A Combined In Vivo and In Vitro Approach to the Study of Endotoxemia in Swine

Importance of Neuropeptides and Implications for Human Adult Respiratory Distress Syndrome

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science
in
Veterinary Medical Sciences

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June 6, 2000
Blacksburg, Virginia

Keywords: endotoxin, ARDS, swine, pathophysiology, lungs, neuropeptides

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Abstract

The cardiopulmonary effects of endotoxin administration (1 microgram/kg) were evaluated in 8-10 week old SPF-derived Yorkshire pigs, both because endotoxemia is a common and important swine problem, and because the pig is a good model for human adult respiratory distress syndrome. Physiological changes included sustained increases in mean pulmonary artery pressure, pulmonary vascular resistance, pulmonary arterial wedge pressure, heart rate, hematocrit, and the arterial partial pressure of carbon dioxide. Transient increases were also observed in central venous pressure and airway pressure. Transient increases, followed by decreases, were observed in mean systemic arterial pressures and systemic vascular resistance. Decreases were seen in cardiac output, cardiac index, arterial partial pressure of oxygen and oxygen saturation. The number of circulating leukocytes, lymphocytes and segmented neutrophils decreased with endotoxin infusion. To investigate the role of airway smooth muscle, bronchial rings were isolated and exposed to contractile agents in tissue baths. A hyperresponsiveness of the third generation bronchi to substance P, carbachol, bradykinin and electric field stimulation was observed. However the increase in response to bradykinin and electric field stimulation were not statistically significant. Histopathology of the lungs demonstrated congestion, hemorrhage and neutrophilic infiltration.

Dedication

I dedicate this work to my father, whose love of life and knowledge inspired me to attempt to understand that which is not understood and to my wife whose understanding and support made this undertaking possible.

Acknowledgements

I wish to thank Dr. Richard Broadstone for his time, effort, insight and patience. With out his guidance and assistance this project would not have been possible.
I also wish to thank Dr. John Robertson for his assistance with and instruction concerning the histopathological analysis of the lungs.
I wish to thank Dr Richard Broadstone, Dr. Peter Eyre and Dr. John Robertson for their diligent editing and support of this work.
I wish to give special thanks to Daniel Ward for his statistical expertise and assistance.
Finally, I wish to thank Kim Jessup for her technical assistance.

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List of Abbreviations

$AaDO_2$	alveolar to arterial oxygen gradient
ACh	acetylcholine
ARDS	adult respiratory distress syndrome
ATP	adenosine triphosphate
BK	bradykinin
C	Celsius
C-fibers	capsaicin sensitive nerve fibers
Ca	calcium
CAMP	cyclic adenosine monophosphate
CBC	complete blood count
CD	cluster of differentiation
CGMP	cyclic guanine monophosphate
CI	cardiac index
CL	chloride
cmH_2O	centimeters of water
CO	cardiac output
COPD	chronic obstructive pulmonary disorder
CVP	central venous pressure
DAG	diacyl glycerol
G protein	guanine nucleotide binding protein
H	hydrogen
H&E	hematoxalin and eosin
HCT	hematocrit
HETE	hydroxyeciosatetraenoic acid

HPETE	hydroperoxyeciosatetraenoic acid
Hr	hour
HR	heart rate
IP_3	inositol triphosphate
K	potassium
Kg	kilogram
LPS	lipopolysaccharide
LT	leukotrienes
M	molar
MCAP	mean central arterial pressure
Mg	milligram
Ml	milliliter
M	molar
MM	millimolar
MmHg	millimeters of mercury
MPAP	mean pulmonary arterial pressure
n	sample size
Na	sodium
NANC	nonadrenergic-noncholinergic nervous system
NEP	neutral endopeptidase
NKA	neurokinin A
NKB	neurokinin B
NSAIDS	nonsteroidal anti-inflammatory drugs
O	oxygen
P	phosphorus
PAF	platelet activating factor
PaO_2	arterial partial pressure of oxygen
$PaCO_2$	arterial partial pressure of carbon dioxide
PG	prostaglandins

PNS	parasympathetic nervous system
Ppaw	pulmonary arterial wedge pressure
PVR	pulmonary vascular resistance
SNS	sympathetic nervous system
SPF	Specific pathogen free
SPO_2	oxygen saturation
T	time
TPR	total peripheral resistance
WBC	white blood cell

Introduction

Endotoxemia causes significant morbidity and mortality in pigs resulting in losses of millions of dollars each year in the swine industry. When exposed to endotoxin the porcine respiratory tract has also been shown to be a good model for human adult respiratory distress syndrome (ARDS) (Borg 1985, Kreimeier 1988, Hardy 1987). For these reasons there has been a great deal of interest in recent years concerning the pathophysiology of endotoxemia in swine.

Pigs are an excellent model for investigating the pulmonary effects of endotoxin, for several reasons. First, the growth pattern of the lungs, the anatomy of the lung parenchyma and the architecture and reactivity of the pulmonary vasculature of swine are similar to those of humans. Second, pulmonary changes seen following endotoxin exposure resemble the human disease syndrome ARDS. The cardiopulmonary and hemodynamic reactions, as well as multiple organ failure, granulocytopenia, thrombocytopenia, pulmonary hypertension, microembolism, and edema which develop in swine administered endotoxin, are also similar to those of humans with ARDS. Third, the anesthetized pig is a large enough species to allow for thorough physiological monitoring, including blood pressures, cardiac outputs, heart rates, airway pressures and blood sampling. Fourth, the porcine respiratory tract is of sufficient size to provide multiple sections of third generation bronchi for in vitro contraction studies. Fifth, the lung lobes of the pig are large enough for fixation and multiple sectioning concurrent with in vitro studies, enzyme assays, bronchial alveolar lavage, etc. This allows for proper histopathologic analysis to occur along with other studies to further characterize the changes that result from endotoxin administration.

These experiments were designed to examine the physiological effects of endotoxin in vivo, and to attempt to correlate these effects with the histopathology and the in vitro changes in contractility of isolated rings of bronchial smooth muscle. To perform these investigations, studies were performed to (1) determine the cardiopulmonary and hemodynamic effects of endotoxin in anesthetized, positive pressure ventilated swine, (2) look for changes in the blood gas and hematological (CBC)

values, (3) determine if there are changes in the responses to various contractile stimuli in vitro, (4) estimate the importance of substance P and bradykinin in endotoxin induced airway hyperresponsiveness, (5) determine the histologic changes induced by endotoxin administration, and (6) to determine if there is acute pulmonary inflammation following endotoxin exposure.

Review of Literature

I. Smooth muscle and pulmonary function.

Smooth muscle, so named because of its lack of obvious transverse striations, is the type of muscle that lines the respiratory tract. Smooth muscle is a so-called involuntary muscle, due to the lack of conscious control over its contractions and relaxations. Contraction instead is controlled by various neural and humoral mediators. The concentrations of these mediators in the lungs is determined by the complex interplay of the animal, the environment and various inflammatory or noxious stimuli. The stimuli, mediators, receptors, and second messenger systems vary, but the final pathway leading to smooth muscle contraction is always the same. The intracellular calcium concentration, which regulates actin-myosin cross-bridge formation, is increased by release from the sarcoplasmic reticulum and by entry of extracellular calcium across the sarcolemma. Calcium thereby binds to calmodulin, a protein that binds to myosin light chain kinase, exposing the catalytic site of the myosin and allowing it to bind adenosine triphosphate (ATP). As ATP is hydrolyzed the myosin ratchets along the actin causing shortening of the cell. The resulting contraction of airway smooth muscle (bronchoconstriction) causes an increase in airway resistance and a decrease in airflow. Poiseuille's law states that by halving the radius of the airway it would take sixteen times as much pressure to maintain the same airflow. Thus, the effects of bronchoconstriction can include increased respiratory effort, decreased ventilation, ventilation-perfusion mismatches and decreased oxygenation of the blood.

