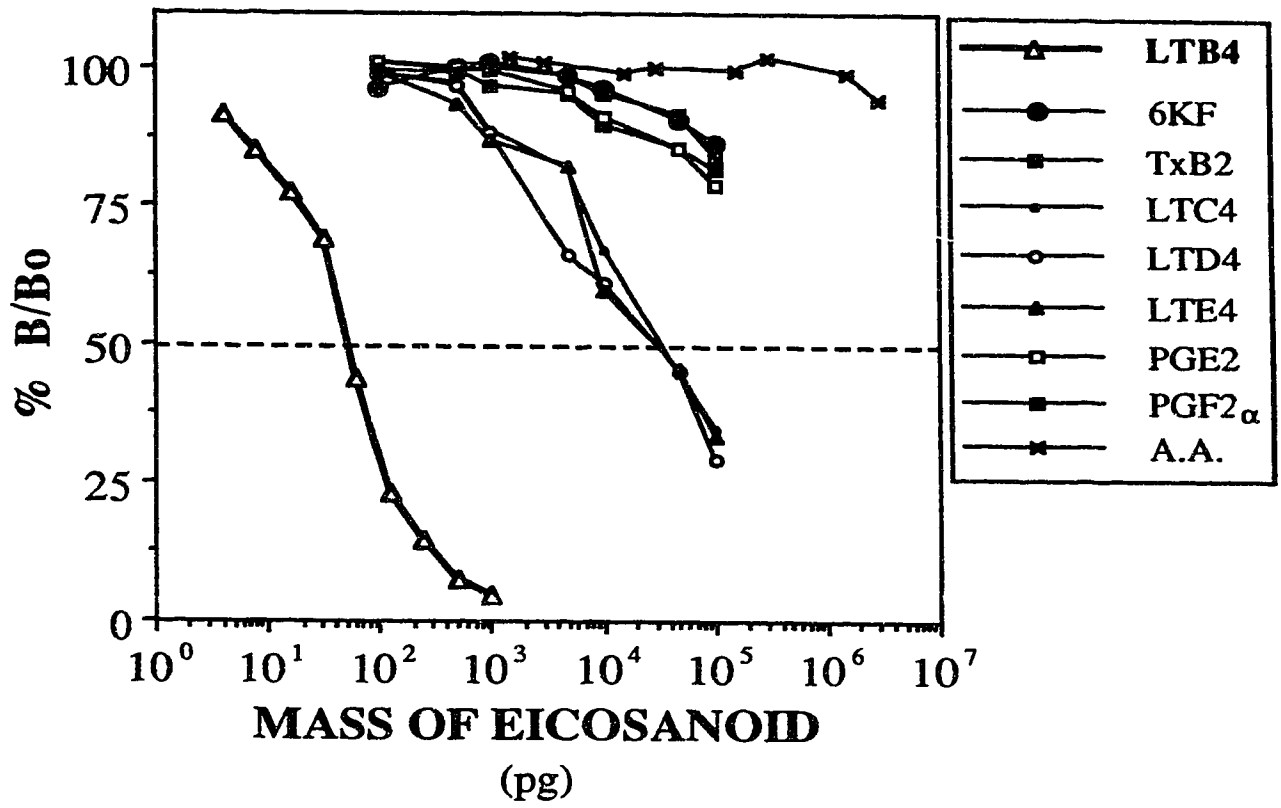


FIGURE 5.6. SPECIFICITY OF LTB4 ANTISERUM.
The procedure used was as described in Figure 5.4.. The radiolabelled eicosanoid used was ^{3}H-LTB4 (>150 Ci/mmol) and LTB4 antiserum was diluted to a working titre of 1:15 000 (v/v). See Table 5.A. for summary of cross-reactivities. Values represent MEAN ± SD of triplicates for one representative experiment (n=3 experiments).

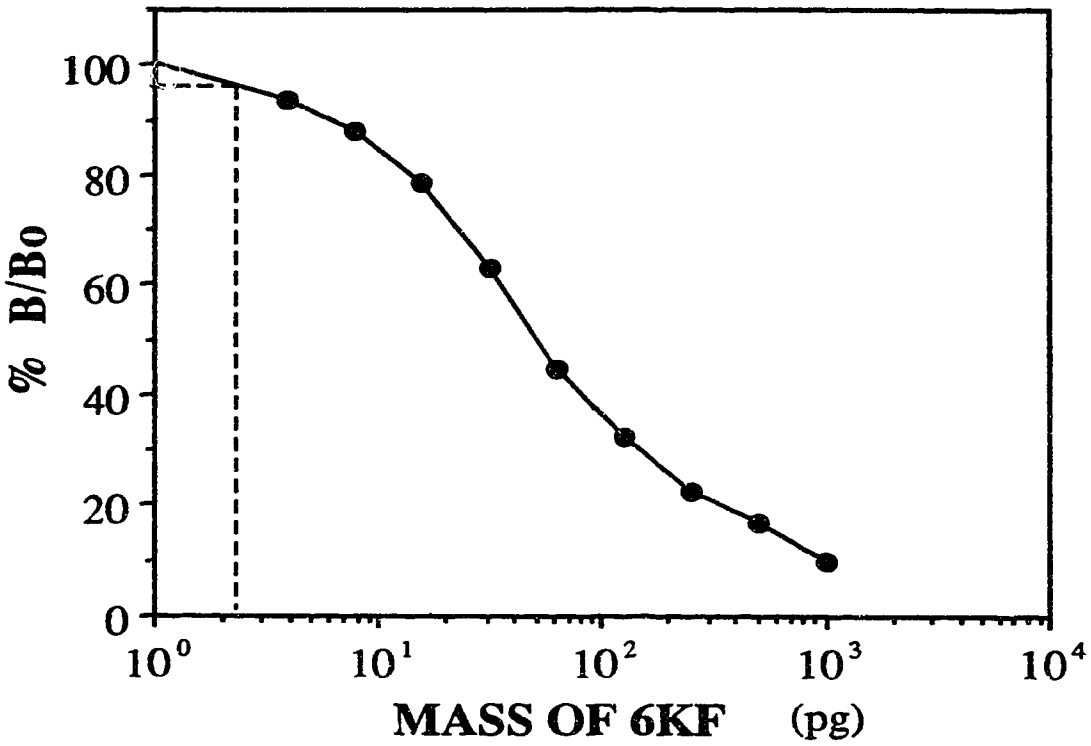


FIGURE 5.7. STANDARD CURVE FOR 6KF AND ASSAY SENSITIVITY DETERMINATION.

^{3}H-6KF (120-180 Ci/mmol) and 6KF antiserum (1:16 000 v/v) were incubated with serial dilutions of unlabelled standards of 6KF (3.9 pg to 1000 pg/0.1 ml). Four replicates of B_o tubes were included for assessment of the assay sensitivity. The lower limit of detection was determined by interpolation as the concentration of standard eicosanoid that was 2 standard deviation units from B_o as calculated by Equation 5.VII. (see text). The lower limit of detection was less than 3.9 pg/0.1 ml. Values represent MEAN ± SD of replicates for one experiment (n=3 experiments).

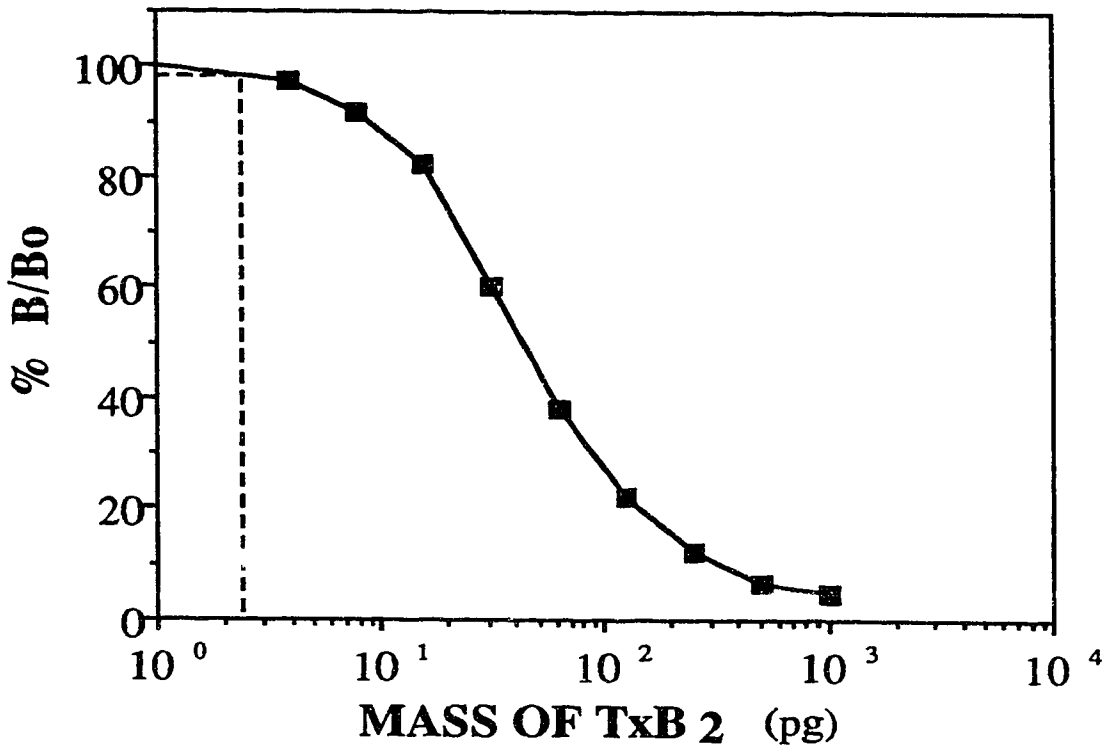


FIGURE 5.8. STANDARD CURVE FOR TxB_2 AND ASSAY SENSITIVITY DETERMINATION.

^3H-TxB_2 (>120 Ci/mmol) and TxB_2 antiserum (1:52 000 v/v) were incubated as described in Figure 5.7.. The lower limit of detection was less than 3.9 pg/0.1 ml. Values represent MEAN ± SD of replicates for one experiment (n=3 experiments).

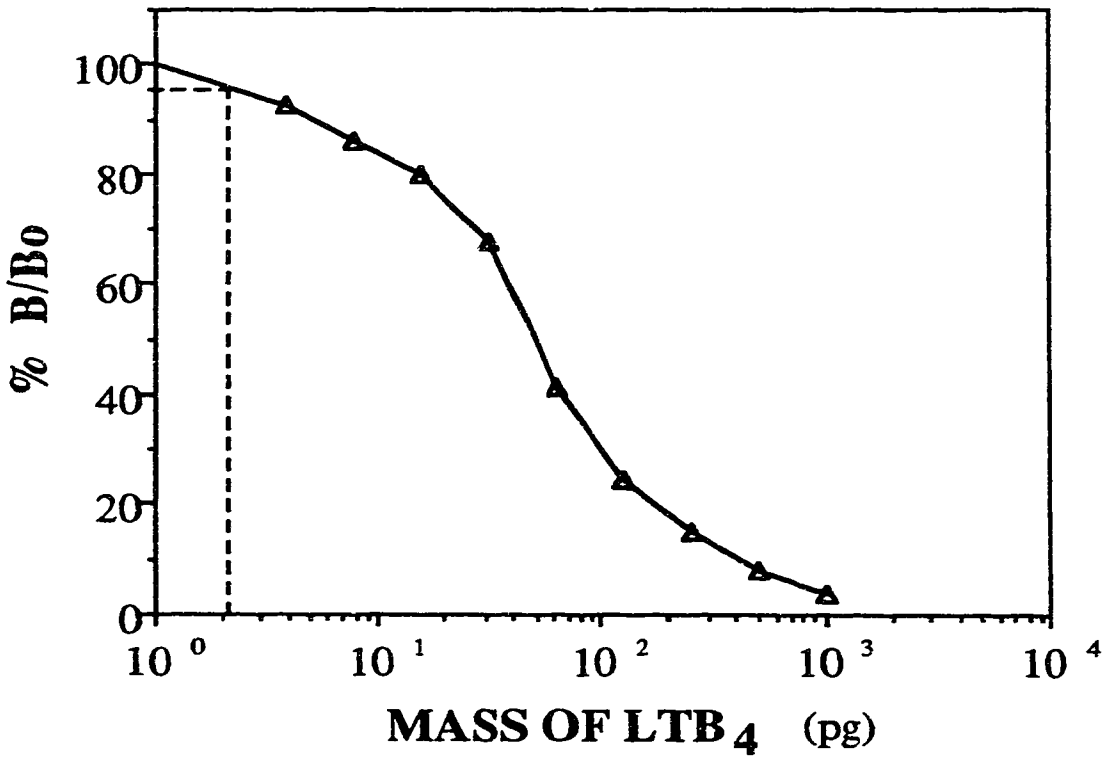


FIGURE 5.9. STANDARD CURVE FOR LTB4 AND ASSAY SENSITIVITY DETERMINATION.
^{3}H-LTB4 (>150 Ci/mmol) and LTB4 antiserum (1:15 000 v/v) were incubated as described in Figure 5.7.. The lower limit of detection was less than 3.9 pg/0.1 ml. Values represent MEAN ± SD of replicates for one experiment (n=3 experiments).

TABLE 5.A.

SUMMARY OF THE PERCENTAGE CROSS-REACTIVITY OF RELATED COMPOUNDS WITH 6KF, TxB_2 and LTB_4 ANTISERA.

The cross-reactivity was determined by the mass of the related compound that displaced 50% of the bound radioactivity. This mass was expressed as a percentage of the mass of the parent eicosanoid that is required to displace 50% of the bound radioactivity (see Equation 5.III. in text). All 3 antisera are specific for their parent eicosanoid since there was negligible cross-reaction of any of the compounds tested. Values represent the MEAN ± SD of triplicates of 3 separate experiments.

ANTIBODY	CROSS-REACTIVITY OF TEST COMPOUNDS (%)								
	6KF	TxB_2	LTB_4	PGE_2	$PGF_{2\alpha}$	LTC_4	LTD_4	LTE_4	A.A.
6KF	100	0.16	0.04	0.13	0.04	0.04	0.04	0.04	0.04
TxB_2	0.21	100	0.12	4.2	0.80	0.12	0.15	0.13	0.04
LTB_4	0.05	0.05	100	0.05	0.05	0.13	0.16	0.14	0.05

TABLE 5.B.

SUMMARY OF INTRA-ASSAY AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR 6KF, TxB_2 AND LTB_4 RADIOIMMUNOASSAY PROCEDURES.

Standard curves were run for each eicosanoid including 6 high pools (HP) at a concentration of 250 pg/0.1 ml and 6 low pools (LP) at 25 pg/0.1 ml. The intra-assay coefficient of variation (% intra-assay C.V.) or the variability within a given assay was determined by Equation 5.IV. (see text). The % inter-assay C.V. or variability between assays performed at different times was calculated by Equation 5.V. (see text). % intra- and %inter-assay C.V.'s were acceptible for the RIA technique (Salmon *et al.*, 1982). Values represent the MEAN ± SD of 3 separate experiments.

	INTRA-ASSAY C.V. (%)		INTER-ASSAY C.V. (%)	
	H.P.	L.P.	H.P.	L.P.
6KF	2.0	7.8	3.3	7.5
TxB_2	5.0	4.1	5.7	5.6
LTB_4	2.8	3.3	3.7	5.6

TABLE 5.C.

ACCURACY OF RADIOIMMUNOASSAYS FOR 6KF, TxB_2 AND LTB_4 USING KNOWN CONCENTRATIONS OF EICOSANOID STANDARDS.

Standard eicosanoids (6KF, TxB_2 and LTB_4) in estimated concentrations of 400 pg, 200 pg, 100 pg, 50 pg and 25 pg/0.1 ml were assayed by RIA. The % binding was interpolated using the standard curves and the calculated concentrations obtained. The estimated concentrations added and the calculated concentrations were not significantly different as assessed by Student's *t*-test ($p<0.05$).

EICOSANOID	ESTIMATED DOSE (pg/0.1 ml)	CALCULATED DOSE (pg/0.1 ml)
6KF	400	410.3 ± 5.6
	200	208.1 ± 4.1
	100	99.2 ± 1.8
	50	51.3 ± 0.9
	25	26.4 ± 0.9
TxB_2	400	408.1 ± 4.5
	200	204.2 ± 2.7
	100	105.1 ± 2.9
	50	52.2 ± 1.8
	25	24.6 ± 1.1
LTB_4	400	398.6 ± 3.4
	200	203.6 ± 2.1
	100	104 ± 2.6
	50	49.7 ± 1.3
	25	26.7 ± 0.8

CHAPTER 6

EXPOSURE EXPERIMENTS

6.1. INTRODUCTION OF EXPOSURE EXPERIMENTS

Prolonged exposure of newborn preterm babies to hyperoxia during oxygen therapy causes lung injury (Frank, 1985). This pulmonary oxygen toxicity has been associated with the development of a syndrome of chronic lung disease in newborn infants called Bronchopulmonary dysplasia (BPD) (Northway *et al.*, 1967).