II. Control of pulmonary smooth muscle function.

The regulators of smooth muscle tone in the respiratory tract include the autonomic nervous system (Chad 1979, Gustin 1989, Moses 1985, Scott 1991, Sulnin

1987), the nonadrenergic-noncholinergic (NANC) nervous system (Ingue 1989, Moses 1985), and an array of local and humoral mediators, including histamine (Chad 1979, Derkson 1985.), eicosinoids (Ito 1982, 1991) and kinins. Generally speaking, autonomic nervous system control predominates in the larger bronchi and the trachea, whereas the NANC system and the local and humoral mediators are of greater importance in determining the diameter of the more distal airways (Matera 1997).

A. Nervous control

The autonomic nervous system is conventionally divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). In the respiratory tract the sympathetic nervous system produces bronchodilation (smooth muscle relaxation) through the activation of β_2 receptors, primarily by epinephrine from the circulation. Because of this effect, epinephrine is used in the treatment of bronchoconstrictive disorders, such as anaphylactic shock. The sympathetic mediators, dopamine, norephinephrine and epinephrine, are all synthesized from a common precursor, the amino acid phenylalanine, by a series of hydroxylase and decarboxylase reactions (Vulliet 1980). These mediators all act at adrenergic receptors but have different affinity for the different types and subtypes of receptors. Norepinephrine acts primarily at α_1 and β_1 receptors and is released from autonomic nerves whereas epinephrine acts with greater affinity at β_1 and β_2 receptors and is released from the adrenal medulla. The activation of these different receptors produces different effects. Beta$_1$ receptor activation causes an increase in heart rate and contractility, whereas β_2 receptor activation causes a relaxation of some vascular and airway smooth muscle. The activation of α_1 receptors causes a vasoconstrictive response and, in some species, causes bronchoconstriction as well (Scott 1991). Alpha$_2$ receptor activation constitutes a neuronal feedback loop and causes a decrease in norepinephrine release.

The effects on smooth muscle of both α and β receptor stimulation are mediated through guanine nucleotide binding proteins (G proteins) (Lambert 1993, Levitzki 1993, Schwinn 1993)). Beta receptor stimulation increases the concentration of cAMP and decreases the concentration of cGMP leading to the activation of protein kinase A (PKA).

Protein kinase A in turn activates the enzymes involved in smooth muscle relaxation, leading to bronchodilitation and vasodilatation. Stimulation of α receptors activates phospholipase C producing inositol triphosphate (IP_3) and diacyl glyceride (DAG). Inositol triphosphate acts by increasing intracellular calcium concentrations and DAG activates protein kinase C, both of which lead to smooth muscle contraction. Alpha receptor activation can also cause a decrease in cAMP, which also causes smooth muscle contraction (Table 1).

The parasympathetic branch of the ANS acts through acetylcholine (ACh) binding to muscarinic receptors and producing bronchoconstriction. The muscarinic receptors, named for the plant alkaloid muscarine which activates them, are found on smooth muscle and in glands, and cause smooth muscle contraction and glandular secretion. The blocking of this cholinergic contractile response is the object of the use of anticholinergic drugs in the treatment of disorders such as chronic obstructive pulmonary disease (COPD). Acetylcholine is released from cholinergic neurons and causes decreased heart rate and contractility, vasodilation (in some vessels), bronchoconstriction and increased mucus secretion. The mechanism of action of ACh differs in different tissues, but in bronchial smooth muscle it acts by decreasing cAMP levels. Nicotinic receptors are found in sympathetic and parasympathetic ganglia as well as at the skeletal neuromuscular junction, and cause stimulation of postganglionic nerves or muscle contraction when activated. These receptors are called nicotinic receptors because they are the site of action of nicotine, an addictive substance found in tobacco. Carbachol is a parasympathomimetic drug that acts at both nicotinic and muscarinic receptors. A major difference between ACh and carbachol is that ACh is rapidly broken down by cholinesterases whereas carbachol is hydrolyzed extremely slowly. This is because ACh is a naturally occurring parasympathomimetic that is released due to depolarization of a cholinergic neuron and whose action is normally terminated by cholinesterases found at the synapse. Carbachol however is a synthetic compound whose structure is similar enough to allow it to activate the same receptors as ACh, but the structure of carbachol is

also different enough from that of ACh to prevent the cholinesterases from degrading it efficiently.

The NANC system, which is considered by some to be a branch of the autonomic nervous system, consists of unmyelinated sensory fibers. These fibers are capsaicin sensitive, meaning that capsaicin can stimulate them, and so are referred to as C-fibers. These C-fibers are found in the respiratory epithelium, blood vessels, tracheobronchial smooth muscle, and in the tracheobronchial ganglia (Martling 1990). The activation of this system is thought to be due to local irritant responses and to be caused by the release of inflammatory mediators. Stimulation of C-fibers leads to the release of a variety of mediators. There appear to be two types of NANC nerves. Inhibitory NANC, or i-NANC, release vasoactive intestinal peptide and nitric oxide and mediate bronchodilation (Barnes 1984, 1986 (1 & 2). The excitatory NANC (e-NANC) effects are produced by neuropeptide Y, calcitonin gene related peptide, and tachykinins. These peptides produce bronchoconstriction, increased mucus secretion, enhanced vascular permeability and vasodilatation (Borson 1984, Lundberg 1987, Matran 1989, Peatfield 1983, Widdicombe 1990). Some effects of NANC activation may be mediated through the activation of the classic autonomic nerves.

B. Local and humoral mediators

Histamine is a biogenic amine, a primary mediator of acute inflammation, anaphylaxis and allergies. Histamine acts through H_1 receptors to contract bronchi and large blood vessels. It relaxes smaller blood vessels leading to a reduction in peripheral vascular resistance and blood pressure by activating both the H_1 and the H_2 receptors. Histamine also contracts endothelial cells and causes increased vascular permeability, leading to vascular leakage and edema. Histamine is produced by the decarboxylation of the amino acid histidine. Histamine within the lungs is mostly stored within the secretory granules of mast cells, and is also found in circulating basophils. The importance of histamine as an inflammatory mediator is best demonstrated by the ability of antihistamines (H_1 antagonists) such as diphenhydramine (Benadryl®) to modulate allergic reactions to drugs, vaccines and inhaled allergens.

Bradykinin (BK), a naturally occurring kinin, causes vasodilatation, increased vascular permeability and bronchoconstriction. It acts by stimulating B_1 and B_2 receptors. It is formed by the proteolytic activity of kininogenases on kininogen, an α-2 globulin found in the plasma. Some of BK's actions may be mediated through the production of prostaglandins and the activation of autonomic nerves (Marceau 1985). To date no direct inhibitors of BK are in common therapeutic use, however they would likely prove very useful in a variety of inflammatory reactions, especially those due to snake bites.

Eicosanoids, some of which are potent mediators of inflammation, can also affect bronchial and vascular smooth muscle. They are formed by the action of phospholipase A_2 on arachidonic acid in cell membranes, with further conversion by cyclooxygenase into prostaglandins (PG) and thromboxanes. Lipoxygenase converts arachidonic acid into leukotrienes (LT), hydroperoxyeicosatetraenoic acids (HPETE) and hydroxyeicosatetraenoic acids (HETE). Leukotrienes C_4, D_4, and E_4 make up the so-called Slow-Reacting Substance of Anaphylaxis, contracting smooth muscle, especially in the bronchi. Eicosanoids have varied effects depending on which one is predominating, what tissues it is acting on, and what other mediators are involved. Eicosanoids' inflammatory and algesic effects are well demonstrated by their inhibition by nonsteroidal anti-inflammatory drugs, which inhibit cyclooxygenase and hence prostaglandin production; and by the new lipoxygenase inhibitors, which inhibit leukotriene synthesis.

All of these aforementioned mediators of inflammation have been shown to be important in, or have the potential to be important in the pathogenesis of endotoxic shock. Their exaggerated effects, due to increased release, decreased degradation, the synergistic effects of other mediators, etc., contribute to the increases in airway pressure, pulmonary artery pressure, vascular permeability, cellular infiltration and the decreases in systemic pressure, myocardial and other organ function that typify endotoxic shock.

III. Effects of endotoxin

Endotoxemia not only causes significant morbidity and mortality in pigs costing the swine industry millions of dollars each year, but also when exposed to endotoxin, the porcine respiratory tract has also been shown to be a good model for human adult respiratory distress syndrome (ARDS). For these reasons there has been a great deal of interest in recent years concerning the pathophysiology of endotoxemia in swine.

Endotoxin, or lipopolysaccharide (LPS) is a structural component of the outer cell wall of Gram negative bacteria which is released during the growth phase as well as during lysis. LPS is composed of a long chain fatty acid (lipid A) and a carbohydrate chain (O antigen). The lipid A portion is the biologically active part of the molecule, which binds to an LPS binding protein in the serum. This complex (LPS-protein) then binds to CD_{14} receptors activating the synthesis and release of a cascade of mediators. Thus endotoxin is said to have two mechanisms of action. The first is a direct action on cells, leading to cellular injury and altered function. The second is the activation of cells via the CD_{14} receptor leading to release of inflammatory mediators and the activation of inflammatory cells. The effects of endotoxin are many-fold. LPS acts on the heart causing myocardial dysfunction; on the vascular system causing vasodilatation (hypotension); on the microcirculation causing endothelial injury and leukocyte extravasation; on the coagulation system causing disseminated intravascular coagulopathy; on the lungs causing airway hyperresponsiveness and impaired oxygenation of the blood; and on the liver and kidneys causing organ failure. Endotoxin also induces fever, leukopenia and a Schwartzman reaction.

In swine the respiratory tract is thought to be the most common route of infection by Gram negative bacteria, although any infection causing septicemia can result in endotoxic shock. Infections of the respiratory tract occur by two methods. The first is droplet/aerosol infection, caused when an infected animal sneezes or coughs, expelling droplets of respiratory secretions containing the organisms. These organisms are then inhaled and the previously uninfected animal is exposed, with the outcome depending on

the number of organisms, their pathogenicity and the immune status of the animal. The other method by which a respiratory infection can occur is via the inhalation of dust (dust-borne infection). This occurs when the environment is contaminated by another animal and the previously uninfected animal is exposed either during the occupancy of the infected animal or at some later time by the inhalation of contaminated dust particles. Most of these infections fall into the opportunistic category: that is exposure is common but infection only occurs at times when there is another insult, such as intercurrent infection, environmental contamination with airborne toxicants, endogenous and immune suppressive stress, and increased organism virulence or increased numbers of pathogenic organisms. However, even focal or subclinical infections may cause reductions in feed efficiency and weight gain, increasing the cost of production and lowering the profit margin for swine producers (Straw 1989).

In humans an endotoxin mediated pneumopathy is known as adult respiratory distress syndrome (ARDS). ARDS is characterized by life threatening respiratory insufficiency, cyanosis and severe arterial hypoxia. This syndrome is caused by damage to the alveolar capillary endothelium and the alveolar epithelium, leading to increased permeability, and edema. It is currently believed that this damage is caused by the interaction of inflammatory mediators, infiltrating/resident inflammatory cells and the oxygen radicals they produce. Endotoxin initiates ARDS by stimulating the release of proinflammatory mediators, fostering the expression of adhesion molecules on endothelial cells and promoting the extravasation of leukocytes. The direct effects of endotoxin result in damage to endothelial cells leading to increased release of inflammatory cells into the tissues. The indirect effects of endotoxin, caused by the release of mediators from those inflammatory cells, will be discussed in the following paragraphs.

IV. Pathophysiology of endotoxin

The pathophysiology of endotoxic shock is extremely complex and involves the interactions of a large array of neural and humoral mediators. These various mediators are released at different times, in different amounts and serve to potentiate as well as antagonize each other. Many of them act indirectly through other mediators and may have different effects when acting on different cells. Normally, all of these mediators are interacting in such a way as to maintain homeostasis and to allow the body to compensate for minor changes. However, when exposed to endotoxin some responses become exaggerated while others may be inhibited, thereby producing the complex and changing clinical picture known as septic shock.

The aforementioned complexity is demonstrated by the interactions of autonomic nerves, C-fibers and pulmonary mast cells. It is known that mast cells and C-fibers are found in close proximity to one another in the porcine respiratory tract (Alving 1991). In other species, such as rats and guinea pigs, a functional relationship, whereby each can induce the release of chemical mediators form the other, exists between the two (Mathison 1992). A similar relationship may exist in the pig as well, and may be involved in a local feedback response. Substance P has been shown to cause mast cell degranulation (Hua 1996, Lilli 1995, Reynier-Rebuffel 1994), and the products of mast cell degranulation have been shown to stimulate C-fiber release (Greene 1988). More specifically it has been shown that histamine acting at H_3 receptors on C-fibers can cause the release of tachykinins (Dimitriadou 1994). Thus, after initiation by some noxious stimuli, or an allergen, this local feed back response could ensure a strong defense response. However in the presence of endotoxin this strong defensive response may be turned into a potentially harmful overreaction. Nicotine has also been shown to cause mast cell degranulation (Hua 1994) and the ability of substance P to cause mast cell degranulation is significantly reduced in the presence of atropine (Hua 1996) . This suggests a functional relationship between autonomic nerves and C-fibers, as well as between autonomic nerves and mast cells. C-fibers have also been shown to synapse directly with autonomic nerves and thus it seems likely that some of their effects are due to depolarization of autonomic nerves and the subsequent release of acetylcholine. Some

of the products released by the stimulation of these interacting cells are, and others may be, important mediators in the pathophysiology of endotoxic shock.

A. Effects of endotoxin on nervous regulation.

The nonadrenergic noncholinergic system (NANC) is composed of unmyelinated capsaicin sensitive sensory neurons or C-fibers. The tachykinins, substance P, NKA, and NKB, are released by C-fibers and, along with other mediators, comprise the excitatory nonadrenergic noncholinergic system. Of these, substance P is the most potent in the porcine respiratory tract (Yohannan 1995). In addition to causing mast cell degranulation, substance P can also act at neurokinin-1 receptors causing vasodilatation (Dreshaj 1994, Haxhiu-Poskurica 1992) and protein extravasation, and also at neurokinin-2 receptors causing tracheobronchial smooth muscle contraction (Martin 1989, Staarne 1994) and glandular secretion. It causes decreases in systemic arterial blood pressure, systemic vascular resistance, the partial pressure of oxygen and dynamic compliance. Substance P causes increases in tracheal resistance, alveolar resistance, lung resistance, tissue resistance and calculated airway resistance (Dreshaj 1994). Some of these actions may be mediated through substance P's interactions with autonomic nerves and mast cells. Contraction studies have shown that atropine, a muscarinic receptor antagonist, significantly diminishes the contractile response to substance P and NKA (Haxhiu-Poskurica 1992). This suggests a receptor mediated stimulation of cholinergic nerves, either directly or indirectly, and a mechanism by which these nerves may become involved in the inflammatory process.

The actions of substance P are regulated by its rate of release from C-fibers, by its potentiation and inhibitory effects on the actions of other mediators, and by angiotensin converting enzyme and neutral endopeptidase, which degrade it after it has been released (Shore 1989). Degradation by these enzymes plays an important role in regulation and maintenance of homeostasis, as inhibition of neutral endopeptidase (NEP) by thiorphan has been shown to cause exaggerated responses to substance P (Haxhiu-Poskurica 1992, Webber 1989). Targeted disruption of the NEP locus in mice results in an enhanced lethality at a given dose of endotoxin compared to mice with the NEP locus intact (Lu

1995). This finding indicates an important protective role of NEP in septic shock. The site of action appears downstream from release of tumor necrosis factor and interleukin-1 since NEP-deficient animals demonstrate increased sensitivity to these mediators as well (Lu 1995). This would potentially allow substance P, and the other mediators degraded by NEP, to build up to higher concentrations and remain active for longer periods. Taking all of these issues into account, substance P would appear to be an important inflammatory mediator with a potential role in the pathogenesis of endotoxic shock.

Capsaicin, an extract from hot peppers, causes release of mediators from C-fibers and, when given as a pretreatment, causes partial C-fiber depletion (Alving 1991). Capsaicin has been used to demonstrate the role of C-fibers in endotoxic shock. When given prior to LPS, capsaicin has been shown to prevent the LPS induced airway hyperresponsiveness and to reduce the movement of inflammatory cells into the lungs (Jarareau 1994). This is presumably due to the lack of tachykinins, especially substance P, and their effects on other cells and tissues.