This progressive lung disorder is initiated by a destructive, exudative phase characterized by hypoplasia of the resident lung cells and pulmonary edema (Clark and Lambertsen, 1971). The lung responds to this initial acute injury with an inflammatory reaction that includes the influx of inflammatory cells. With prolonged exposure to the hyperoxic stimulus, the original oxidant-induced injury is excessive and the inflammatory reaction is inappropriately regulated (Konig *et al.*, 1990). When the neonate is weaned from

oxygen, there is an aberrant reparative response following the extensive lung injury which results in remodelled pulmonary vasculature, airways and parenchyma. This is particularly detrimental to the lung of the neonate which is still developing at the time when pulmonary injury has occurred.

Oxidants that are generated during hyperoxia are capable of influencing the eicosanoid production by affecting enzymes in the eicosanoid cascade such as PLA2 (Iwata *et al.*, 1986), PGHS (Hemler and Lands, 1980), 15-OH-PGDH (Chaudhari *et al.*, 1979) and 5-lipoxygenase (Sun and McGuire, 1984). Cells within the lung have been shown to be sensitive to changes in oxygen tension and respond by altered prostaglandin output (Olson *et al.*, 1987, Lee *et al.*, 1990).

The broad range of physiological and pathological activities that eicosanoids are known to mediate and modulate is suggestive of a possible involvement in lung injury (Olley and Coceani, 1984). For example, LTs may be involved in the inflammatory reaction during hyperoxic lung injury due to their potential to increase vascular permeability (Matthay and Berger, 1981), and to induce chemotaxis and degranulation of inflammatory cells (Hopkins *et al.*, 1984, Martin *et al.*, 1989). LTC4 may stimulate fibrosis through its ability to cause fibroblast proliferation and induce aberrant collagen synthesis (Phan *et al.*, 1988, Baum *et al.*, 1978). Prostacyclin antagonizes the actions of the LTs since PGI2 inhibits the influx of activated PMNLs (Doukas *et al.*, 1988) and inhibits fibroblast proliferation and collagen synthesis (Fine *et al.*, 1989).

Previous studies in adult animals have examined the effects of exposure to oxidant-stimuli on eicosanoid production in the lungs in relation to pulmonary injury. Oxidant-induced lung injury produced by tert-butyl hydroperoxide increased the levels of all eicosanoids (6KF, TxB_2, LTB_4 and LTC_4) in bronchoalveolar lavage fluid (Farrukh *et al.*, 1988). These biochemical changes correlated with increased pulmonary arterial pressure and increased vascular permeability. With decreased levels of 6KF and TxB_2, following inhibition of PGHS with indomethacin, there was a coincidental decrease in pulmonary arterial pressure but no change in the oxidant-induced increase in vascular permeability (Hageman *et al.*, 1988). This may be explained by the rise in LTs that is observed when PGHS was inhibited (Dworski *et al.*, 1989). When LTs were blocked during hyperoxic exposure, there was a decrease in PMNL influx and a decrease in mortality (Taniguchi *et al.*, 1986).

It has been postulated that PGI_2 may play a protective role during oxidant-induced lung injury and that levels may be relatively diminished during hyperoxia (Hageman *et al.*, 1988). TxA_2 may be the potential mediator of the increased arterial pressure if present in proportionally elevated concentrations in comparison to the antagonistic PGI_2. The leukotrienes are likely candidates to be the mediators of the increased vascular permeability and the inflammatory response since they are produced in increased amounts during hyperoxic lung injury. It is hypothesized that an important determining factor in the pathogenesis of hyperoxic lung

injury is the balance of the individual eicosanoids, in relation to each other.

These previous studies examined eicosanoid production by the lung in bronchoalveolar lavage fluid which limited assessment to the airways (Taniguchi *et al.*, 1986). Other studies have used vascular perfusion techniques which allows examination of the blood side of the lung. No studies have examined the dynamics of the eicosanoid production within the lung tissue as an entire unit. Therefore, a lung explant system was developed that would more closely depict the eicosanoid profile as may be occurring *in vivo*. Also, the majority of studies examined the effect of hyperoxia on adult animals and it has been shown that adult animals are less tolerant to hyperoxic exposure than are newborn animals (Holtzman *et al.*, 1989). Therefore conclusions from adult studies must be extrapolated to the neonate with caution.

In the present study, a model of pulmonary oxygen toxicity in the neonatal rat was used to explore the temporal association of lung injury with changes in the eicosanoid profile of the lung. The objective was to determine whether altered eicosanoid synthesis was responsible, in part, for the pathological events occurring in the lung during neonatal pulmonary oxygen toxicity.

6.2. MATERIALS AND METHODS

6.2.1. Materials

Arachidonic Acid (Nu-chek-prep, Elysian, MN)
A23187 (Sigma Chem. Co.-St. Louis, MO)
DNA Standard calf thymus (Type I) (Sigma Chem. Co.-St. Louis, MO)
Hoechst 33258 Dye (200 µg/ml) (Amer. Hoechst Corp., Somerville, NJ)
Ketamine HCl (100 mg/ml) (MCT Pharmaceuticals, Cambridge, ON)
Neutral Buffered 10% Formalin (Sigma Chem. Co.-St. Louis, MO)
Rompun (20 mg. ml) (Chemagro Ltd., Etobicoke, ON)

Solutions

Hanks' Balanced Salt Solution (HBSS)

9.5 g	HBSS, Modified (Sigma Chem. Co.-St. Louis, MO)
15 mM	HEPES (Sigma Chem. Co.-St. Louis, MO)
1.67 mM	$CaCl_2$ (Sigma Chem. Co.-St. Louis, MO)
1 litre	DDH_2O
pH=7.38	

Ammonium Hydroxide-Triton X-100 Extraction Solution (AT)

1 M	NH_4OH (Sigma Chem. Co.-St. Louis, MO)
0.2%	Triton X-100 (Fisher Scientific Co.-Fair Lawn, NJ)

DNA Assay Buffer

100 mM	NaCl (BDH Inc.-Toronto, ON)
10 mM	EDTA (Sigma Chem. Co.-St. Louis, MO)
10 mM	Trizma Base/Trizma HCl (Sigma Chem. Co.-St. Louis, MO)
pH=7.0	

6.2.2. Exposure Protocol

Sprague-Dawely females (250-275 g) were mated with Long-Evans males (Charles River, St. Constant, Quebec). Timed-pregnant females were placed in exposure chambers in the early morning of the expected delivery date (term=22 days). Pups were born into atmospheres of either hyperoxia or normoxia. The hyperoxic regime consisted of >95% oxygen for 7 days and 60% oxygen from day 7 to 28. Normoxic gas tensions were composed of 21% oxygen and served as a control. Carbon dioxide was maintained at <0.5% by passing gas through soda lime. Odours were controlled by the use of carbon filters and pathogens eliminated using HEPA filters. Gas tensions were sampled and recorded daily using a Beckman Blood-Gas Analyzer. The temperature was maintained at 25°C and a 12 hour light-dark cycle was used. Paired litters exposed to O_2 or air were used and culled to 10 pups/litter at the time of birth. A third litter born in normoxia was used on day 1 to cull the experimental litters. Chambers were opened daily for approximately 10 minutes to give food and water and change bedding. The pups were counted to record mortality and the paired litter was adjusted accordingly. The dams were switched daily between the normoxic and hyperoxic litters since the adult females were more susceptible to hyperoxia (Holtzman *et al.*, 1989). After 21 days, pups were weaned from their mother, and males and females were separated. At exposure durations of 1, 7, 17 and 28 days entire paired litters were used for

experimental determinations. Five replicate, paired litters were used at each time point.

6.2.3. Dissection

Pups were sacrificed by anesthetic overdose using an intraperitoneal injection of a 1:3 mixture of Ketamine and Rompun at a dose of 1.0 mg Ketamine/g body weight : 0.6 mg Rompun/g body weight. Effective anesthesia was assessed by the absence of the withdrawal reflex. The pups were weighed to determine total body weight, in grams. Mid-line and transverse incisions were made through the skin and underlying muscle layer. The diaphragm was punctured and the thorax opened. The trachea was severed and the lungs removed *en bloc*. The lungs were placed on an ice-filled petri dish and dissected free from the heart and major vessels. The left and right lobes of the lungs were separated. They were either blotted on filter paper (3 to 4 pups) and placed in a pre-weighed, sealed test tube for weight determinations or placed in chilled HBSS for explant preparation (6 pups). All lobes of the liver were removed from the abdomen, blotted and placed in sealed test tubes.

For lung fixation, the thoracic cavity was opened as described above. The trachea was exposed and 3 ligatures of surgical silk were placed loosely around the circumference. An incision was made on the ventral surface of the trachea between the cartilagneous rings. A polyethylene catheter was inserted 1 to 2 cm into the trachea and

secured with the ligatures. The catheter was pre-filled with neutral buffered 10% formalin and attached to a biuret. The animal was positioned so that the chest wall was at the zero level. The biuret was calibrated by bringing the level of the fixative to 21 to 25 cm above the zero point. The stop-cock was opened for 10 minutes which was determined to be sufficient time for filling of the lungs to equilibrate to a final pressure of 20 cm H_2O. The volume instilled was recorded. The catheter was removed and the trachea was tied off with a ligature. The lungs remained *in situ* for 60 minutes. Then, they were removed *en bloc* and placed in a vial containing neutral buffered 10% formalin for at least 1 week. The tissue was then processed by standard histological techniques and stained with hematoxylin and eosin.

6.2.4. Hyperoxic Injury Determination

Lung and Liver Weight Ratios

The whole lungs and liver were weighed in the sealed test tube to preserve the tissue moisture. The wet weight of the lungs and liver were recorded after subtracting the test tube and parafilm weights. The seal was removed from the test tubes and the lungs were placed in an oven at 37°C and dried until a constant weight was attained. The wet lung weight-to-body weight (WL/BW), dry lung weight-to-body weight (DL/BW), wet lung weight-to-dry lung weight

(WL/DL), wet liver weight-to-body weight (LIV/BW) ratios were calculated (Kitterman, 1986).

Histological Assessment of Lung Specimens

Histological examination of the lungs was made by a descriptive assessment of various parameters of lung pathology. Each slide was viewed with the observer blinded to the duration of exposure and the treatment group. Each slide was surveyed and described on 3 different occasions. A minimum of 20 fields per slide were surveyed, firstly at a low magnification (40 X) and then at a higher magnification (63 X). At each magnification, ten fields were examined from the left lobe of the lung and 10 fields from the right lobes in order appraise the entire lung. Assessements were made from 3 paired hyperoxic and normoxic litters at each exposure duration and 2 to 3 pups were used from each litter. The epithelium and the walls of the large airways and blood vessels were examined for alterations caused by hyperoxia. The general appearance of the lung tissue, such as the width of the interstitial space and the intercellular material and the numbers and types of cells of the interalveolar septae, the types of cells present within the alveoli, presence of pulmonary edema, the overall appearance of the parenchyma, and assessment of the interstitial capillaries were examined.

6.2.5. Eicosanoid Determinations

The left lobe was carefully dissected free of visible blood vessels and airways and placed on ice in chilled HBSS. Lung explants were prepared by slicing the lung to a thickness of 500 µm using a McIllwain tissue chopper and separated with paint brushes. Slices from the 6 pups were pooled and mixed in order to get a representative sample of the litter as a whole. This procedure eliminated differences between the individual pups. Approximately 40 to 50 mg of lung tissue (8 to 10 slices) were placed on each nylon mesh grid (original design KSK) and inserted into 12-well culture plates. It was imperative to ensure that the tissue slice was not folded onto itself. Eighteen replicate wells were studied for each litter. A volume of 700 µl of pre-warmed HBSS at 37°C was added to each well, bringing the liquid level to the bottom surface of the explant. Each slice was kept moist via capillary action and agitation of the water bath which alternately exposed the upper surface of the explant to the media and air. The plates were covered to maintain the humidity of the ambient air and incubated in a water bath at 37°C.

Explants were incubated to basal eicosanoid release for 150 minutes with fresh, pre-warmed media replaced every 30 minutes. Samples of the media were placed in chilled test tubes and immediately flash frozen. These were used to determine when the eicosanoid release reached basal levels. Explants were then stimulated with 100 µM A.A. or 50 µM A23187 in pre-warmed HBSS

for a 30 minute incubation peroid. Media were aspirated, flash frozen and stored at -70°C. Within one week, eicosanoids were determined by direct RIA for 6KF, TxB_2 and LTB_4. The tissue explants were frozen at -70°C until DNA determinations were made.

Media samples were thawed, mixed thoroughly and centrifuged at 2500 rpm for 30 minutes at 4°C. The supernatants were decanted and placed on ice. Eicosanoids, 6KF, TxB_2 and LTB_4 were measured by RIA as described in section 5.2.2.. Duplicate samples were assayed at two different concentrations in every assay. Media blanks of HBSS, HBSS + 100 μM A.A. and HBSS + 50 μM A23187 were assayed in triplicate. Also high pools (250 pg/0.1 ml) and low pools (25 pg/0.1 ml) were included in triplicate in every assay to monitor inter-assay variation. Total eicosanoid levels per well were calculated from RIA values, media backgrounds were subtracted and standardized to total explant DNA.

Basal eicosanoid release was determined as the levels measured in the 120 minute to 150 minute interval and background HBSS media levels were subtracted. Stimulated eicosanoid release was determined during the 150 minute to 180 minute interval and background A.A. and A23187 media levels were subtracted. The eicosanoid production that was caused exclusively by A.A. or A23187 stimulation was determined by subtracting the basal output levels.

6.2.6. DNA Determinations

The DNA quantitation technique was adapted from Downs and Wilfinger (1983). Explants were thawed and blotted on filter paper. All tissue from one insert was placed in 1.0 ml of ice cold AT extraction solution. The tissue was homogenized using a Potter-Elvehjem tissue grinder that was connected to a drive motor. The tissue was homogenized in four, 20 second periods during which the glass vessel was kept in an ice bath. The homogenate was aspirated using a Pasteur pipette, placed in a test tube, capped immediately and placed on ice. When all tissues were processed, the homogenates were incubated at 37°C in a shaking water bath for 10 minutes. A 50 µl aliquot of the homogenate was added to 2.0 ml of DNA assay buffer. The mixture was centrifuged at 2500 rpm for 30 minutes at 4°C. The supernatants were capped, placed on ice and DNA was quantitated as described below.

The stock solution of the DNA standard was made to a concentration of 50 µg DNA/ml H_2O. The standard curve was constructed in triplicates of DNA concentrations of 300 ng, 250 ng, 200 ng, 150 ng, 100 ng, 75, ng, 50 ng, 25 ng, 12.5, 0 ng/ml. The standards were diluted with DNA assay buffer containing the same concentration of AT solution that was present in the tissue extract (25 µl AT solution/1.0 ml DNA assay buffer).

A 50 µl aliquot of DNA standard or sample was added to a 12 x 75 mm test tube. Then, 1.5 ml of Hoechest 33258 dye working solution (100 ng/ml) was added. The mixture was incubated at room

temperature for 30 minutes. The DNA assay buffer containing 25 µl AT/ml was utilized as a reference assay blank. One standard curve was read at the beginning of the assay and the second at the end, in order to ensure precision within the assay.

Calibration curves were constructed by plotting fluorescence units against the concentration of DNA standard. The sample DNA concentrations were determined using the linear regression equation.

6.2.7. Statistical Analysis

All exposures were performed by the pairing of two litters, one in hyperoxia and the control in normoxia. The 2 litters were considered to be paired because they were exposed simultaneously, shared mothers, had equal numbers and experimental manipulations were performed at the same time. A two-way analysis of variance (ANOVA) with replication was used to determine significance (Sokal and Rohlf, 1981). One factor was oxygen tension and the second factor was duration of exposure. If significance was found ($F<0.05$), then the individual means were assessed for significant differences using Tukey's honestly significant difference method for unplanned comparisons (Sokal and Rohlf, 1981). Significant difference was achieved at $p<0.05$. All data are reported as mean ± standard deviation (SD).

6.3. RESULTS

6.3.1. Normal Growth and Development

Body, lung and liver weight ratios were calculated in order to assess the changes that occurred in the growth and development of the neonatal rat pups. Examining the ratios of the normoxic group indicates the normal pattern of growth changes that occur with postnatal development (Table 6.A.).

Comparing day 1 air exposed animals with day 7 showed that there was a 6% ($p<0.05$) postnatal increase of the WL/BW. This may be due to the rapid growth of the lung tissue components relative to the body since a 21% ($p<0.05$) increase in the DL/BW from day 1 to 7 was also seen. In contrast, the fluid components of the lung decreased from day 1 to 7 as indicated by the 16% ($p<0.05$) decrease in the WL/DL. Following this normal postnatal clearance, the fluid content of the lung was unchanged from day 7 through to day 28 (Bland, 1986). However from day 7 to 28, there was a 63% ($p<0.05$) decrease in the WL/BW. The growth rate of the lung tissue components progressively slowed down relative to the rate of body growth since the DL/BW decreased by 63% ($p<0.05$) from day 7 to 28. Being able to effectively describe the normal pattern of lung growth illustrated the accuracy and sensitivity of these ratios in reflecting processes occurring in the lung.

6.3.2. Effects of Hyperoxia on Overall Growth

The body weights of the pups in the hyperoxic group were not significantly different from the controls at any of the exposure durations (Table 6.A.).

6.3.3. Effects of Hyperoxia on Wet Lung Weight-to-Body Weight Ratios

The WL/BW ratio reflected the total lung weight in relation to body weight (Table 6.A.). Changes in the wet lung weight may be due to changes in the water content of the lung and/or changes in the tissue of the lung, including cellular and connective tissue matrix components. One day of hyperoxia did not alter the WL/BW ratio when compared to controls, however, 7 days of hyperoxia caused a 10% ($p<0.05$) decrease. On day 17, the WL/BW ratio was elevated by 16% ($p<0.05$) in the hyperoxic group compared to control animals and on day 28, the ratio was 25% ($p<0.05$) higher.