B. Role of enzymatic degradation of mediators in endotoxemia

Neutral endopeptidase is a membrane bound zinc metalo-endopeptidase which degrades neuropeptides, including tachykinins and enkephalins, and humoral mediators, including bradykinin, atriopeptin and endothelins (Gafford 1983), all of which have been shown to play a role in the pathogenesis of endotoxic shock. Contraction studies using tracheal smooth muscle in vitro have shown that inhibition of NEP with thiorphan potentiated the contractile response to substance P (Haxhiu-Poskurica 1992).

C. Effects of endotoxin on local and humoral mediators

Mast cells release a wide variety of mediators including histamine, serotonin, and prostaglandins (depending on the species), all of which have been shown to play important roles in endotoxic shock. Some of these mediators, such as histamine and serotonin are preformed and stored in granules, whereas others such as the prostaglandins are synthesized at the time of insult. Histamine causes a variety of effects including vasodilation (Martin 1989), bronchoconstriction, both directly (through the activation of H_1 and H_2 receptors on smooth muscle) and indirectly through activation of the sensory arm of the cholinergic autonomic nervous system (Dreshaj 1996), and plasma

extravasation. These actions when produced in excess lead to the same pathological changes as substance P and, due to the interaction of C-fibers and mast cells, their concomitant release probably potentiates their effects during endotoxemia. Serotonin causes vasoconstriction, leading to increases in pulmonary vascular resistance, pulmonary arterial pressure, and bronchoconstriction, as demonstrated by blocking these effects with ketanserin, a serotonin antagonist (Olson 1986). The increased pulmonary arterial pressure, coupled with the leaky junctions between and the sloughing of endothelial cells, leads to further losses of plasma and proteins into the surrounding tissues, causing pulmonary edema and acts as a potent stimulus for the margination and infiltration of various inflammatory cells as well. Pulmonary edema produces an extra layer of fluid between the air in the alveoli and the hemoglobin in the blood vessels. This leads to an increased diffusion distance for oxygen and causes an increased partial pressure of oxygen difference between the alveoli and the blood. The pulmonary hypertension and increased pulmonary vascular resistance also lead to increased venous shunting, allowing unoxygenated blood to bypass the lungs and further reducing the PaO_2.

Prostaglandins are formed by the sequential activity of enzymes, including phospholipase A_2 and cyclooxygenase, in a variety of cells, such as mast cells (Peters 1984), epithelium, and pulmonary endothelial cells. Pulmonary endothelial cells exposed to LPS show increases in arachidonic acid metabolism and in cyclooxygenase production (Suttorp 1987), suggesting a possible role for these mediators in endotoxic shock. This role is further demonstrated by the assaying of prostaglandin levels during endotoxemia, the addition of exogenous prostaglandins or their antagonists, and the ability of non steroidal anti-inflammatory drugs and steroids to block some of the effects of endotoxin administration. Thromboxane A_2 levels peak at approximately thirty minutes after administration of endotoxin, whereas prostaglandin F_1 alpha and F_2 alpha peak around four and a half hours, suggesting a progression of mediators in the pathogenesis of endotoxic shock (Hardie 1987). Nonsteroidal anti-inflammatory drugs block the increases in pulmonary vascular resistance, pulmonary hypertension, alveolar to arterial oxygen gradient and the decreases in cardiac output and lung dynamic compliance (Borg 1986,

Modig 1985, Olson 1985), suggesting that these effects are mediated directly or indirectly by or through prostanoids. These effects may be due to a reduction in airway relaxing prostaglandins, such as PGE_2 (Folkerts 1989), as well as due to increases in proinflammatory prostaglandins, such as PGD_4 and PGE_4 which cause bronchoconstriction and pulmonary edema (Fink 1991). Leukotriene B_4 plays an important role in inflammatory cell margination (VanderMeer 1995) into the lungs, which in turn can lead to the production of more inflammatory mediators and the production of oxygen radicals which directly damage the lungs. In granulocyte depleted animals exposed to endotoxin there is significantly less pulmonary hypertension, and extravascular lung water; and significantly better cardiac output and arterial oxygenation (Modig 1987), demonstrating the importance of these migrating cells, as well as the mediators released by them.

Other mediators such as platelet activating factor (PAF) and BK, from platelets, macrophages, and neutrophils respectively, are also important mediators in endotoxic shock. PAF inhibitors attenuate the decreases in cardiac output and stroke volume, pulmonary hypertension, vasoconstriction, bronchoconstriction, hypoxemia and mortality caused by endotoxin administration (Kruse-Elliot 1996, Siebeck 1994). Bradykinin-2 receptor antagonists have been shown to improve the arterial PaO_2 and decrease margination of inflammatory cells into the lung (Ridings 1995). In the normal porcine aorta there are primarily B_2 receptors and very few B_1. However in LPS exposed pigs there is a three-fold increase in B_1 receptors (Schremmer-Danninger 1996, 1998), which appears to occur in the smooth muscle layer of the aorta. Bradykinin-1 receptor stimulation during endotoxic shock attenuates systemic hypotension (Eich-Rathfelder 1997). Antagonism of the B_1 receptor on the other hand causes increased mortality (Siebeck 1996), whereas antagonism of the B_2 receptor causes a decrease in systemic hypotension, as well as a reduction in mortality. Thus, bradykinin has contradictory actions depending on which receptor subtype it is acting. Much is known about the vascular effects of bradykinin, however significantly less is known about the effects of bradykinin on the airways during endotoxemia. In species such as guinea pigs and

humans, BK has been shown to be a potent bronchoconstrictor in addition to its vascular effects. BK production has also been shown to be increased in some inflammatory processes, including those caused by endotoxin administration. Bradykinin's actions appear to occur later in the progression of mediators because bradykinin antagonists attenuate the reduction in systemic arterial pressure beginning at two hours post endotoxin administration (Ridings 1995). The vasodilatory effects of bradykinin appear to be mediated by activation of nitric oxide production and prostacyclin (PGI_2) synthesis (Flemming 1992). Thus, even though bradykinin synthesis is not inhibited by NSAIDS some of its actions may be due to activation of prostacyclin synthesis. Herein lies the principle difficulty in determining exactly which mediators are causing which stages and symptoms of endotoxic shock.

V. In vitro studies and the pharmacology of endotoxemia

Due to the complexity of the interactions involved in the production of endotoxic shock, the inhibition or antagonism of any mediator may have profound effects on pathways in which it is not directly involved. This has lead to much experimentation in vitro, attempting to determine the direct effects of these agonists while blocking some of their indirect effects. A major problem is that effects in vitro do not always mimic the in vivo picture. The advantages of using an in vivo model followed by an in vitro preparation are obvious. It allows direct comparison of in vitro contraction with in vivo changes in pressure and resistance, while still allowing for the identification of the actions of the individual mediators.

The concepts of receptor binding and the dose-response relationship, central to pharmacology, play an important role in the pathogenesis of endotoxic shock as well. Many of the mediators, whose activities lead to the pathological changes seen during endotoxemia, act by binding to specific receptors to produce their effects. The chemical mediator itself, however, does not cause specific changes, it is the receptors that the mediator activates that determine what changes will occur.

Agents that bind to receptors and activate them to produce a cellular response are called agonists. These agonists also have characteristic properties, such as receptor specificity, potency, efficacy and mechanisms of degradation or elimination, which determine the specific changes that occur due to its presence. The potency of an agonist is determined relative to other agonists, and is a measurement of the concentration of that agonist required to produce a measurable change. Efficacy is also a comparative term, but it describes the maximum biological change that an agonist can produce. For example if agonist A and agonist B produce the same change, but agonist A produces the change at a lower concentration, then agonist A is more potent than agonist B. However, if agonist B produces a higher maximum change than agonist A then agonist B is more efficacious than agonist A.

Another factor which may determine what changes are seen in the presence of an agonist is the simultaneous presence of an antagonist. An antagonist is an agent that binds to a receptor, does not elicit a cellular response, but inhibits the activity of an agonist. Antagonists may be of the competitive or the noncompetitive type.

Competitive antagonists, like agonists, are in a constant state of dynamic equilibrium between being bound to a receptor and being dissociated in solution. Their concentration and affinity determine the number of receptors being occupied by the drug at any given time. Antagonists act by binding to receptors and not activating them, thus leaving fewer receptors remaining to be bound by the agonist. The greater the number of receptors occupied by the antagonist, the fewer remain to be activated by the agonist and so the cellular response is diminished. This is the concept of the dose dependency of drug action. The concentration of the drug at the site of the receptor is dependent on the dose given; the number of receptors bound is dependent on the concentration of the agent at the site of receptors, and the level of cellular response is determined by the number of receptors activated. An antagonist or a decrease in the number of receptors decreases cellular response, whereas an increase in the concentration of agonist or the number receptors increases cellular response.

A noncompetitive antagonist acts by binding irreversibly to the receptor, thereby permanently inactivating that receptor. This irreversible binding to the receptor negates

the concept of dynamic equilibrium between the bound and unbound states for the noncompetitive antagonist. Thus the concentration relative to the concentration of the agonist is not as important for noncompetitive antagonists as it is for competitive antagonists. For the competitive antagonist to work, assuming its affinity for the receptor is approximately equal with the agonist, it must be present in a higher concentration then the agonist so that it binds a greater number of receptors and blocks their activation. However, once the noncompetitive antagonist is bound to a receptor, that receptor can not be stimulated regardless of the concentration of agonist and thus effect of noncompetitive antagonists is not dose dependant, assuming that the minimal effective concentration has been met.

Recent studies of endotoxin effects involve the use of selective receptor antagonists to determine which effects are prevented: thus determining by inference the type of mediators involved in the process. Selective antagonists are also important in understanding in vitro studies. An agonist is given which produces contraction of smooth muscle. This is recorded as active tension (difference in tension from baseline) and expressed as a percentage of the maximum effect. Thus, one agonist can be compared to others. These agonists act by binding to receptors in a concentration dependent fashion to produce their cellular response, i.e. contraction. Thus, in vitro studies can be used to determine the effects of a particular agent on smooth muscle tone and these changes can be extrapolated and compared to changes occurring in the live animal.

VI. Pathology and changes in physiologic parameters due to endotoxin administration.

There have been relatively few studies of the pathologic changes in the pig lung during endotoxemia. When endotoxin is instilled in the trachea, it produces focal areas of atelectasis, consolidation and necrosis. Accompanying these changes is an increased cellularity, due to the accumulation of neutrophils and macrophages, and an increase in the thickness of the alveolar septa. These changes suggest that when endotoxin is present

in the lungs (such as due to a Gram negative bacterial infection of the lungs) it may produce pathologic changes in pig lungs similar to those seen in human ARDS. Similar findings are reported in animals receiving intravenous endotoxin, suggesting that septicemia can produce pathologic changes in the lungs.

Anesthetized swine administered endotoxin intravenously also show similar pathophysiological changes to those expressed by humans with ARDS. There are decreases in PaO_2, mean arterial pressure, oxygen saturation, cardiac output and increases in pulmonary vascular resistance, mean pulmonary artery pressure, tracheal pressures and extravascular lung water. Anesthetized swine also demonstrate significant decreases in circulating leukocytes and platelets.

We hypothesize that intravenously administered endotoxin will cause significant changes in hematological and physiological parameters over a six hour period and that these changes will resemble those that occur when a human or a pig suffers from an infection with a Gram-negative organism. We also hypothesize that the expected changes in pulmonary parameters are the result of changes that can be demonstrated on a histological level as acute inflammation and in vitro as a hyperresponsiveness of bronchi when exposed to certain contractile agents.

Materials and Methods

Ten SPF derived Yorkshire pigs (Archer Farms, Bellcamp MD) weighing 17 $\pm$ 4 kg were divided into two treatment groups. Treatment group 1 (n=5) served as controls and received only physiological saline intravenously during the six hour protocol. Treatment group 2 (n=5) received *E. coli* derived LPS (Sigma, St. Louis MO) during the six hour protocol.

In vivo protocol - The pigs were premedicated with an injection of sodium pentobarbital 25 to 35 mg/kg intraperitoneally. Pentobarbital was then given to effect in order to reach a surgical plane of anesthesia through an intravenous catheter placed in the auricular vein. The animal was then placed in dorsal recumbency and a midline incision was made on the ventral neck. A tracheotomy was performed and a 7.0 mm id endotracheal tube inserted, inflated and held in place by a ligature. The pig was then ventilated with room air using a Harvard ventilator, with a tidal volume of 10-15 ml/kg to achieve a starting airway pressure of 15-17 cmH_2O. Airway pressures were measured by a pressure transducer (Gould P23ID) attached to the endotracheal tube and were recorded on a Grass polygraph (model 7D). A 4700 Oxicap end-tidal carbon dioxide monitor (Omeda, Louisville CO) was also attached and the rate of ventilation was set to produce a baseline end-tidal CO_2 of 36 $\pm$ 2 mmHg. The carotid artery and jugular vein were then isolated and catheterized using polyethylene tubing (Becton Dickinson, Sparks MD) with I.D. 1.67 mm and 0.86 mm, respectively. A pressure transducer (Gould P23ID) was attached to the carotid catheter and the central arterial pressure was recorded on the Grass polygraph. Both of the jugular catheters were connected to syringe pumps, one containing LPS and the other containing pentobarbital. The femoral vein was isolated via a medial cut down and a 7 French triple-lumen, thermistor-tip Swan Ganz catheter (Baxter, Santa Ana CA) was inserted and advanced into the pulmonary artery. Pulmonary artery pressure, pulmonary wedge pressure and central venous pressure, were measured and recorded on the polygraph. The Swan Ganz catheter was also used to monitor body

temperature (which was maintained at 37.5 $\pm$ 0.5 C) and to perform cardiac output measurements by thermal dilution using an Edwards COM 2 cardiac output computer (Baxter, Santa Ana CA). Blood samples were obtained for complete blood counts and blood gas determinations from the carotid catheter at 0, 2, 4, and 6 hours.

Physiological measurements were taken at T = 0 and every 30 minutes thereafter until the animal was euthanitized by overdose with pentobarbital at 6 hours. For treatment group 2, LPS was infused intravenously at a rate of 1 micrograms/kg/hr, and all groups received lactated Ringers solution at 10 ml/kg/hr. Immediately following euthanasia the thorax was opened and the trachea was clamped off upon maximum inflation of the lungs via the ventilator and the trachea and lungs were removed together. The two caudal and the accessory lobes were removed for histopathology and assays for neutral endopeptidase. Both the cranial and the middle lung lobes were used for contraction studies of the bronchi.

Tissue bath - The middle and both cranial lung lobes were removed and immediately immersed in 37.4°C Krebs solution (NaCl 118.0 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 0.5 mM, NaH_2PO_4 1.0 mM, $NaHCO_3$ 25.0 mM, glucose 11.0 mM). The third generation bronchi were then cut into rings and placed within a tissue bath filled with 37.4°C Krebs solution and bubbled with 95% O_2 and 5% CO_2. They were attached to a force transducer (Grass force-displacement transducer FT03C) and the contractile force was recorded on a grass polygraph. Three grams baseline tension was applied and the tissues were allowed to equilibrate for one hour. All rings were initially contracted with 60mM potassium chloride (KCl), which produced a maximum contractile response to KCL. Then the bath was changed to rinse the tissues with fresh Krebs several times. After the KCl was washed out and relaxation had occurred, the baseline was returned to three grams tension. Then a dose response curve was performed using substance P(10^{-9} to 10^{-5} M), bradykinin (10^{-9} to 10^{-5} M) and carbachol (10^{-9} to 10^{-3} M) each on a different pair of bronchial rings. A fourth pair received electrical field stimulation at 10 volts, 0.3 ms duration and a frequency of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 13, 26, and 52 per second. This was followed by thiorphan at 10^{-5} M and, after a 20 minute period, electrical field

stimulation was repeated using the same stimulation parameters. The contraction of each ring was recorded as active tension, the total grams of tension minus the base line tension. After dose response curves were completed, 10^{-3} M carbachol was administered to produce maximal contraction. All responses were calculated as a percentage of the maximal contraction to both KCl and carbachol.