6.3.4. Effects of Hyperoxia on Dry Lung Weight-to-Body Weight Ratios

The DL/BW ratio demonstrated changes in the tissue components of the lung relative to the body weight and was used to provide information about the growth of the lung, both normal and pathological (Table 6.A.). Changes in the dry tissue weight may reflect alterations in the cellular populations and/or connective tissue matrix components of the lungs. After 1 day of hyperoxia, the DL/BW ratio was slightly higher than normoxic controls but did not reach significance. The effect of 7 days of hyperoxia, was a 20% ($p<0.05$) decrease in the ratio compared to the normoxic group. However, after 17 days and 28 days of hyperoxia, the DL/BW was increased by 9% ($p<0.05$) and 28% ($p<0.05$) versus control values.

The normal age related increase observed in both the WL/BW and DL/BW ratios was not seen in the hyperoxic group from day 1 to day 7. From day 7 to day 28, the hyperoxic animals did demonstrate the same significant decrease in the WL/BW and DL/BW that occurs with advancing age.

6.3.5. Effects of Hyperoxia on Wet Lung Weight-to-Dry Lung Weight Ratios

The WL/DL reflected changes in the fluid content of the lungs and was used as an indicator of pulmonary edema (Table 6.A.). There was no change in the ratio after 1 day of hyperoxia, in comparison to day 1 control pups. Exposure to 7 and 17 days of hyperoxia produced 9% ($p<0.05$) and 8% ($p<0.05$) increases in the WL/DL, respectively. Then after 28 days of hyperoxic exposure, the ratio was again not different from controls.

6.3.6. Effects of Hyperoxia on Liver Weight-to-Body Weight Ratios

The liver weight-to-body weight ratio (LIV/BW) reflected the relative growth of the liver in relation to the body. The hyperoxic group showed no difference in the LIV/BW in comparison to the normoxic group (Table 6.A.). This data indicated that the effect of hyperoxia was specific to the lung and did not affect other organs such as the liver.

6.1. Histological Descriptions of Lung Specimens

Day 1

Following 1 day of exposure, there appeared to be no identifiable differences between the hyperoxic and normoxic lungs. The epithelium of the large airways were lined by low cuboidal cells with round, pale nuclei that were scattered unevenly on the basement membrane. The endothelium of the large blood vessels had thin, ovoid nuclei and the lumens were tightly packed with red blood cells (RBCs). The walls of the large airways and blood vessels did not have much smooth muscle and did not have well-developed outer layers.

The appearance of the parenchyma at a low magnification (40 X through the microscope = 180 X in the Figures) showed that the peripheral airspaces were mainly alveolar ducts and sacs that had very few septae (Figures 6.1.A. & 6.1.B.). The remaining airspace was composed of isolated, large, round alveoli. The overall pattern was disorganized and appeared lacy with thick walls. The terminal airspaces were lined by very thin Type I epithelial cells. The surface on the air side appeared smooth. The overall appearance of the interstitium was uniform. The interstitial space was 2 to 3 cell layers thick with the nuclei tightly packed together so that there was not much cytoplasm visible. The main cell nuclei identified were from endothelial cells and fibroblasts. Cuboidal Type II cells, with large, pale nuclei were seen in localized groups. Half the cell population of

the interstitial space was RBCs that were contained within the abundant capillary network. The intraalveolar space was relatively clear but a few RBC and small, pale staining macrophages were seen.

Day 7

Following 7 days of exposure, differences became evident between the hyperoxic and normoxic lungs. The large airways of the normoxic lungs were lined by a 1-cell thick layer of cuboidal cells that possessed large, round nuclei (Figure 6.2.B.). A slight difference was seen in the hyperoxic lungs where the conducting airways had an epithelium composed of 1 to 2 layers of columnar cells with pale staining, ovoid nuclei (Figure 6.2.A.). The outer layers of the vessels did not have much connective tissue development in either group (Figures 6.2.A. & 6.2.B.). The large blood vessels were different in the hyperoxic group where the endothelium had darker nuclei that were more rounded in appearance than those seen in the normoxic lungs (Figures 6.2.A. & 6.2.B.). A striking difference was seen by the presence of large clear spaces around the large blood vessels in the oxygen exposed lungs (Figure 6.2.A.). Within the vascular lumens of the hyperoxic group, PMNLs were present in substantially increased numbers (Figure 6.2.A.).

At low magnification, the overall appearance of the parenchyma was distinctly altered by O_2 where the alveolar walls were very attenuated and lacy as opposed to the normoxic lungs. The control lungs appeared lacy but were thicker and coarser looking

(Figure 6.2.B.). The alveolar sacs in the hyperoxic group did not possess the degree of septation that was demonstrated by the normoxic lungs (Figure 6.2.A.).

A closer look with a higher magnification (63 X through the microscope = 280 X in the Figures), the interstitium of the normoxic group showed this barrier to be 2 to 3 cells thick consisting of both endothelial cells and fibroblasts with their nuclei tightly packed (Figure 6.3.B.). In contrast, the O_2 group's interstitium was only 1 cell layer thick with very sparse nuclei of proportionally more fibroblasts and less endothelial cells (Figure 6.3.A.). In many stretches of the interstitium only a thin layer of foamy, pink material was seen. There was a marked reduction in the number of capillaries and RBCs seen in the interstitial spaces of the O_2 exposed lungs (Figure 6.3.A.). The most striking change in the cellular population in the hyperoxic group was the appearance of many PMNLs and large, vacuolated macrophages in the capillaries, interstitial space and in the alveoli (Figure 6.3.A.). Also within the alveoli of the hyperoxic group, there was light pink staining along the walls.

Days 17 and 28

Both 17 and 28 days of hyperoxic exposure produced morphological alterations in the lungs. The large airways of the O_2 group showed focal metaplasia of the epithelial lining where it was 2 to 3 scattered layers thick (Figures 6.4.A. & 6.6.A.) as opposed to the

uniform, single layer of the normoxic lungs (Figures 6.4.B. & 6.6.B.). There were also localized increases in the amount of smooth muscle in the medial layer of the hyperoxic groups. The large blood vessels of the O_2 treated lungs were surrounded by large clear areas that contained diffuse pink strands and dark staining nuclei of fibroblasts (Figures 6.4.A. & 6.6.A.). There was more fibrous connective tissue surrounding the blood vessels after 28 days of hyperoxia than 17 days.

The parenchyma of the hyperoxic group demonstrated focal areas of emphysema alternating with areas of atelectasis giving an overall disorganized appearance (Figures 6.4.A. & 6.6.A.). Although the general pattern was delicate and lacy as were the normoxic lungs, there were focal thickenings. The parenchymal area of the normoxic lungs was composed of equal amounts of well-expanded alveolar sacs with many sepate and clusters of medium, polyhedral alveoli (Figures 6.5.B. & 6.7.B.). In the O_2 group, the majority of the peripherial airspaces were crumpled, irregularly shaped alveolar ducts that had club shaped projections for septae (Figures 6.5.A. & 6.7.A.). There were very few small, isolated, irregularly shaped alveoli that had strikingly thick walls and areas of clumped cells and debris.

The interstitial space of both the O_2 and air groups was about 1 to 2 cells thick. But in the O_2 group, the cellular distribution was not uniform because there were localized clumps of irregularly shaped nuclei (Figures 6.5.A. & 6.7.A.). The terminal airspaces in hyperoxic

lungs that resembled large alveolar ducts and sacs were lined by a fragmented or absent layer of the typical Type I cells. In other areas that appeared as small alveoli, the surface was lined by cuboidal Type II cells. This was more pronounced following 28 days of hyperoxia than after 17 days. There were very few capillary endothelial cells seen and the ones that were identified had irregularly shaped nuclei. The majority of the nuclei seen were large, pale clusters of Type II cells and small, dark nuclei of fibroblasts. In both the hyperoxic and normoxic lungs, the thin portions of the interstitium were composed of only pink staining material and capillaries. However in the hyperoxic lungs of the day 17 animals, the interstitial tissue appeared very foamy and contained fibrous looking pink material. In the day 28 O_2 lungs, the interstitium was even more fibrous looking. There was also a noticeable decrease in the number of capillaries and RBCs in the interstitium of the O_2 treated groups.

6.3.8. Eicosanoid Determinations from Lung Explants

Eicosanoid levels from the lungs of the neonatal rats were determined using a tissue explant technique. 6KF, TxB_2 and LTB_4 levels were determined under basal and maximally stimulated conditions. The basal release values reflect the resting activity of the various enzymes in the eicosanoid pathway, i.e. phospholipase A_2

(PLA2), prostaglandin endoperoxide H synthase (PGHS), 5-lipoxygenase, prostacyclin synthetase, thromboxane A2 synthetase, LTA4 hydrolase. Also, resting levels reflect the availability of endogenous substrate and the proportions of different cell populations that are present in the lung. The total stimulated values represent the amounts of eicosanoid that are produced by a maximally saturating dose of arachidonic acid or optimal stimulated eicosanoid synthesis from maximum amounts of endogenous substrate release with A23187.

Normal Developmental Lung Eicosanoid Production

A normal postnatal developmental increase in the basal release of eicosanoids from the lung was observed in the air exposed animals. This was evident by the basal release of 6KF which progressively increased at each stage of the exposure (Figures 6.8., 6.10., 6.12., 6.14.). The lungs of 28 day old animals released basal levels of 6KF that were 4 fold higher ($p<0.05$) than day 1 pups (Figures 6.8. and 6.14.). Basal release of TxB_2 remained at low, steady state levels from day 1 to day 17 but then increased 3 fold ($p<0.05$) from day 17 to day 28 (Figures 6.8, 6.12. and 6.14.). The basal release of LTB_4 increased 4 fold ($p<0.05$) between day 7 and day 17 (Figures 6.10. and 6.12.).

Upon stimulation with exogenous A.A., the normoxic lungs demonstrated a different response of eicosanoid release than with the basal release. The capacity to synthesize 6KF and TxB_2 from

exogenous A.A. decreased by 45% ($p<0.05$) and 50% ($p<0.05$), respectively (Figures 6.9 and 6.11) in day 7 lungs compared to day 1 lungs in the normoxic animals. Then from day 17 to day 28, there was an increased ability to metabolize exogenous substrate with a 2.5 fold ($p<0.05$) increase in levels of 6KF and a 3 fold ($p<0.05$) elevation for TxB_2 (Figures 6.13. and 6.15.). A23187-stimulated LTB_4 production increased progressively with advancing postnatal age with a 4 fold ($p<0.05$) increase comparing day 1 to day 28 (Figures 6.9. and 6.15.).

Effect Of Hyperoxia On Lung Eicosanoid Production

Day 1

One day of hyperoxic exposure caused a decrease in the ability of the lung explants to produce PGHS metabolites. Basal amounts of 6KF were 50% ($p<0.05$) lower from hyperoxic explants than from controls (Figure 6.8.). The capacity to synthesize 6KF and TxB_2 from exogenous A.A. was greatly blunted by hyperoxia since the levels of 6KF and TxB_2 were decreased by 30% ($p<0.05$) and 46% ($p<0.05$), respectively (Figure 6.9.), compared to controls. A similar effect of hyperoxia was seen with A23187 stimulation but the decrease was not significant (Figure 6.9.). Basal LTB_4 release from the hyperoxic explants was elevated by 35% ($p<0.05$) over control levels (Figure 6.8.). A23187 stimulation produced levels of LTB_4 in the hyperoxic exposed explants that were 66% ($p<0.05$) greater than the levels released from normoxic explants (Figure 6.9.).

Day 7

Hyperoxia had a different effect on eicosanoid production by the lung after 7 days of exposure. There was an increase in the capacity to produce both 6KF and TxB_2 by the hyperoxic exposed lung explants over controls. Basal levels of 6KF and TxB_2 were 40% ($p<0.05$) and 42% ($p<0.05$) higher in hyperoxic explants over controls (Figure 6.10.). In the presence of saturating levels of A.A., the hyperoxic exposed lungs appeared to preferentially direct PGHS metabolism toward PGI_2 synthesis over TxA_2 since 6KF, but not TxB_2, output was significantly elevated in the hyperoxic group over the controls (Figure 6.11.). A23187 stimulation demonstrated a similar trend but was not significant (Figure 6.11.). Hyperoxia stimulated a 62% ($p<0.05$) elevation in basal LTB_4 release in hyperoxic explants over controls (Figure 6.10.). Stimulation with A23187 produced a 50% ($p<0.05$) increase in LTB_4 levels in the hyperoxic exposed explants compared to normoxic explants (Figure 6.11.).

Day 17

On day 17, the reduced hyperoxic environment of 60% O_2 had a selective depressant effect on eicosanoid release from the lungs. Basal output of 6KF was decreased by 16% ($p<0.05$) in the hyperoxic explants but basal TxB_2 release did not change (Figure 6.12.). Upon stimulation with exogenous substrate, the 6KF release was not different between the hyperoxic and normoxic groups but there was

a 22% ($p<0.05$) decrease in TxB_2 levels in the hyperoxic group (Figure 6.13.). On day 17, basal LTB_4 levels were higher in the hyperoxic group although this did not reach a significant difference from controls (Figure 6.12.). However, following stimulation with A23187, there was a 48% ($p<0.05$) increase in the amounts of LTB_4 produced by the hyperoxic exposed explants compared to the normoxic explants (Figure 6.13.).

Day 28

After 28 days of hyperoxic exposure, the eicosanoid profile of the lung again had a different response. Basal 6KF release from hyperoxic lungs was decreased by 17% ($p<0.05$) compared to controls. In contrast, 28 days of hyperoxia caused lungs to release TxB_2 so that levels were elevated by 51% ($p<0.05$) compared to controls (Figure 6.14.). A.A. stimulated levels of 6KF were 24% ($p<0.05$) lower in the hyperoxic explants whereas the production of TxB_2 from exogenous A.A. was not altered (Figure 6.15.). Following A23187 stimulation, 6KF synthesis by hyperoxic explants was lower than controls but differences did not reach significance. Basal LTB_4 levels were higher in hyperoxic animals compared to controls but were not significantly different (Figure 6.14.). After stimulation with A23187, the hyperoxic explants produced 41% ($p<0.05$) higher levels of LTB_4 than normoxic explants (Figure 6.15.).

6.4. DISCUSSION

6.4.1. Effects of Hyperoxia on Lung Growth and Development

Prolonged exposure to hyperoxia produces a toxic reaction by the lungs (Clark and Lambertsen, 1971). The lung of the preterm neonate is especially susceptible to the deleterious effects of hyperoxia due to its immaturity (Frank, 1985). This study demonstrated that the exposure model used, induced lung injury that followed the known pathogenesis of pulmonary oxygen toxicity (Kapanci *et al.*, 1969, Kaplan *et al.*, 1969, Crapo *et al.*, 1980).

Day 1

There was no evidence of overt injury observed after 1 day of hyperoxia (Table 6.A.). This corresponds to a 24 hour lag-phase that has been observed to occur during the genesis of oxygen toxicity (Fanburg *et al.*, 1986). During this time, there is an excessive production of intracellular oxygen free radicals which overwhelms the intracellular antioxidant defense systems (Freeman and Tanswell, 1985). Oxygen free radicals then react with the cellular components such as DNA, structural proteins (i.e. collagen and elastin), functional proteins (i.e. enzymes and receptors) and with membrane phospholipids causing lipid peroxidation (Cross *et al.*, 1987). These

events cause subtle injury which results in aberrations of cell functioning.

Day 7

After 7 days of hyperoxic exposure, evidence of a pulmonary hypoplastic response in conjunction with pulmonary edema was observed. The hypoplasia of the lung may be the result of cellular destruction of resident lung cells that occurs during the acute phase of oxygen toxicity. Endothelial cell numbers decrease first because of their less resilient AOE system and compromised antiprotease levels (Bowden and Adamson, 1974, Jongkind *et al.*, 1989, Cheronis *et al.*, 1987). This may explain the observed decrease in the number of capillaries in the lung parenchyma following hyperoxia. A substantial reduction in the numbers of Type I cells may also contribute to the lung hypoplasia that was observed (Kapanci *et al.*, 1969). Fibroblasts (Tanswell *et al.*, 1990) and smooth muscle cells (Palmberg *et al.*, 1989) may be reduced in numbers as suggested by the acellular appearance of the interstitium in comparison to the control lungs.

In combination with the destructive effects of oxygen toxicity, there was a cessation of the normal postnatal lung growth and development. The lungs normally increase in weight as a result of postnatal alveolarization from day 1 to day 7 (Meyrick and Reid, 1982). Comparing the hyperoxic exposed pups on day 1 to the day 7 pups showed that the DL/BW ratio did not change. The day 7 oxygen

exposed lungs appeared to have fewer distinct alveoli and less septae than the controls, which may be indicative of the arresting of pulmonary development. Although, it is not simply a suppression of alveolarization that has occurred because the day 7 hyperoxic lungs have thin, acellular walls whereas the day 1 hyperoxic lungs have thick, cellular walls.