Histopathology - One caudal lung lobe was fixed by infusion of 10% formalin into the secondary bronchus to a pressure of 10 –15 cm H_2O: then placed in 10% formalin and sectioned at 2cm intervals from cranial to caudal following the main bronchi. The sections were stained with H&E and the slides were quantitatively evaluated for edema, vascular congestion, lymphoid aggregates, alveolar hemorrhage, neutrophils and macrophages.

Calculations - Resistance was calculated using the formula: resistance = (arterial pressure – venous pressure) / flow. Mean arterial pressures were calculated using the formula 1/3 (systolic – diastolic pressure) + diastolic pressure. Cardiac index was calculated by dividing the cardiac output by the weight in kilograms.

Statistics - Endotoxin treated pigs were compared to time matched controls using unpaired t-tests, using a significance level of $p < .05$. All figures were created using mean values and the error bars represent the standard error of the mean.

Results

In vivo–

Mean Central Arterial Pressure (MCAP)- At time zero the MCAP was 98.3 ± 3.58 mmHg and 97.4± 4.29 mmHg in the endotoxin group (Figure 1). These values were not significantly ($p \geq .05$) different between groups. The MCAP of the LPS treated group showed an increase after 0.5 hr, but the difference in pressures was not statistically significant ($p \geq .05$). The MCAP of the endotoxin treated group at 1 hr dropped sharply and by 1.5 hr reached a level significantly ($p < .05$) below that of the control group. MCAP of the endotoxin treated group remained significantly ($p < .05$) below that of the control group through time 2.5 hr. After time 2.5 hr the MCAP of the LPS group began to rise slowly and was not significantly different from the control group throughout the remainder of the six hour protocol.

Mean Pulmonary Arterial Pressures (MPAP)- At time zero the MPAP was 13.0 ± 2.50 mmHg for the control group and 14.7 ± 1.41 mmHg for the endotoxin group (Figure 1). These values were not significantly different between groups. The MPAP for the endotoxin treated group rose sharply at 0.5 hr to 46.2 mmHg, at which point it was significantly ($p < .005$) higher than the control group. The MPAP of the group receiving endotoxin then fell slightly but remained significantly ($p < .05$) greater than that of the control group for the remainder of the six hours.

Total Peripheral Resistance (TPR)- At time zero the TPR was 41.1 ± 8.18 mmHg/l/min for the control group and 32.1 ± 1.92 mmHg/l/min for the endotoxin treated group. These values were not significantly different between the two groups (Figure 2). However, at 0.5 hr the group receiving endotoxin showed a rapid rise to a point significantly ($P < .05$) above the control group. At 1 hr the TPR of the endotoxin treated group tended to drop to a point below that of the control group and remained there throughout the rest of the six hour protocol, although these differences were not statistically significant.

Pulmonary Vascular Resistance (PVR)- At time zero the PVR was 3.6 ± 0.51 mmHg/l/min for the control group and 3.21 ± 0.21 mmHg/l/min for the endotoxin group

(Figure 3). These differences were not statistically significant. In the endotoxin treated group there was a dramatic rise in PVR during the first 0.5 hrs. This was significantly ($p < .005$) greater than that of the control group. The PVR of the endotoxin treated group remained statistically significantly ($P < .05$) greater than the controls for the duration of the experiment.

Pulmonary Arterial Wedge Pressure (Ppaw)- The Ppaw was 5.0 $\pm$ 1.34 mmHg for the control group and 6.6 $\pm$ 0.38 mmHg for the endotoxin treated group at time zero. These values were not significantly different between the two groups (Figure 4). At 0.5 hrs the Ppaw of the endotoxin treated group rose very significantly ($p < .005$) above the control group and remained significantly ($p < .05$) higher through 1.5 hrs. Ppaw of the endotoxin treated group remained measurably above the control group throughout the rest of the six hour protocol, but was only statistically significant ($p < .05$) at times 3.5 hr and 5.5 hr.

Central Venous Pressure (CVP)- At time zero the CVP was 3.5 $\pm$ 0.55 mmHg for the control group and 6.3 $\pm$ 0.58 mmHg for the endotoxin group. These values were within the normal range for both groups (Figure 5). There was a spike in CVP in the endotoxin treated group at 0.5 hr to a point that was statistically ($p < .05$) higher than it was at time zero. This rapid rise in CVP did not occur in the control group.

Heart Rate (HR)- The HR was 131.2 $\pm$ 9.36 bpm for the control group and 144.8 $\pm$ 3.97 bpm for the endotoxin group at time zero. These values were not significantly different (Figure 6). The endotoxin group at 0.5 hr reached a HR significantly ($p < .05$) greater than the control group and maintained an upward trend for the remainder of the six hour protocol. Statistical significance ($p < .05$) was maintained from 0.5 hr through 6 hrs and at some points reached the significance level of ($p < .0005$).

Cardiac Output (CO) and Cardiac Index (CI)- Both cardiac output (control = 2.91 $\pm$ 0.44 l/min, LPS = 3.06 $\pm$ 0.21 l/min) and cardiac index (control = 2.58 $\pm$ 0.36 l/min/kg, LPS = 2.86 $\pm$ 0.12 l/min/kg) were calculated and neither showed a significant ($p \geq .05$) difference at time 0 (Figure 7 & 8). CO and CI in the endotoxin treated both showed a significant decline below the levels of the control group at 0.5 hr. In the endotoxin treated group both CO and CI remained at lower levels then in the control group for most

of the remainder of the six hours. However, only the CI was significantly ($p < .05$) less then control at time 4.5 hr.

Airway Pressure- At time zero the airway pressure was 15.7 $\pm$ 0.44 cmH_2O for the control group and 16.1 $\pm$ 0.46 cmH_2O for the endotoxin group (Figure 9). The airway pressures in the endotoxin treated group rose to a level significantly ($p < .005$) greater than the control group at 0.5 hr and maintained significance ($p < .05$) throughout the first 4.5 hours. After 4.5 hours the airway pressures of the endotoxin treated group remained measurably higher than those of the control group, but not significantly ($p \geq .05$) so.

Alveolar-arterial Oxygen Gradient ($AaDO_2$)- At time zero the $AaDO_2$ was 60.93 $\pm$ 3.48 mmHg for the control group and 60.03 $\pm$ 0.90 mmHg for the endotoxin group. These differences were not statistically significant (Figure 10). The $AaDO_2$ of the endotoxin treated group was significantly ($p < .005$) higher than that of the controls for all three samples at 2, 4, and 6 hrs thereafter.

Blood gas- The partial pressure of oxygen in the arterial blood (PaO_2) of control pigs and endotoxin treated pigs showed no significant differences at time zero (Figure 11). A significant ($p < .0005$) decline below control levels occurred in the endotoxin treated group at 2, 4, and 6 hrs.

The partial pressure of carbon dioxide in arterial blood ($PaCO_2$) for both groups at time zero was 39 mmHg (Figure 11). The endotoxin treated group showed increases in $PaCO_2$, which became significant ($p < .005$) at 4 hrs and remained so ($p < .05$) at 6 hrs.

The oxygen saturation (SPO_2) of both groups at baseline was 97.2% (Figure 12). In the endotoxin treated group the SPO_2 dropped precipitously at 2 hr to a level significantly ($p < .005$) below that of the control group and at 4 and 6 hrs remained significantly ($p < .0005$) below the level of the control group.

Complete Blood Count- The hematocrit (HCT) was not significantly different between the two groups at time zero (Figure 13). The control group showed a slight but insignificant downward trend, whereas the endotoxin treated group showed a slight upward trend which reached statistical significance ($p < .05$) at time 6 hr.

The white blood cell count (WBC) for both groups was within normal range at time 0 (Figure 14). However, the WBC of the endotoxin treated group dropped to a level

significantly ($p < .05$) below the control group by 2 hr. The 4 hr and 6 hr samples were significantly ($p < .0005$) less than those of the control group as well.

The numbers of segmented neutrophils were not significantly ($p \geq .05$) different between the two groups at time zero (Figure 15). The control group showed a slight upward trend, whereas the endotoxin group showed a rapid decline in the number of circulating segmented neutrophils. This decline brought the endotoxin treated group to a level significantly ($p < .05$) below that of the control group where it was maintained it for all three of the remaining samples.

The numbers of banded neutrophils were not significantly different between the two groups throughout the six hour protocol (Figure 16), however the group receiving endotoxin showed an upward but insignificant trend at the six hour sample that was not seen in the controls.

The number of lymphocytes was significantly ($p < .05$) higher at time zero in the endotoxin treated group when compared to the control group (Figure 17). However, the number of lymphocytes in the endotoxin treated group dropped to a level significantly below that of the control group at 4 and 6 hrs ($p < .0005$).

The levels of plasma proteins in both groups showed a slight downward trend throughout the entire protocol and at no point were they significantly ($p \geq .05$) different (Figure 18).

In vitro–

Contractile studies- All bronchi responded in a dose dependant manner. The active tension produced upon the addition of substance P was significantly greater in the endotoxin treated group when compared to the control group throughout the entire range of doses used (Figures 19 & 20). This was true whether expressed as a percentage of the maximum contraction to carbachol $1x10^{-3}$ M or 60 mM KCl.

Bronchi of neither the control group nor the endotoxin treated group contracted to $1x10^{-9}$ M through$1x10^{-6}$ M bradykinin (Figure 21 & 22). None of the control group bronchi contracted to $1x10^{-5}$ M bradykinin either. The endotoxin treated group's bronchi

contracted to $1x10^{-5}$ M bradykinin, but not to a level that was significantly ($p \geq .05$) different from the control group. This was true whether compared to carbachol or KCl as the maximum contracting agent.

Carbachol produced contraction in the bronchi of endotoxin treated pigs at doses of $1x10^{-9}$ M and $1x10^{-8}$ M, but not in those of control pigs (Figure 23 & 24). However, this difference was not statistically significant ($p \geq .05$). At doses of $1x10^{-7}$M to $1x10^{-5}$M a significantly ($p < .05$) greater contraction occurred in the endotoxin treated bronchi when compared to those of the control group, whether compared to KCl or carbachol as the maximum contracting agent.

Electrical field stimulation produced contraction in the bronchi of both groups throughout the entire range of frequencies used. The bronchi from the endotoxin treated group tended to contract to a higher percent maximum when compared to KCl or carbachol than did the control group bronchi (Figures 25 & 26). These differences were not statistically significant ($p \geq .05$). When thiorphan and captopril were added prior to electric field stimulation the control group bronchi showed a higher percent maximum contraction to carbachol, whereas the endotoxin treated group bronchi showed a higher percent maximum to KCL (Figures 27 & 28). These differences were not statistically significant ($p \geq .05$).

Histopathology–

Lung sections from pigs treated with endotoxin had significantly ($p< 0.005$) more congestion, hemorrhage and infiltration of neutrophils then did sections from the control group. Edema was present more often and was more severe in sections from the endotoxin treated pigs, but these changes were not statistically significant. No statistically significant differences in thrombus formation, mixed cell infiltration, lymphocytic infiltration, enlarged lymphoid aggregates, foamy macrophages, fibrosis, foreign debris, or atelectasis were present.

Table 1. Receptors and their actions

Receptor type	Receptor sub-type	Endogenous agonist	Action
Adrenergic	α_1	Norepinephrine	Vasoconstriction
	β_1	Epinephrine	Increase heart contractility and rate
	β_2	Epinephrine	Broncho/Vasodilation
Cholinergic	Muscarinic	Acetylcholine	Bronchoconstriction, mucus secretion, vasodilation, decreased heart rate and contractility
Histamine	$H_{1\&2}$	Histamine	Bronchoconstriction, vasoconstriction of large blood vessels, vasodilation of small blood vessels and increased vascular permeability
	H_3	Histamine	Effects on the nervous system, including activation of C-fibers and autonomic nerves
Bradykinin	$B_{1\&2}$	Bradykinin	Bronchoconstriction, vasodilation, increased vascular permeability and chemotaxis
Neurokinin	$NK_{1\&2}$	Substance P & neurokinin A	Bronchoconstriction, mucus secretion, vasodilation, increased vascular permeability