The higher pulmonary fluid content of the hyperoxic exposed lungs on day 7 (Table 6.A.) is a demonstration of the acute exudative phase of oxygen toxicity (Clark and Lambertsen, 1971). From the WL/DL, it is difficult to determine whether the edema is interstitial, intraalveolar or both. Perivascular edema was noticed around the large blood vessels. It has been shown that during the acute phase, interstitial edema may be caused by an increase in vascular permeability of the endothelial and epithelial barriers (Staub, 1981, Jefferies *et al.*, 1984) and/or a decrease in lymphatic drainage (Bland, 1986). Eventually, there may be a complete breakdown of the integrity of these barriers which allows the free flow of vascular components into the interstitium and alveolar spaces.

The normal postnatal drainage of fluid from the lung appeared to be retarded by hyperoxia since the WL/DL from day 1 to day 7 although decreasing, does so to a lesser extent than the air pups. A possible explanation may be due to aberrations in the functioning of the epithelial cells which interferes with the fluid transport mechanism across the epithelium from the air side to the interstitial space (Eling *et al.*, 1988). This would result in the stagnation of

pulmonary fluid in the alveolar spaces. The slow clearance of fluid from the hyperoxic lungs may be due to impaired lymphatic drainage which would contribute to accumulation of fluid in the interstitium (Bland, 1986).

Days 17 and 28

Following 7 days of the lethal dose of 100% O_2, the hyperoxic environment was reduced to 60% O_2 to allow the lung to repair. On day 17, there was still evidence of pulmonary edema in the hyperoxic lungs, as on day 7. However, this occurred in conjunction with the appearance of lung hyperplasia which may be indicative of aberrant repair processes that were occurring in the lungs. By day 28, the edema seems to have resolved however the lung remained hyperplastic. This hyperplastic phenomenon appeared to occur in focal areas of the parenchyma since increased numbers of Type II cells and fibroblasts appeared in clusters particularly in relation to small alveoli. The parenchyma had an overall pattern of regions demonstrating emphysema and atelectasis which is characteristic of the later stages of BPD (Northway *et al.*, 1967). Emphysematous regions were identified by the presence of the club shaped projections which indicate the breakage and subsequent recoil of an alveolar wall. Atelectic regions occurred where the alveolar walls appeared to be thickened but may be a collapsed alveolus with the two alveolar walls stuck together. The increase in lung weight in the hyperoxic lungs may be due to an increase in the amount of

connective tissue deposition. There appeared to be an alteration in the amount of fibrous looking material in the interstitial spaces of the O_2 treated lungs which may be indicative of an increase in collagen. There did appear to be an increase in the muscularity and evidence of fibrosis of the large airways and blood vessels.

6.4.2. Effects of Hyperoxia on Eicosanoid Production in the Lung

The eicosanoid pathways, both PGHS and 5-lipoxygenase cascades, are sensitive to hyperoxia (Warso and Lands, 1985). This study demonstrated that the exposure model induced progressive alterations in the pulmonary eicosanoid profile throughout the duration of the exposure protocol.

Normal Developmental Eicosanoid Production

An increasing postnatal ability of the lung to produce eicosanoids was demonstrated by the progressively greater basal levels produced as the control pups matured. The developmental changes in the pulmonary cellular populations with maturation of the lung may be responsible for the elevated synthetic capability. The extensive alveolarization and vascularization that occurs in the lung postnatally would translate into an increase in the absolute number

of epithelial cells and endothelial cells that would contribute to the overall eicosanoid pool.

Day 1 normoxic pups had an extraordinarily high capacity to convert exogenous substrate to 6KF and TxB_2 in comparison to day 7 normoxic pups. It is postulated that immediately post-partum there is a large reserve pool of PGHS that is not utilized under basal conditions and A.A. release is at non-saturating levels. Day 1 animals may possess this large amount of resting enzyme just following birth in association with the rapid pulmonary vasodilation that occurs upon air breathing (Leffler *et al.*, 1978).

Effects Of Hyperoxia On Lung Eicosanoid Production

Day 1

Pups exposed to hyperoxia for 1 day demonstrated a depressed capacity to make the PGHS metabolites, 6KF and TxB_2, but an increased ability to produce LTB_4. Under basal conditions, 6KF release decreased, but TxB_2 levels were not affected by hyperoxia. This may indicate that the highly susceptible endothelial cells which make the majority of the PGI_2 were preferentially exhibiting injury (Gryglewski *et al.*, 1978). In turn, the more resistant interstitial cells, which are a large source of TxA_2, did not appear to be affected by hyperoxia since they did not appear different from control lungs.

Also platelets, which can be a major contributor of TxA_2, may not be present to a large extent in the lung at this stage of lung injury.

Following 1 day of hyperoxia, the lungs had a decreased capability to convert exogenous A.A. to 6KF and TxB_2, in comparison to day 1 controls. The reduction in the synthesis of these PGHS metabolites may result from the direct inactivation or destruction of the PGHS protein by the excessive oxygen radicals that are present within the resident lung cells.

The cellular source of leukotrienes at this stage of the exposure was postulated to be resident macrophages. These cells respond to the hyperoxic stimulus by releasing LTB4 (Ida *et al.*, 1988). The increase in LTB4 may be due to increased A.A. release within the macrophages as a result of membrane lipid peroxidation. Also, LTB4 synthesis may be increased due to the stimulation of the 5-lipoxygenase enzyme by elevated levels of lipid peroxides produced by hyperoxia (Sun and McGuire, 1984).

Exposure to 1 day of hyperoxia caused an alteration in the balance of eicosanoids, such that the ability of the lung to produce 6KF and TxB_2 was decreased but was increased for LTB4. This shift may be an early signal that the resident lung cells have sustained damage which cannot yet be seen as overt injury, but is manifested as biochemical aberrations.

Day 7

After 7 days of hyperoxic exposure, overall eicosanoid production in the lung was increased, as demonstrated by the elevated basal outputs of 6KF, TxB_2 and LTB_4. The elevation of all 3 eicosanoids may reflect an increase in the basal pool of free A.A. caused by enhanced release from the membrane phospholipids due to lipid peroxidation or increase PLA_2 activity (Warso and Lands, 1985, Iwata *et al.*, 1986). The increase in overall eicosanoid synthesis may be reflective of a change in the relative cellular proportions in the lung that has occurred in response to hyperoxia. There did appear to be an increase in the populations of cells in the hyperoxic exposed lungs since the alveolar walls appeared acellular in comparison to controls. The enhanced basal and A23187-stimulated levels of LTB_4 were mostly due to an increase in the population of oxidant-injured macrophages that was observed in the lung parenchyma and in the alveolar spaces. The increased basal levels of eicosanoids following hyperoxia may result from an inhibition in the metabolism of eicosanoids by the lung tissue (Nowak, 1984). It has been shown that the enzymes that metabolize eicosanoids such as 15-hydroxy-prostaglandin dehydrogenase (15-OH-PGDH), are specifically inhibited by hyperoxia (Chaudhari *et al.*, 1979).

The alterations in the pulmonary eicosanoid profile after 7 days of hyperoxic exposure are in agreement with the observations of many past studies. It was shown that eicosanoid levels (6KF,

TxB_2, LTB_4, and LTC_4) measured in bronchoalveolar lavage fluid (BALF) are generally elevated following an acute dose of hyperoxia (Hageman *et al.*, 1986, Taniguchi *et al.*, 1986). This elevation of eicosanoid levels was found to occur in conjunction with increased BALF albumin concentrations and increased WL/DL, both indicating the presence of pulmonary edema and increased numbers of inflammatory cells.

It has been suggested that the increase in PGI_2 is not causing the injury but may be an attempt of the lung to protect itself during oxygen-induced lung injury. This was demonstrated in studies where administration of indomethacin (a PGHS inhibitor) or dexamethasone (A.A. release and PGHS inhibitor) during hyperoxia produced lower levels of PGF in the lungs in conjunction with higher mortality and elevated BALF protein concentrations, indicating worsening edema (Smith *et al.*, 1986).

It is spectulated that elevated levels of LTB_4 may be the mediator that is responsible for promoting hyperoxic lung injury. LTB_4 is detected in elevated concentrations in BALF of hyperoxic exposed adult rats and correlates with a marked increase in the number of inflammatory cells (Taniguchi *et al.*, 1986). The role of LTB_4 in mediating the increased presence of inflammatory cells was elucidated by the administration of a 5-lipoxygenase inhibitor during hyperoxic exposure. There was a reduction in the amounts of LTB_4 that were produced and a coincidental attenuation of the hyperoxia-induced infiltration of phagocytes (Taniguchi *et al.*, 1986).

It is possible that the elevation in overall eicosanoid production by the lung may explain the lung injury that was observed following 7 days of hyperoxia. Pulmonary edema that was detected at this point in the exposure may be the result of the increase in LTB_4 promoting an increase in vascular permeability (Bray *et al.*, 1984). LTB_4 may in turn cause an increase in TxA_2 which compounds the increased vascular leakage (Farrukh *et al.*, 1988). The increase in PGI_2 would cause vasodilation of the pulmonary blood vessels resulting in an increase in the blood flow through the lungs. This would cause an increase in the vascular hydrostatic pressure which would favour the transudation of fluid out of the vasculature into the interstitial spaces.

Day 17

On day 17 the hyperoxic environment was reduced in order to allow the lung to repair. The lung's eicosanoid production responded to this change with an inhibition of the PGHS metabolism but a sustained elevation of LTB_4.

The lower basal output of 6KF from endothelial cells may indicate that the endogenous A.A. stores had been exhausted due to the elevated output on day 7. The PGHS enzyme did not appear to be the step that was affected by hyperoxia because with the addition of exogenous A.A., there was no difference in 6KF output between hyperoxia and normoxia. With regard to TxB_2 release by platelets, it appeared that hyperoxia did not affect the A.A. release step since

basal release was not different between the two groups. However, the PGHS step may be inactivated by high peroxide tone since the hyperoxic group produced less TxB_2 following the addition of exogenous A.A. (Warso and Lands, 1983).

Basal LTB_4 output in the hyperoxic group was higher than controls but did not attain significance. This may indicate that there was an increase in the population of inflammatory cells that were capable of synthesizing LTB_4. It is not only the augmented presence of these cells that leads to increased LTB_4 levels but their activation is also necessary. This was supported by the finding that upon activation with A23187, there was a significant increase in LTB_4 levels caused by the oxidant stimulus (Sun and McGuire, 1984). From these studies, it was difficult to determine whether the increases were due to increased populations of cells that synthesize LTB_4, or if there was a stimulation of the enzymes in the LT synthetic cascade or both.

After 17 days of exposure, it was postulated that repair of the initial lung injury was taking place. The persistent hyperoxic environment may have an effect on these repair processes, by the direct actions of O_2 free radicals and indirectly by mediators of the chronic inflammatory response. Eicosanoids are a potential group of these mediators. The hyperplasia of Type II pneumocytes and fibroblasts may be mediated by the elevated levels of LTs due to their mitogenic effects on fibroblasts, epithelial cells, and smooth muscle cells (Phan *et al.*, 1988, Palmberg *et al.*, 1989). PGI_2 is anti-

mitogenic through its ability to elevate intracellular cAMP levels and hence decreased concentrations of PGI_2 would allow for unopposed cell proliferation (Rabinovitch, 1987).

A portion of the increased DL/BW may be caused by an increased number of PMNLs in the interstitium and alveoli, which has been shown to occur as the secondary inflammatory response (Barry and Crapo, 1985). An increase in the dry lung weight may also be due to increased connective tissue deposition in the airways, blood vessels (Wilson *et al.*, 1985), interstitium (Clark and Lambertsen, 1971) and alveolar spaces (Reid, 1979). The accumulation of collagen and elastin may result from the stimulation of the enlarged population of fibroblasts by elevated levels LTs (Phan *et al.*, 1988).

The pulmonary edema that was observed at this stage may be due to injury to the endothelium and epithelium caused by the proteolytic actions of the inflammatory cells (Royston *et al.*, 1990). The increased LTB_4 production would promote chemotaxis and activation of these cells (Borgeat and Naccache, 1990). The decreased levels of PGI_2, which normally inhibits PMNL activity, would permit the genesis of a chronic inflammatory reaction (Doukas *et al.*, 1988).

Day 28

The hyperoxic environment on day 28 was the same as on day 17 however the eicosanoid profile of the lung was different. After 28 days of exposure, basal 6KF release was decreased but basal TxB_2 release was markedly elevated. The decrease in 6KF was speculated to be due to a decrease in the activities of both PGHS and PGI_2 synthetase as a result of inactivation by chronically elevated levels of lipid peroxides (Wang *et al.*, 1988). The marked increase in TxB_2 may indicate a stimulation of A.A. release and stimulation of PGHS activity in an increased number of platelets and fibroblasts. Endothelial cells and platelets may exhibit a preferential shunting toward TxA_2 synthesis over PGI_2 which occurs at high levels of substrate since PGI_2 synthetase may be saturated at lower substrate concentrations than TxA_2 synthetase (James and Walsh, 1988).

The elevated LTB_4 on day 28 was most likely due to the increased population of phagocytes and from the proliferating alveolar macrophage population that was induced during adaptation to hyperoxia (Crapo *et al.*, 1980). The 5-lipoxygenase enzyme within these cells may have be stimulated by the hyperoxic stimulus through elevated levels of stimulatory lipid peroxides and activating free calcium (Sun and McGuire, 1984, Iwata *et al.*, 1986).

The eicosanoid environment of elevated TxA_2 over PGI_2 would result in a net vasoconstriction within the pulmonary vessels (Nowak, 1984). This would decrease the blood flow through the vasculature and hence may serve to explain the resolution of

pulmonary edema observed on day 28. The pulmonary hyperplasia that was evident by the elevated DL/BW may be promoted by the elevated LTB4 in conjunction with decreased 6KF as explained for day 17.

Similar alterations in eicosanoid levels (i.e. decreased PGI_2 and increased LTs) in other models of lung injury have been previously demonstrated. In patients with pulmonary alveolar proteinosis where a proteinlike material accumulates in the alveoli, large amounts of LTB4 and LTC4 in conjunction with lower amounts of PGHS products were detected in BALF (Zijlstra *et al.*, 1987b). These findings lead to the speculation that the development of lung injury occurs when the balance of eicosanoids is shifted towards increased amounts of LTs and decreased amounts of PGI_2.

6.5. SUMMARY OF EXPOSURE EXPERIMENTS

The pathological sequence of events that occur in the lungs during acute pulmonary oxygen toxicity and the final processes resulting BPD of the neonate are well-documented (Clark and Lambertsen, 1971, Northway *et al.*, 1967). As well, an acute dose of hyperoxia has been shown to induce alterations in the eicosanoid cascade in the lung (Hageman *et al.*, 1986, Taniguchi *et al.*, 1986). However, the link between an altered pulmonary eicosanoid profile and the entire spectrum of hyperoxic lung injury has not been established. In particular, the neonatal lung may have a different eicosanoid response to hyperoxia than the adult lung and the ensuing lung pathology will have different consequences since the organ is still developing at the time of the insult.

The hyperoxic regime used in this study induced the characteristic sequence of lung injury of an acute exudative, hypoplastic phase followed by a reparative, hyperplastic phase. In conjunction with this, the eicosanoid production by the pre-exposed lung tissue was altered in response to hyperoxia. More importantly, this study demonstrated a temporal association between differential alterations in the eicosanoid profile and the various stages of the hyperoxic lung injury. From the findings of this study a direct causal role for the individual eicosanoids cannot be established but there is evidence that eicosanoids participate in the pathogenesis of hyperoxic lung injury. The observation of changes in the pulmonary

profile of eicosanoids occurs before the appearance of overt injury may implicate them as mediators of lung injury. The altered production of the different eicosanoids, relative to each other, has been hypothesized to be the important determinant in controlling the events that occur within the microenvironment where they are released. From the known biological actions of the individual eicosanoids, it was possible to speculate that the observed changes in the levels of a particular eicosanoid may be responsible for a physiological event that will lead to the observed lung injury.

The value of this study was that it established the validity of the hyperoxic exposure regime and the lung explant technique for the evaluation of the pulmonary eicosanoid profile. The information that was gained provides a basis for further studies that will investigate the causal role of altered eicosanoid environment in the pathogenesis of neonatal hyperoxic lung injury.

FIGURE 6.1. LOW MAGNIFICATION VIEW OF DAY 1 NEONATAL RAT LUNGS.

Lung specimens from day 1, **A)** hyperoxic and **B)** normoxic exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 40 X and represented in the photographs at 180 X (i.e., 1.8 mm in picture=10 μm actual). The parenchyma was composed of mainly alveolar ducts (**) and sacs (□). The alveolar walls were 2 to 3 cells thick. There was no difference between the hyperoxic and normoxic lungs. These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=7 animals in each group).


A

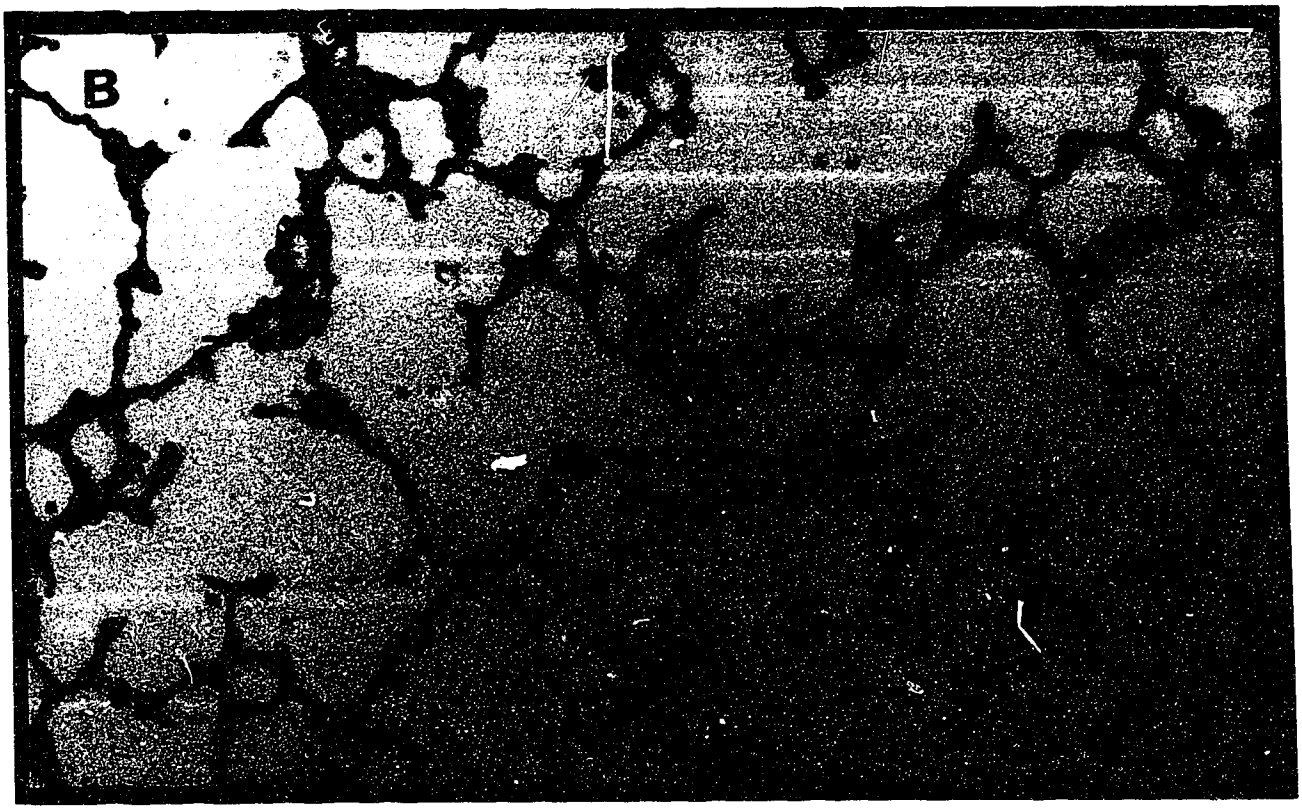

B

FIGURE 6.2. LOW MAGNIFICATION VIEW OF DAY 7 NEONATAL RAT LUNGS.

Lung specimens from day 7 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 40 X and represented in the photographs at 180 X (i.e., 1.8 mm in picture=10 μm actual).

A) hyperoxic group. Large blood vessels (arrowhead) had perivascular edema. The parenchyma was mainly alveolar ducts (**) and alveolar sacs (□). The interstitium was attenuated and acellular. Macrophages were seen in the alveoli (m). B) normoxic group. Large blood vessels (arrowhead) were lined by 1 layer of cuboidal epithelial cells and thin, ovoid endothelial cells, respectively. The parenchyma was composed of more alveoli (*) and alveolar sacs (□) with more septation than the hyperoxic lungs. The alveolar walls were 2 to 3 cells thick. These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=6 animals in each group).

FIGURE 6.3. HIGHER MAGNIFICATION VIEW OF DAY 7 NEONATAL RAT LUNGS.

Lung specimens from day 7 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 63 X and represented in the photographs at 280 X (i.e., 2.8 mm in picture=10 μm actual).

A) hyperoxic group. The interstitium was very attenuated. Capillaries were almost absent and few RBCs were seen. Macrophages (m) and PMNLs (◆) were seen in the alveoli. **B)** normoxic group. The interstitium contained tightly packed nuclei of endothelial cells (e), interstitial cells (i), intact Type I cells (I) and Type II cells (II) were seen in the corners of the alveoli. Many capillaries (c) containing RBCs were seen in the interstitium. These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=6 animals in each group).

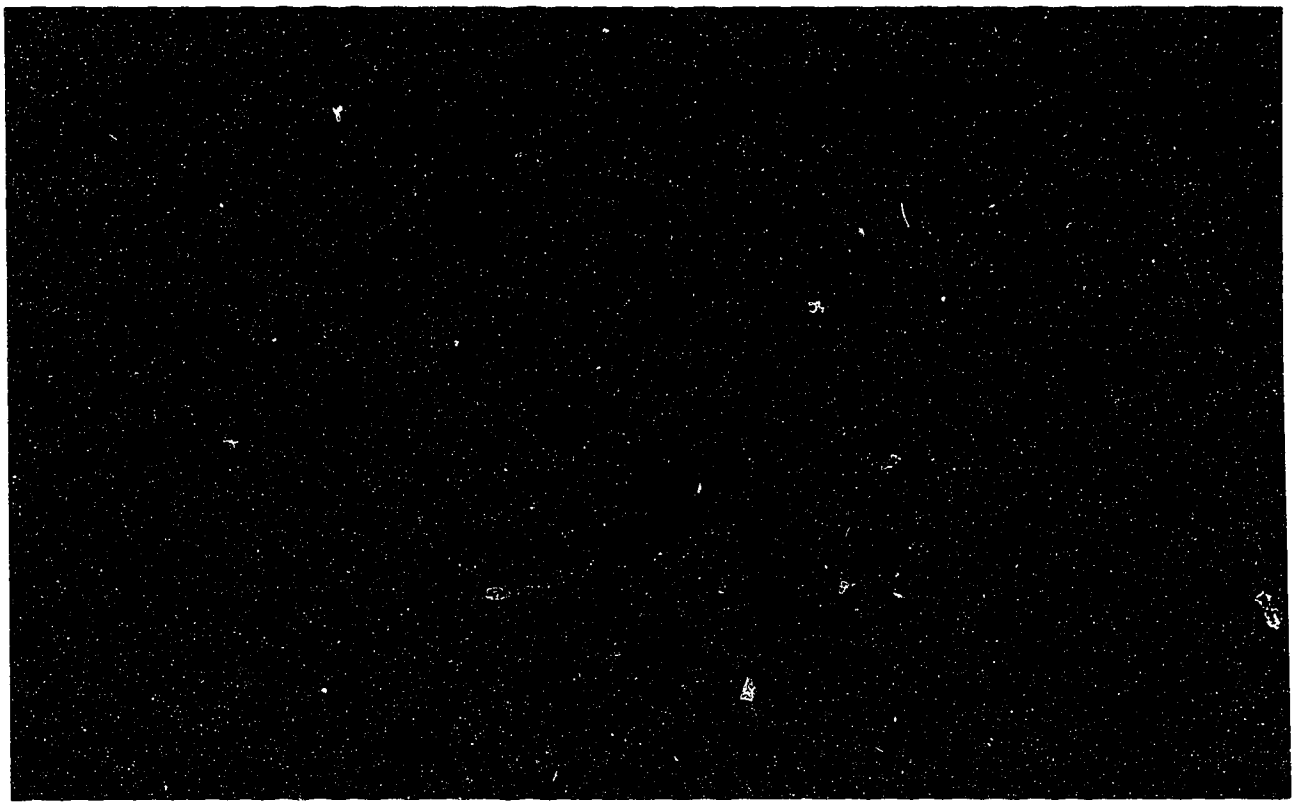
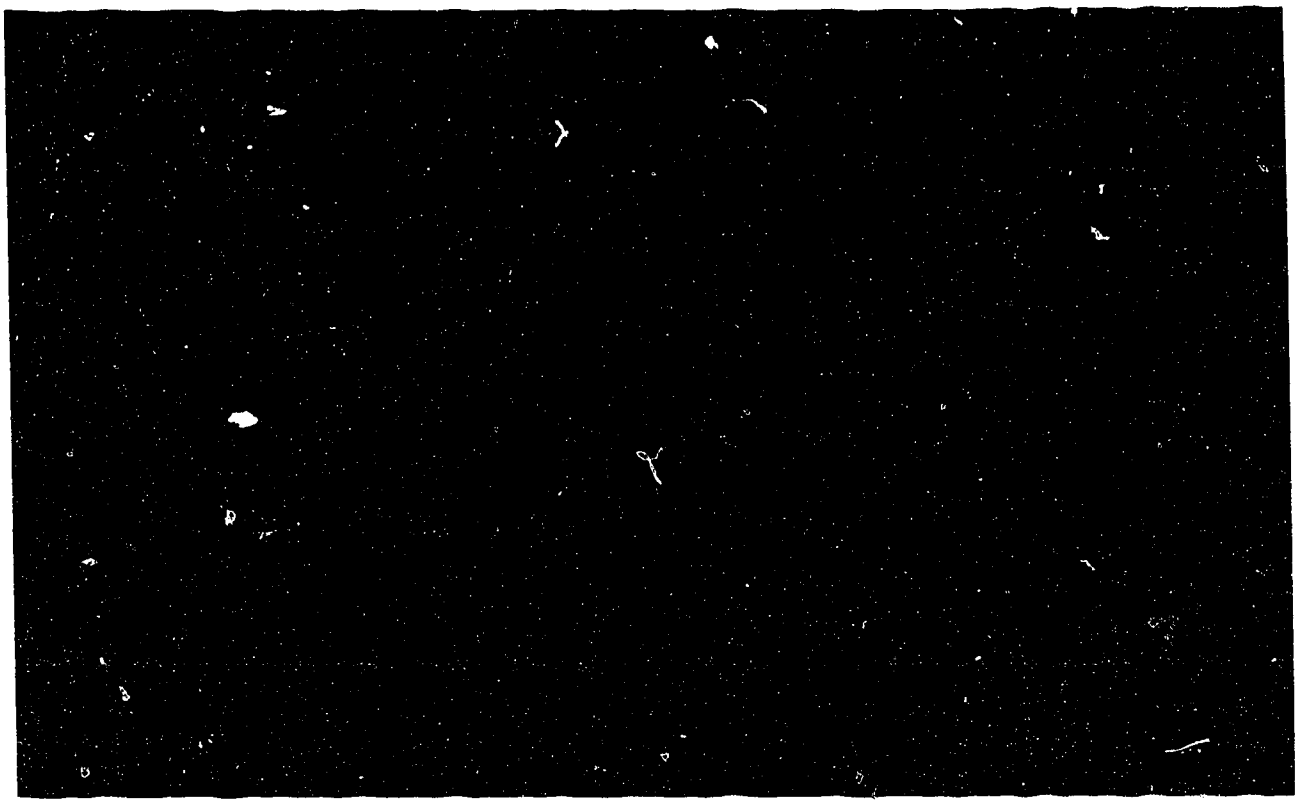


FIGURE 6.4. LOW MAGNIFICATION VIEW OF DAY 17 NEONATAL RAT LUNGS.

Lung specimens from day 17 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 40 X and represented in the photographs at 180 X (i.e., 1.8 mm in picture=10 μm actual).

A) hyperoxic group. The parenchyma demonstrated alternating regions of emphysema and atelectasis (arrow) and the airspaces were mainly irregularly shaped alveolar sacs (□) with club shaped projections (p). **B)** normoxic group. The parenchyma had a more uniform appearance than the hyperoxic lungs and was composed of more well-developed alveoli (*). These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=6 animals in each group).



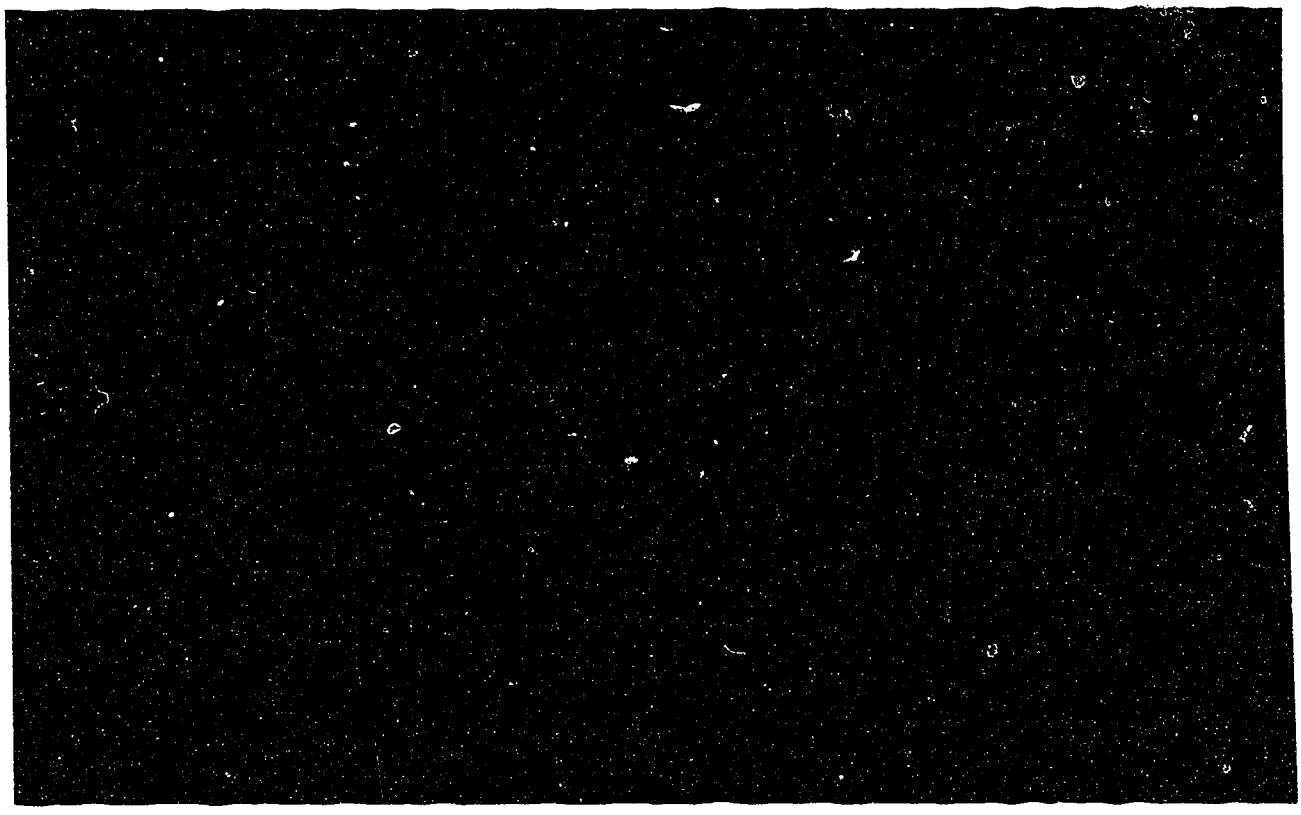
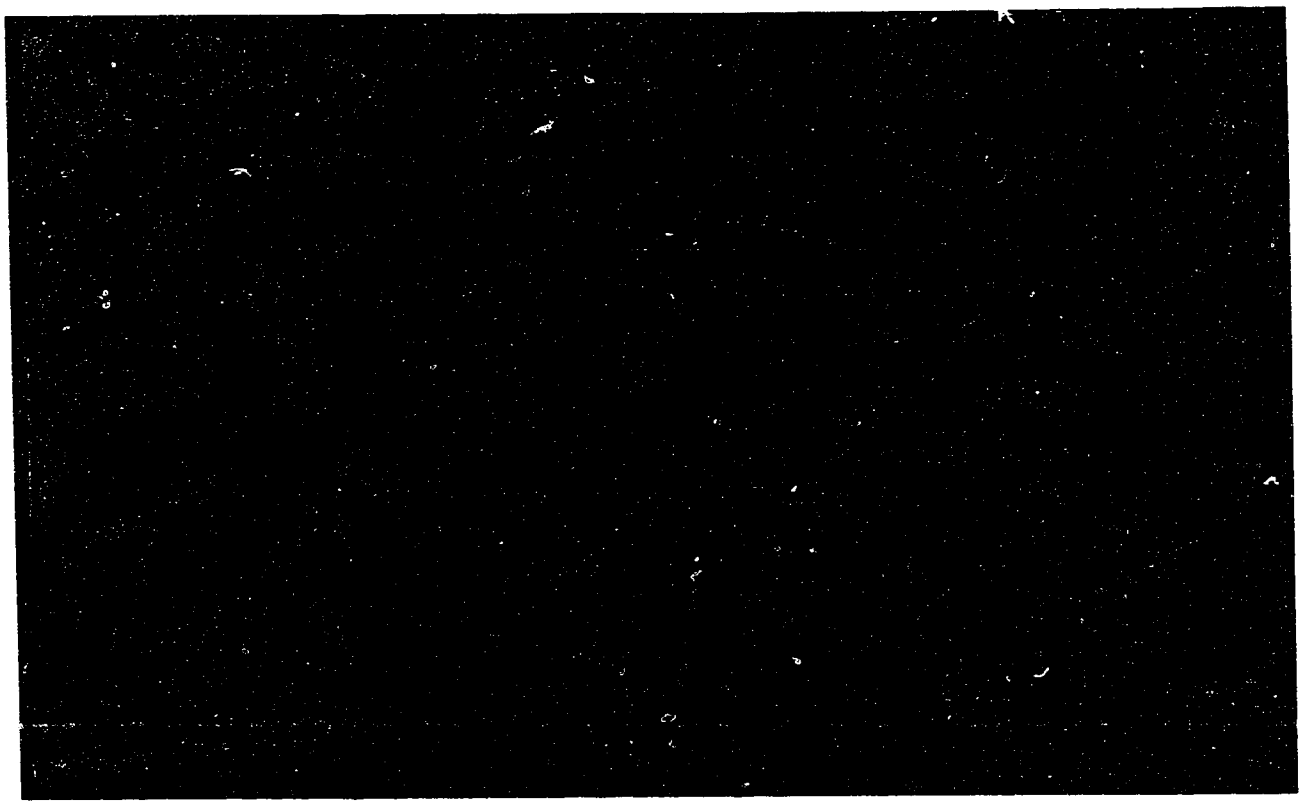


FIGURE 6.5. HIGHER MAGNIFICATION VIEW OF DAY 17 NEONATAL RAT LUNGS.

Lung specimens from day 17 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 63 X and represented in the photographs at 280 X (i.e., 2.8 mm in picture=10 μm actual).

A) hyperoxic group. There were focal clumps of cells (arrow). The interstitium was more cellular than in normoxic lungs and as a result the width was increased. Capillaries were almost absent and few RBCs were seen. **B)** normoxic group. The parenchyma was composed of mainly well-expanded alveoli (*). The interstitium contained uniformly distributed nuclei and was functionally very thin. Many capillaries containing RBCs were seen in the interstitium (c). These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=6 animals in each group).



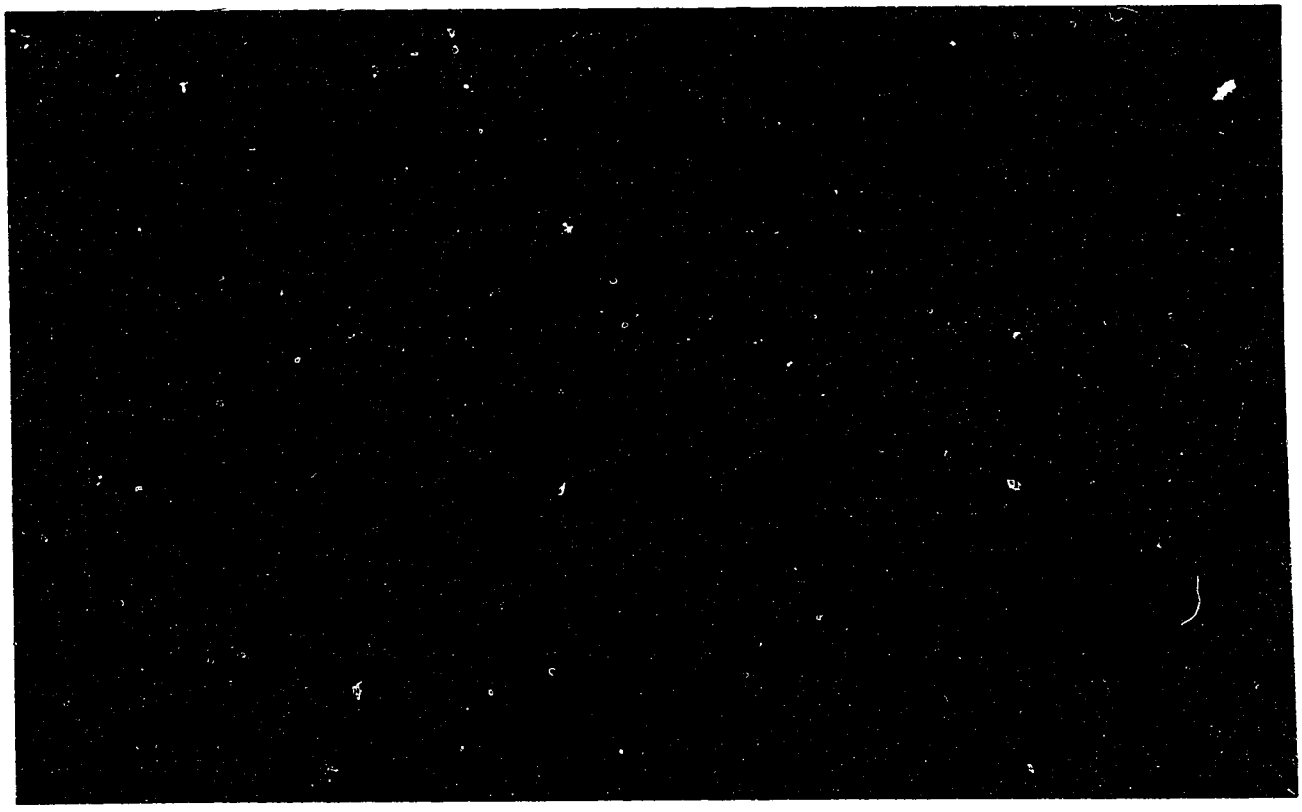


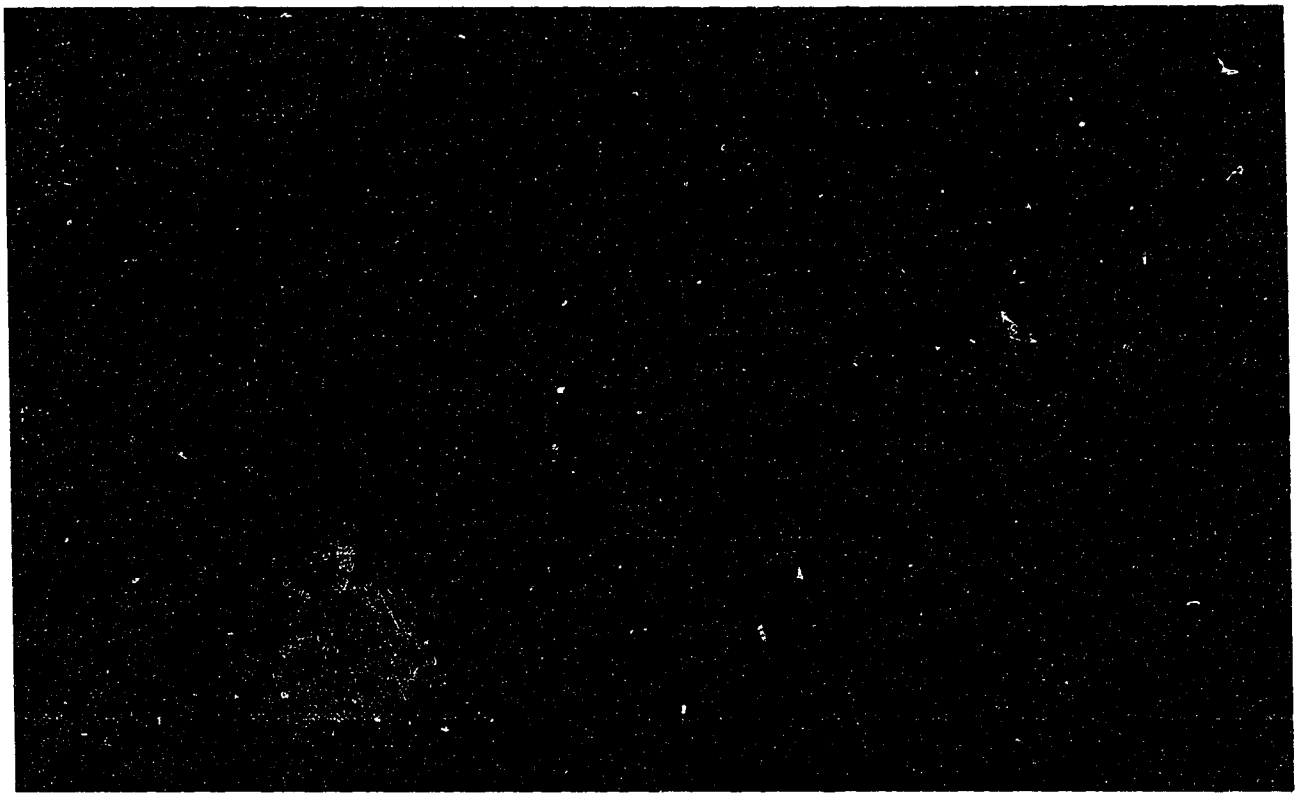
FIGURE 6.6. LOW MAGNIFICATION VIEW OF DAY 28 NEONATAL RAT LUNGS.

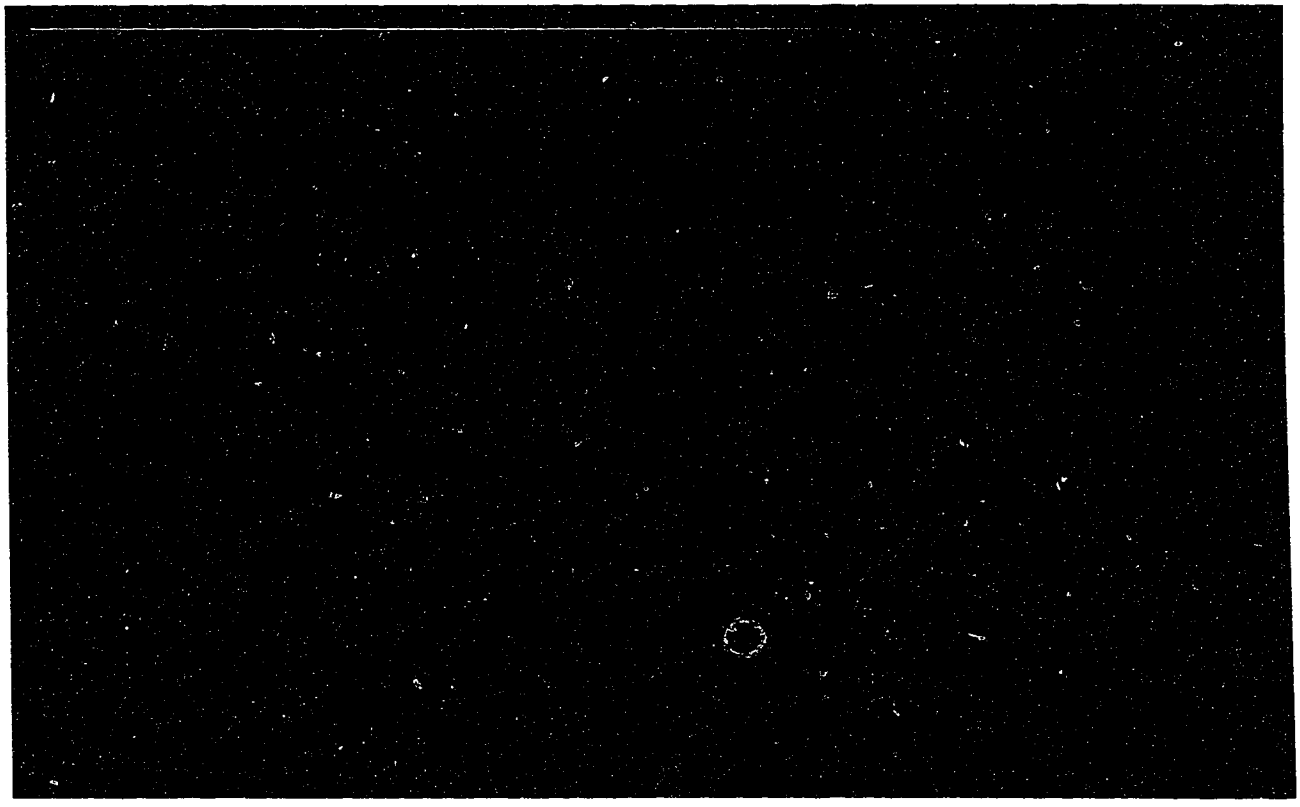
Lung specimens from day 28 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 40 X and represented in the photographs at 180 X (i.e., 1.8 mm in picture=10 μm actual). **A**) hyperoxic group. Large blood vessels (bv) had localized increases in muscularization and marked fibrosis. The parenchyma had alternating regions of emphysema and atelectasis (arrow) and the airspaces were mainly irregularly shaped alveolar sacs (□). The alveolar wall appear more cellular. **B**) normoxic group. The parenchyma had a more uniform appearance than the hyperoxic lungs and was composed of more well-expanded alveoli (*). The interstitium was very thin. These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=7 animals in each group).


A

FIGURE 6.7. HIGHER MAGNIFICATION VIEW OF DAY 28 NEONATAL RAT LUNGS.

Lung specimens from day 28 exposed neonatal rat lungs stained with hematoxylin and eosin. Sections were viewed with a light microscope at a magnification of 63 X and represented in the photographs at 280 X (i.e., 2.8 mm in picture=10 μm actual). **A)** hyperoxic group. The alveolar walls appear fragmented and debris was seen in the intraalveolar spaces (open arrowhead). The interstitium was very cellular and contained focal clumps of markedly increased numbers of Type II cells (II). **B)** normoxic group. The interstitium contained uniformly distributed nuclei and was functionally very thin. Many capillaries containing RBCs were seen in the interstitium (c). These are representative sections from 1 hyperoxic and 1 normoxic exposed animal (n=7 animals in each group).





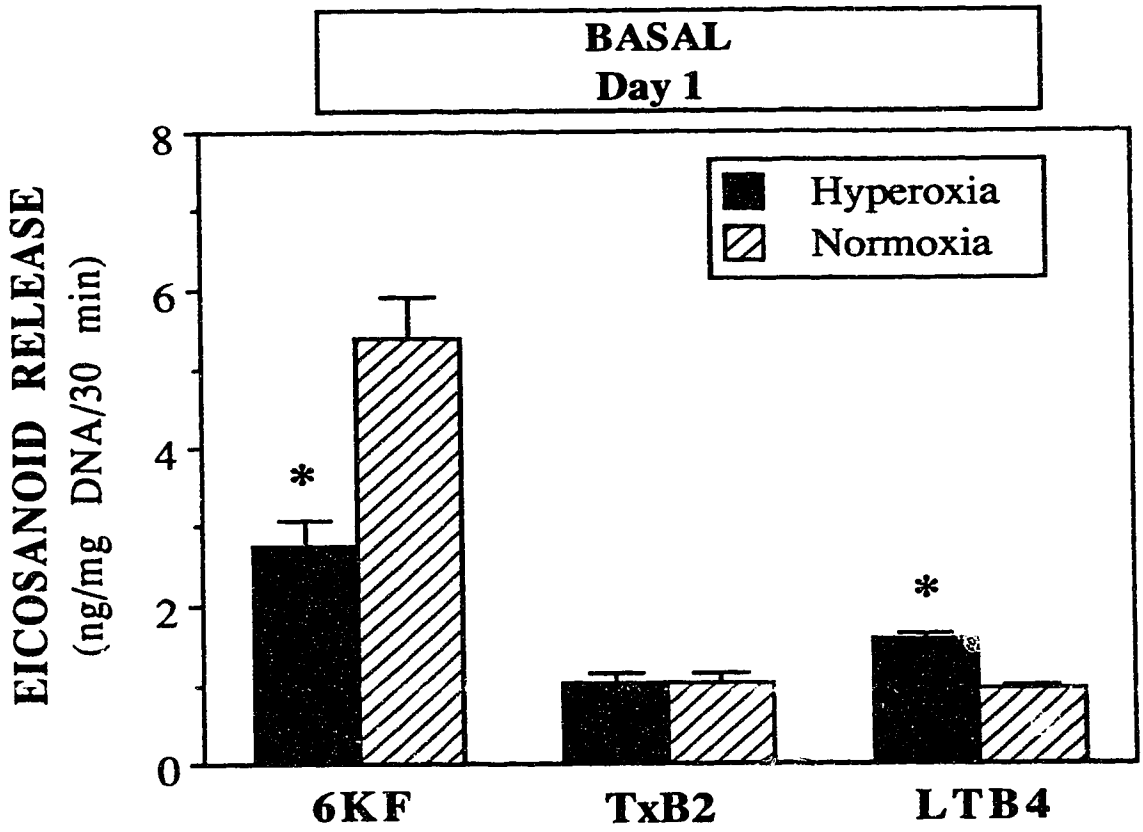


FIGURE 6.8. BASAL EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING ONE DAY OF EXPOSURE.
Lung explants were prepared (6 pups/litter) following one day of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Basal release of 6KF, TxB_2 and LTB_4 for a 30 minute interval was assessed after a 120 minute pre-incubation. One day of hyperoxic exposure caused a decrease in basal 6KF production and an increase in basal LTB_4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

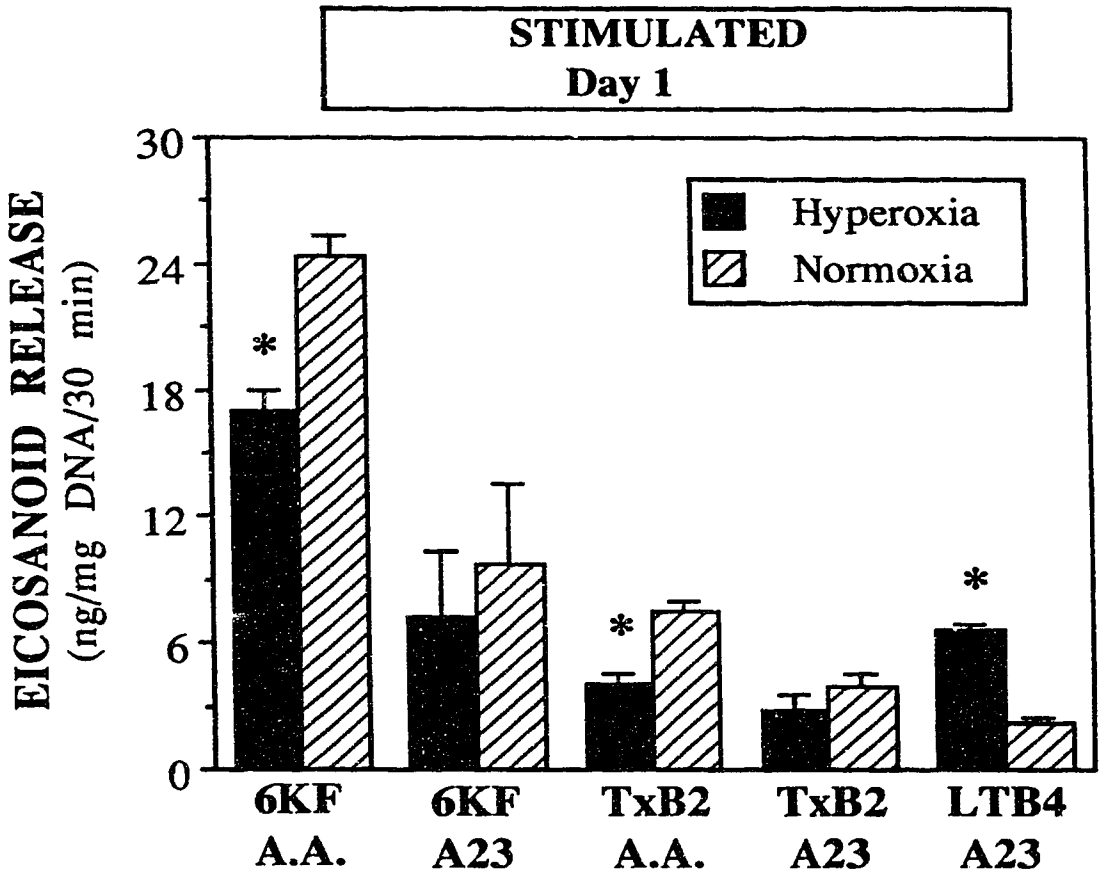


FIGURE 6.9. STIMULATED EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING ONE DAY OF EXPOSURE.

Lung explants were prepared (6 pups/litter) following one day of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Following 150 minutes of pre-incubation, 6KF and TxB_2 production was maximally stimulated with 100 μM A.A. and LTB4 production with 50μM A23187 for a 30 minute interval. 6KF and TxB_2 production from exogenous A.A. was determined after correction for eicosanoid production from endogenous A.A.. One day of hyperoxic exposure caused a decrease in 6KF and TxB_2 production from exogenous A.A. and an increase in A23187 stimulated LTB4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

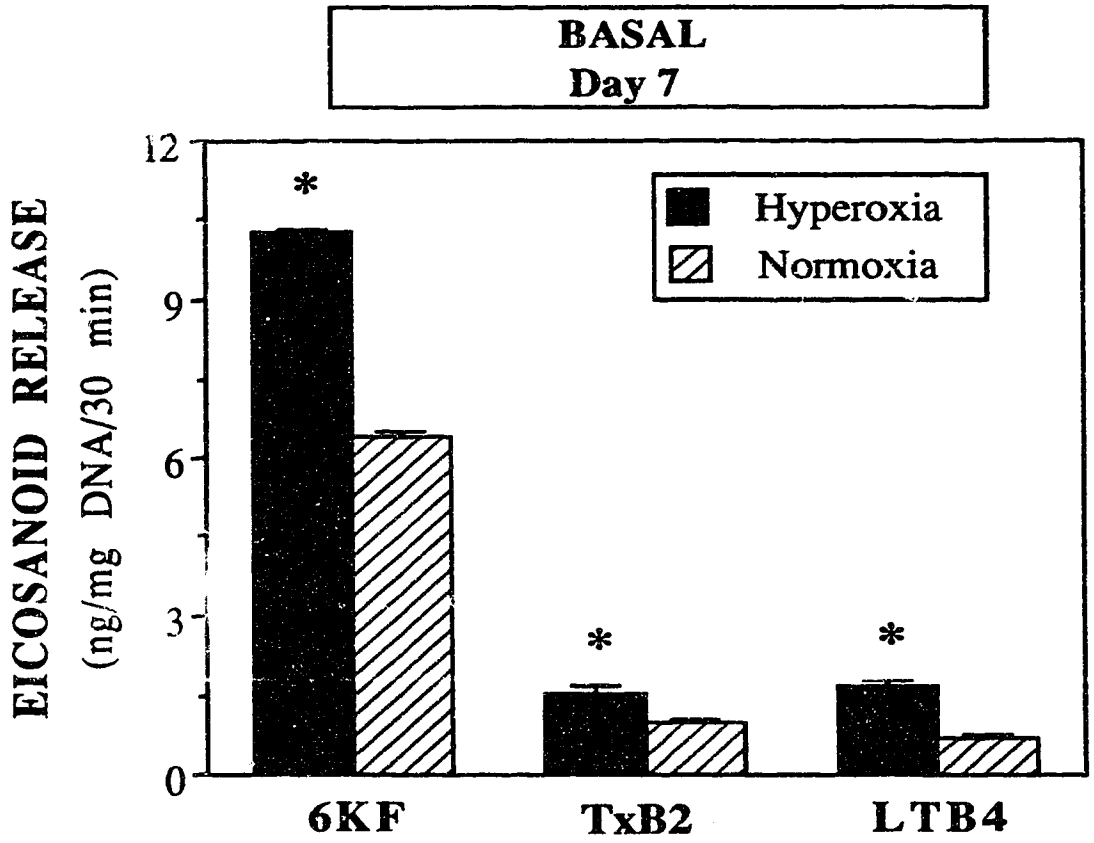


FIGURE 6.10. BASAL EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING SEVEN DAYS OF EXPOSURE.
Lung explants were prepared (6 pups/litter) following seven days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Basal release of 6KF, TxB_2 and LTB_4 for a 30 minute interval was assessed after a 120 minute pre-incubation. Seven days of hyperoxic exposure caused an increase in basal 6KF, TxB_2 and LTB_4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

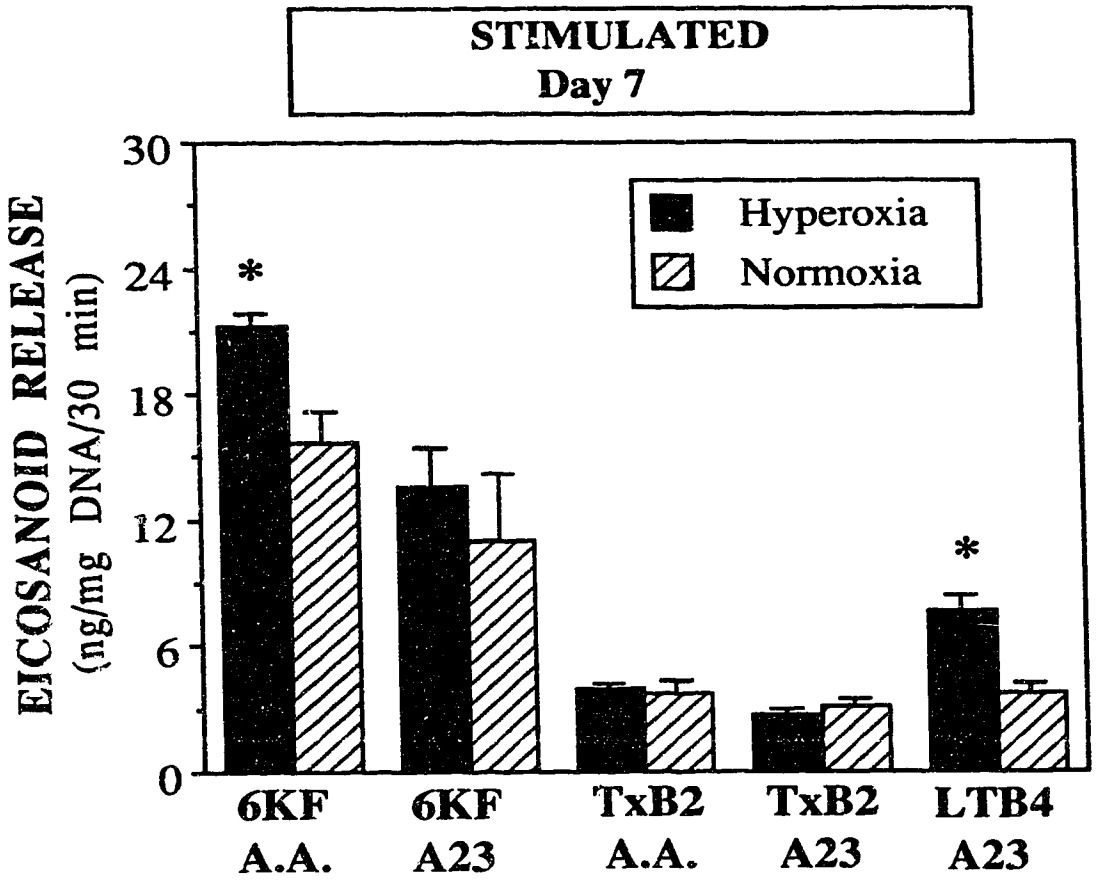


FIGURE 6.11. STIMULATED EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING SEVEN DAYS OF EXPOSURE.

Lung explants were prepared (6 pups/litter) following seven days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Eicosanoid release was determined as described in Figure 6.9.. Seven days of hyperoxic exposure caused an increase in 6KF production from exogenous A.A. and an increase in A23187 stimulated LTB4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

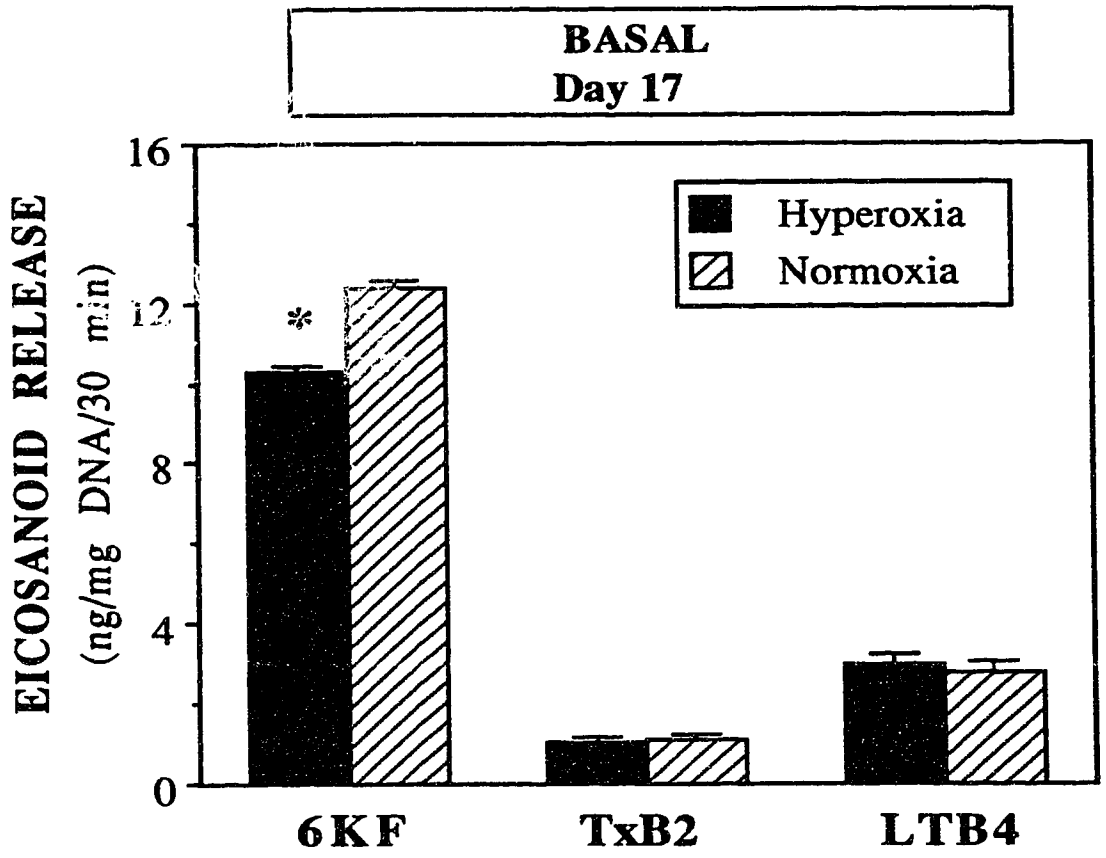


FIGURE 6.12. BASAL EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING SEVENTEEN DAYS OF EXPOSURE.
Lung explants were prepared (6 pups/litter) following seventeen days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Basal release of 6KF, TxB_2 and LTB_4 for a 30 minute interval was assessed after a 120 minute pre-incubation. Seventeen days of hyperoxic exposure caused a decrease in basal 6KF production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

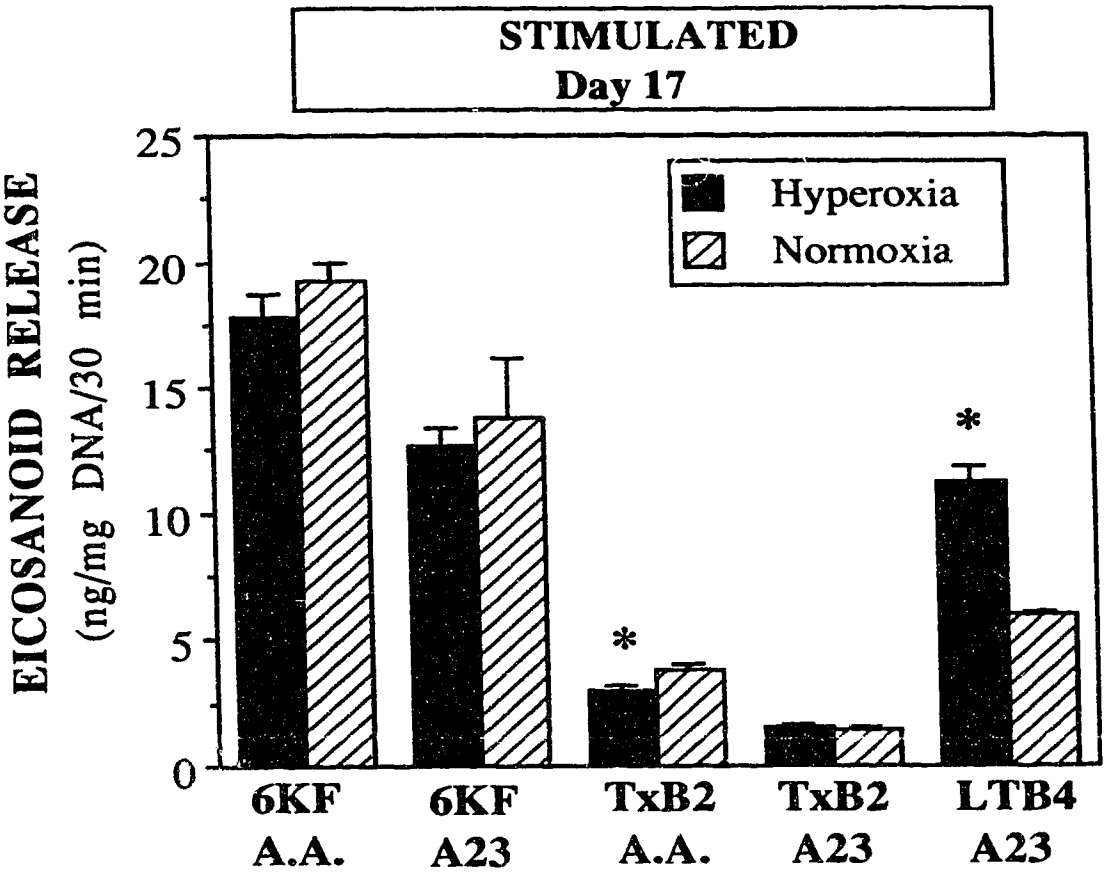


FIGURE 6.13. STIMULATED EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING SEVENTEEN DAYS OF EXPOSURE.

Lung explants were prepared (6 pups/litter) following seventeen days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Eicosanoid release was determined as described in Figure 6.9.. Seventeen days of hyperoxic exposure caused a decrease in A.A. stimulated TxB2 release and an increase in A23187 stimulated LTB4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

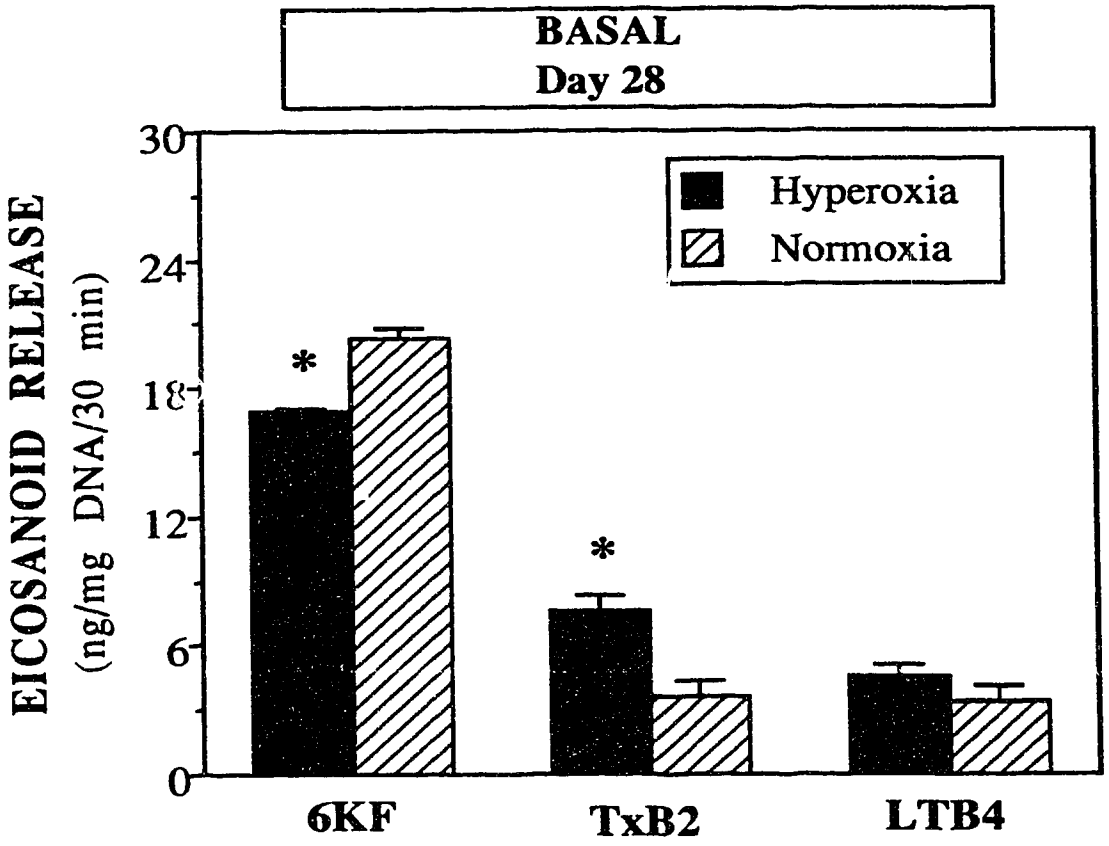


FIGURE 6.14. BASAL EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING TWENTY-EIGHT DAYS OF EXPOSURE. Lung explants were prepared (6 pups/litter) following twenty-eight days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Basal release of 6KF, TxB_2 and LTB_4 for a 30 minute interval was assessed after a 120 minute pre-incubation. Twenty-eight days of hyperoxic exposure caused a decrease in basal 6KF production and an increase in basal TxB_2 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

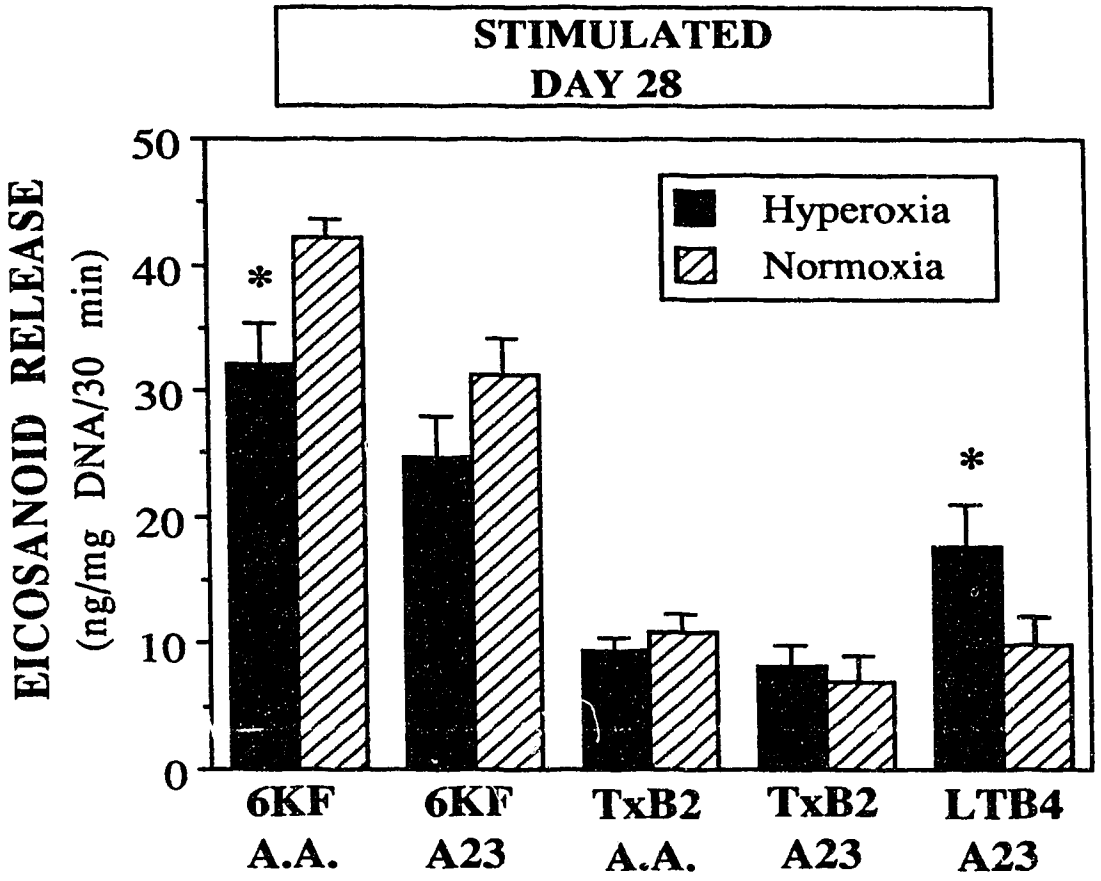


FIGURE 6.15. STIMULATED EICOSANOID RELEASE FROM NEONATAL RAT LUNG EXPLANTS FOLLOWING TWENTY-EIGHT DAYS OF EXPOSURE.

Lung explants were prepared (6 pups/litter) following twenty-eight days of *in vivo* pre-exposure to hyperoxia (solid bars) or normoxia (hatched bars). Eicosanoid release was determined as described in Figure 6.9.. Twenty-eight days of hyperoxic exposure caused a decrease in A.A. stimulated 6KF release and an increase in A23187 stimulated LTB4 production. Results represent MEAN ± SD of 5 replicate litters. The * indicates a significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

TABLE 6.A.

EFFECTS OF HYPEROXIA ON BODY WEIGHTS, LUNG WEIGHT RATIOS AND LIVER WEIGHT RATIO.

Body weight, wet lung weight, dry lung weight (dried to constant weight at 37°C) and wet liver weight were determined for neonatal rat pups (3 pups/litter) after 1, 7, 17 or 28 days of exposure to hyperoxia (O_2) or normoxia (AIR). Hyperoxia had no effect on body weight. Wet lung weight-to-body weight ratios (WL/BW X 1000) indicate changes in lung fluid and tissue components of the lung. Dry lung weight-to-body weight ratios (DL/BW X 1000) indicate changes in the tissue components of the lung. Wet lung weight-to-dry lung weight (WL/DL) indicates changes in the water content of the lungs. Wet liver weight-to-body weight ratios (LIV/BW X 1000) were determined to examine the organ specific effect of hyperoxia on the lungs. See text for details of results. Values represent MEAN ± SD of 5 replicate litters. The * indicates significant difference between hyperoxia and normoxia at $p<0.05$ (see text, 6.2.7. for details of statistical analysis).

WEIGHTS & RATIOS	EXPOSURE CONDITIONS							
	DAY 1		DAY 7		DAY 17		DAY 28	
	O_2	AIR	O_2	AIR	O_2	AIR	O_2	AIR
BW (g)	**6.4** ±0.2	**6.4** ±0.2	**13.4** ±0.8	**13.6** ±0.8	**29.7** ±0.9	**29.6** ±1.1	**68.4** ±1.8	**68.6** ±1.5
WL/BW (X1000)	**16.6** ±0.3	**16.1** ±0.3	**15.4** * ±0.7	**17.1** ±0.06	**12.2** * ±0.04	**10.2** ±0.3	**8.7** * ±0.1	**6.4** ±0.1
DL/BW (X1000)	**2.9** ±0.1	**2.8** ±0.1	**2.8** * ±0.1	**3.5** ±0.1	**2.3** * ±0.04	**2.1** ±0.05	**1.8** * ±0.06	**1.3** ±0.02
WL/DL	**5.8** ±0.2	**5.8** ±0.2	**5.4** * ±0.1	**4.9** ±0.1	**5.3** * ±0.1	**4.9** ±0.1	**4.8** ±0.2	**5.1** ±0.1
LIV/BW (X1000)	**56.3** ±2.4	**57.3** ±1.7	**30.1** ±1.2	**29.2** ±2.0	**31.1** ±0.8	**31.6** ±0.9	**47.5** ±1.9	**49.0** ±2.1

CHAPTER 7

FUTURE STUDIES

The findings of this study indicate that the exposure model and the techniques used are valid tools to determine the lung eicosanoid profile during the progression of hyperoxic lung injury. Utilizing this protocol, there are many new questions that may be asked and answered.

The sulfidopeptide leukotrienes may be important in the pathogenesis of hyperoxic lung injury and therefore should be quantitated utilizing the same procedures of this study. To determine the total amount of this group of leukotrienes, it has been shown that an enzymatic conversion of LTC4 and LTD4 to the end product of LTE4 is useful (Hoppe *et al.*, 1986). It is necessary to assess the requirement of extracting these peptide-containing eicosanoids (Miyamoto *et al.*, 1987).

Questions at the cellular and molecular levels also arise.

What are the effects of hyperoxia at the ultrastructural level using this hyperoxic protocol?

Electron microscopic evaluation of the lungs at the various exposure durations will answer this question.

Are there differential effects of hyperoxia on the various cell populations of the lung?

Following pre-exposure to hyperoxia, the lung tissue is enzymatically digested and the various cell populations are separated by Percoll fractionation (Holt, 1985). The number cells of each population are determined to appraise changes between hyperoxia and controls. The eicosanoid profile and enzyme assays (see below) of each cell population are determined to assess alterations caused by hyperoxia.

At what enzymatic steps is oxygen having an effect?

Enzyme assays for PLA2 (Leslie, 1991), PGHS (Olson lab protocol), 5-lipoxygenase and LTA4 hydrolase (Rouzer *et al.*, 1988) and 15-OH-PGDH (Chaudhari *et al.*, 1979) are assessed to determine if pre-exposure to hyperoxia has altered the activities of these enzymes. Then it is necessary to relate changes in the enzyme activities with the levels of the individual eicosanoid that were measured.

Is this effect produced on the protein structure of the enzyme?
The mass of the individual enzymes may be determined by western blot analysis. In the Olson laboratory a protocol for PGHS quantitation is currently in use.

Does hyperoxia affect the gene expression of the various enzymes of the eicosanoid pathway?
Following pre-exposure to hyperoxia, northern blot analysis may be used to determine changes in the levels of mRNA for the various eicosanoid enzymes in the lung tissue.

In order to establish a cause and effect relationship for eicosanoids in causing lung injury, it is necessary to perform experiments where the eicosanoid pathways are exogenously manipulated.

1) The hypothesis that elevated leukotrienes may promote lung injury can be evaluated by blocking this pathway during hyperoxic exposure. Eicosanoids are determined to verify the effectiveness of the blocking compound and the effect on lung injury is assessed.
2) In conjunction with the above experiments, studies should examine the ability of exogenous administration of leukotrienes in normoxia to mimic the lung injury as seen with hyperoxia.

3) The hypothesis that prostacyclin may be a protective mechanism can be tested by exogenous administration of prostacyclin during hyperoxic exposure and determining if lung injury is lessened.

These are a few suggestions for future directions that this study should take in an attempt to elucidate the role of eicosanoids in mediating and modulating hyperoxic lung injury. Also to examine the specific actions of oxygen on the various levels of the eicosanoid cascade in order to determine where pharmacological intervention may be of most benefit.

CHAPTER 8

SUMMARY

The main study was undertaken to investigate the participation of the eicosanoids in hyperoxic lung injury in the neonatal rat lung. Preceding this study, development of the techniques that were to be utilized was required. A lung explant technique was developed in order to determine the eicosanoid profile from the lung using a model that would mimic the *in vivo* environment. Radioimmunoassays were characterized so that eicosanoids could be reproducibly quantitated. With these tools confidently established and characterized, the hyperoxic exposures were conducted.

The lung tissue explant technique was chosen as the method to examine the eicosanoid profile of the lung. The major advantage is that tissue slices maintain the *in vivo* architecture thereby keeping the cell interactions intact. This was considered to be important in the assessment of the eicosanoid profile since their production is very dependent upon the interactive relationships of the various cell types with each other.

Similar techniques using lung explants have been used previously but were longer term cultures that examined development of the perinatal lung (Black *et al.*, 1951, Gross *et al.*, 1978, Davis *et al.*, 1990). However, for this study it was desired to determine the production of eicosanoids by the lung as close in time as to what was occurring in the animal. *In vitro* developmental changes were not to be examined as in these studies. Therefore, a short-term incubation procedure was developed. The first obstacle encountered was the large, artifactual release of eicosanoids following tissue slicing. This was overcome by a 150 minute pre-incubation so that the tissue was at basal release levels. Then it was necessary to determine the concentrations of the stimulators exogenous A.A. and A23187 that would be optimal with the use of whole tissue slices. As was expected, the concentrations were larger, 100 μM for A.A. and 50 μM for A23187, than for cell culture since the compounds would have to diffuse through the thickness of the tissue to reach all the cells. The time that these stimulators must be given in order to maximally activate eicosanoid production was determined and was found to be 30 minutes. The explants were found to be viable throughout the duration of the incubation. LDH which was found to be high following cell disruption from slicing but then the remaining cells did not release significant amounts of LDH. Now with the explant technique well-characterized it was ready for use in the hyperoxic exposure studies.

Radioimmunoassay was chosen as the methodology for measuring the eicosanoids, 6KF, TxB_2 and LTB_4 due to its specificity and sensitivity and practicality in determinations of the large number of samples generated. Other techniques such as gas chromotography/mass spectrometry and high pressure liquid chromatography lack the sensitivity of RIA although they have a greater specificity (Murphy, 1985). Other drawbacks of these techniques are that they are more technically involved thus are able to process a small number of samples for immediate analysis also they were not routine procedures in our laborabory.

It was necessary to characterize the antisera that were used in this quantitative method since they were new batches. The detailed characterization of the RIA technique illustrated and confirmed that it was specific since the antisera did not cross-react with other related compounds. It was reproducible since the intra- and inter-assay % C.V.'s were generally lower than the acceptable 10% (Pegg, 1976) and was sensitive since the lower limit of detection was found to be lower than the smallest dose of the standard curve of 3.9 pg/0.1 ml. This agreed with findings of others using similar assays (Salmon *et al.*, 1982, Hageman *et al.*, 1986). The assays were also accurate as indicated by the recovery of known amounts of added eicosanoids.

The TxB_2 and LTB_4 radioimmunoassays had not been previously used in our laboratory and therefore required assessment as to whether the procedure as used for 6KF was suitable. Many

details such as determination of the composition of the charcoal solution and the quantity to be used in order to efficiently separate the free and bound eicosanoid was essential. The pH of the assay system had to be investigated since other procedures suggest for LTB_4 the pH should be 8.6 to prevent lactonization (Salmon *et al.*, 1982). However, it was found in this assay system adjustment of the pH did not make a difference. It was found that the procedure used for the 6KF assay was optimal for the other two assays.

Therefore, following this detailed characterization, the RIAs for 6KF, TxB_2 and LTB_4 were now ready to be used to determine eicosanoid production by the neonatal rat lung explants.

Neonatal rat pups were exposed to a hyperoxic regime that induced lung injury which followed the pathogenic sequence of an acute phase followed by a repair phase. The acute phase was evident by day 7 of >95% oxygen as indicated by the decreased DL/BW ratio which was suggestive of hypoplasia of the lung in comparison with age matched normoxic-exposed controls. Histological analysis demonstrated that hyperoxia produced alveolar walls that were attenuated and acellular as compared to age matched controls. In conjunction with hypoplasia, there was evidence of pulmonary edema since the WL/DL ratio was higher in the hyperoxic group. The eicosanoid profile of the lung responded differently to hyperoxia on day 7. 6KF, TxB_2 and LTB_4 were all elevated. This may be a response of the lung to protect itself from oxygen toxicity.

On day 17 and 28 with 60% oxygen, the lung demonstrated the repair response. The repair phenomena were aberrant as seen by the higher DL/BW suggesting lung hyperplasia. Histological analysis demonstrated focal increases in Type II cells and fibroblasts and perivascular and peribronchiolar fibrosis. Residual edema still existed on day 17 but after 28 days this resolved. The eicosanoid profile changed again with hyperoxia where PGHS metabolites were generally decreased but LTs were still elevated.

From this series of exposure experiments a direct causal relationship cannot be established for the individual eicosanoids. What has been demonstrated is that pre-exposure of the neonatal rat to hyperoxia does produce the characteristic lung injury. In conjunction with this, the eicosanoid profile of the lung responds to the hyperoxic stimulus and is altered progressively throughout the exposure protocol. It has been speculated that the homeostatic balanced levels of the various eicosanoids in relation to each other are an important determinant. These findings point to the need for further investigation into the specific roles of the eicosanoids in the pathogenesis of hyperoxic lung injury.

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