Figure 1. Mean central and mean pulmonary arterial pressures

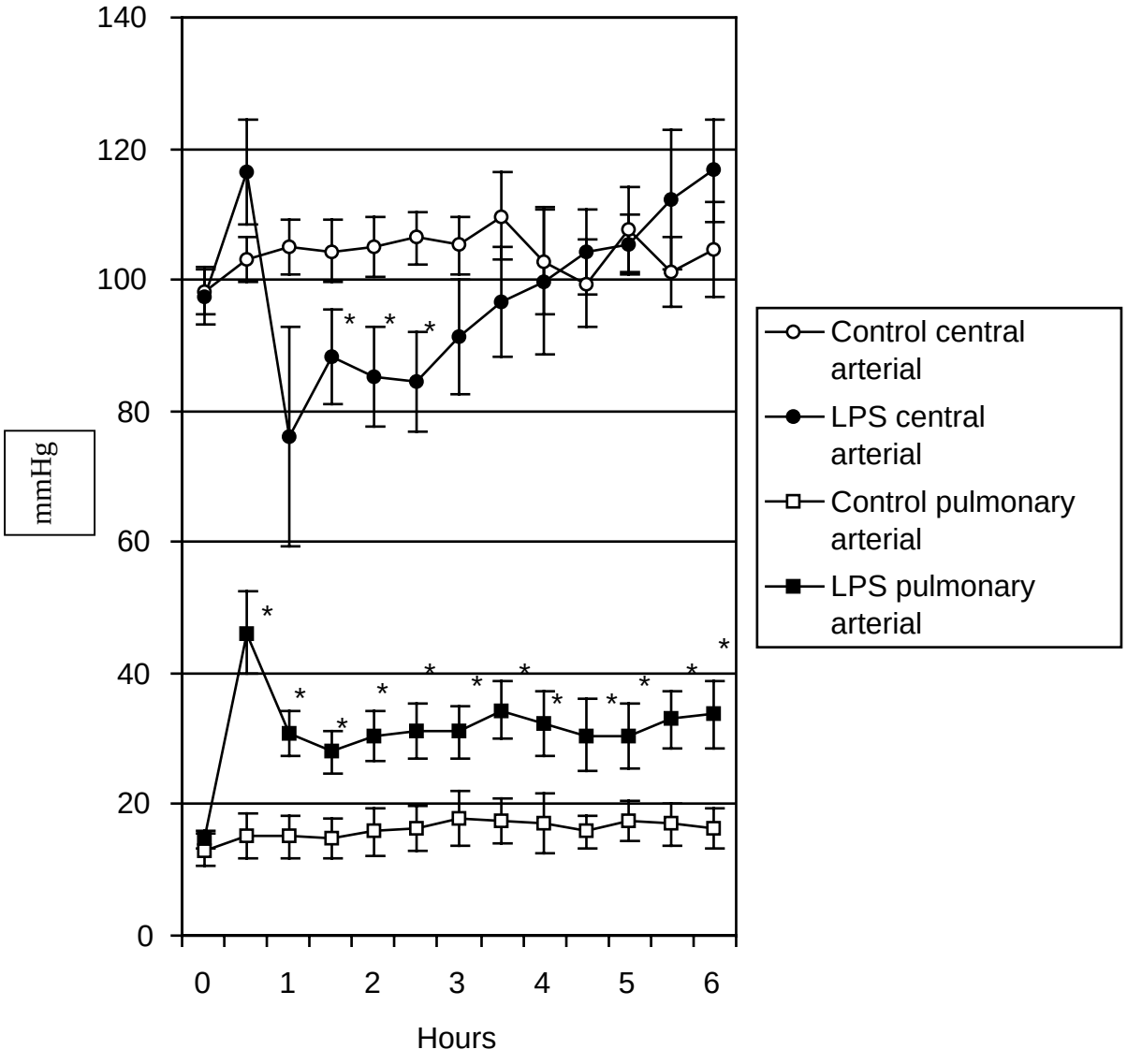


Figure 2. Total peripheral resistance

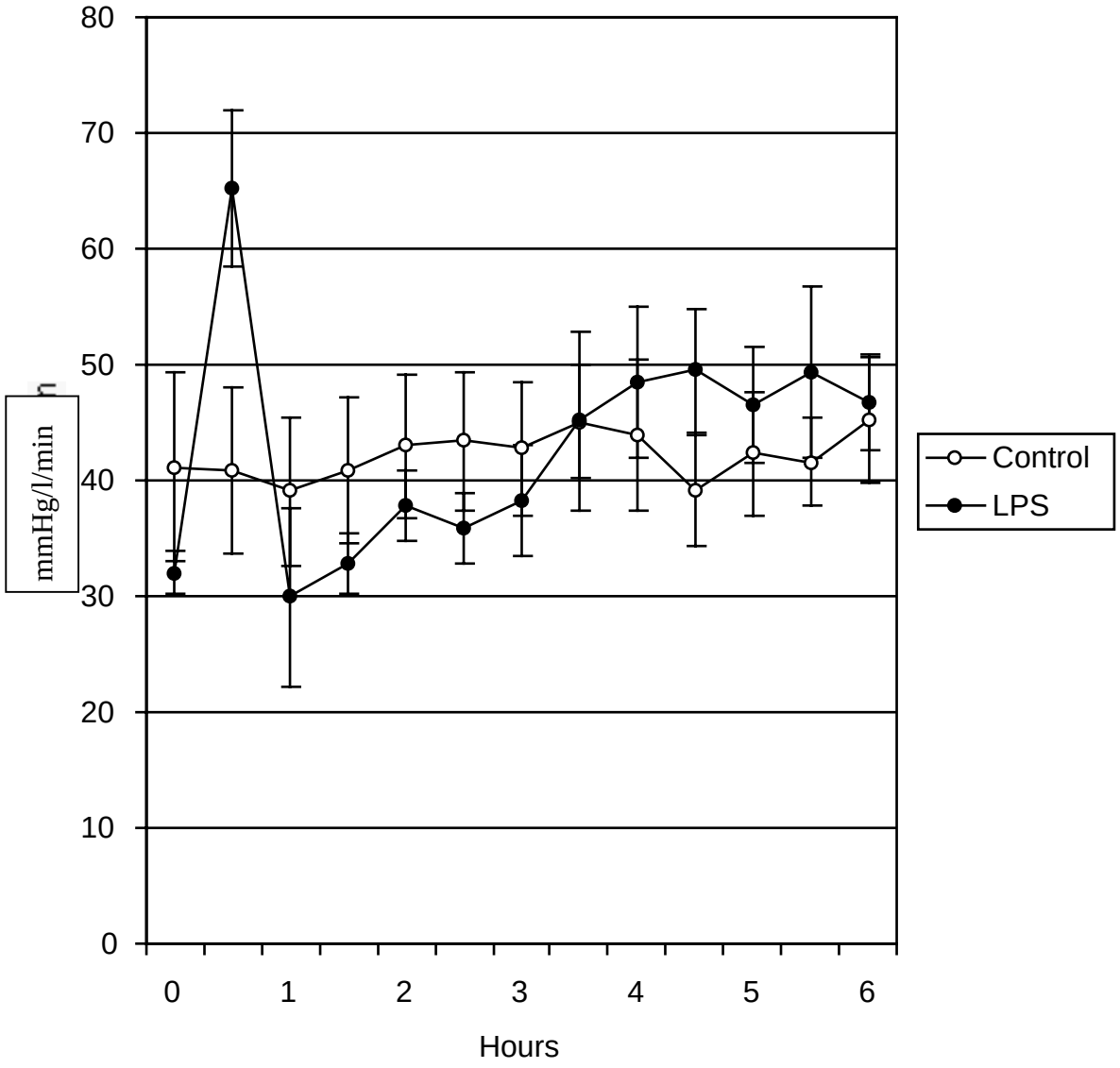


Figure 3. Pulmonary vascular resistance

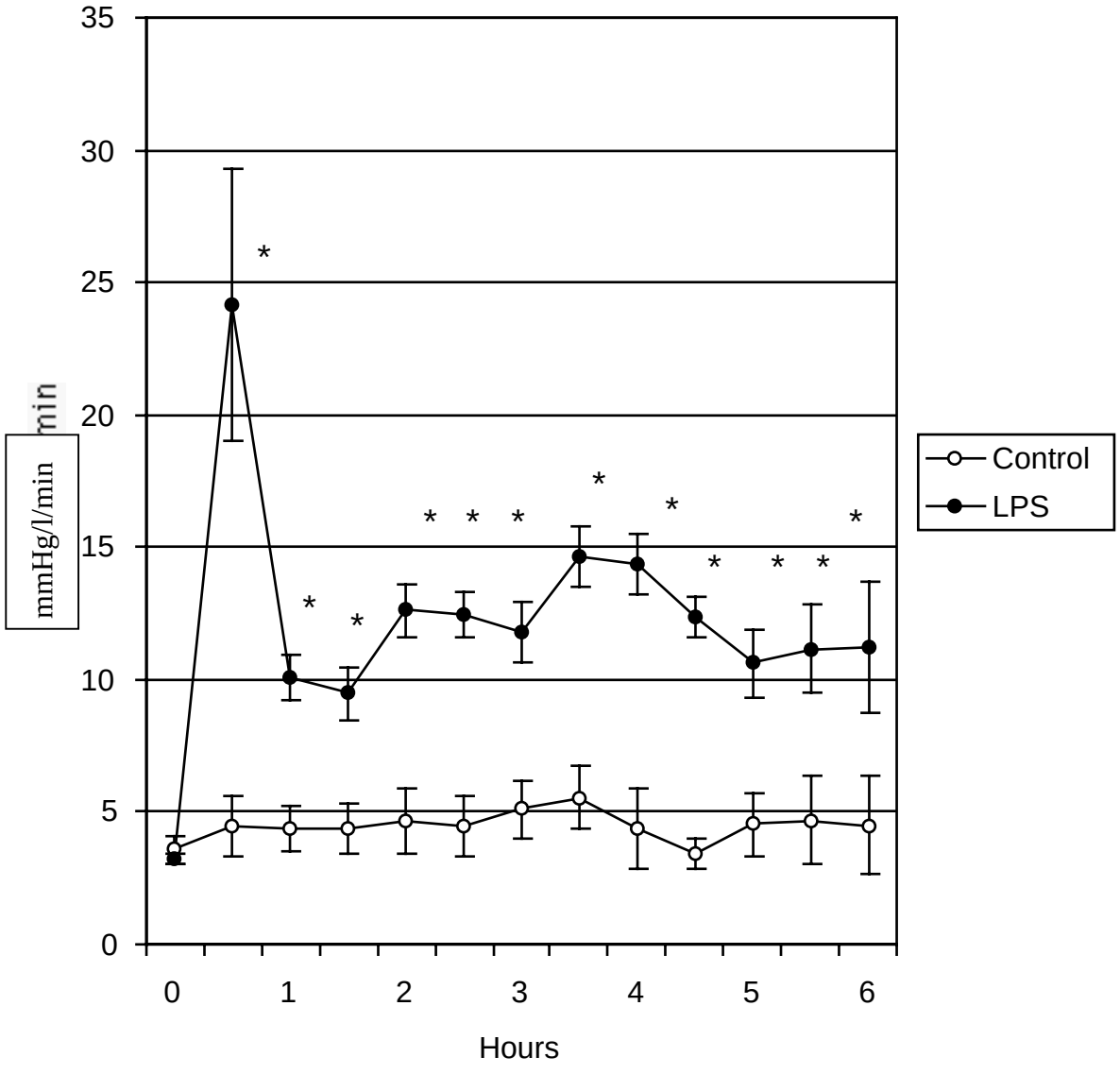


Figure 4. Pulmonary arterial wedge pressure

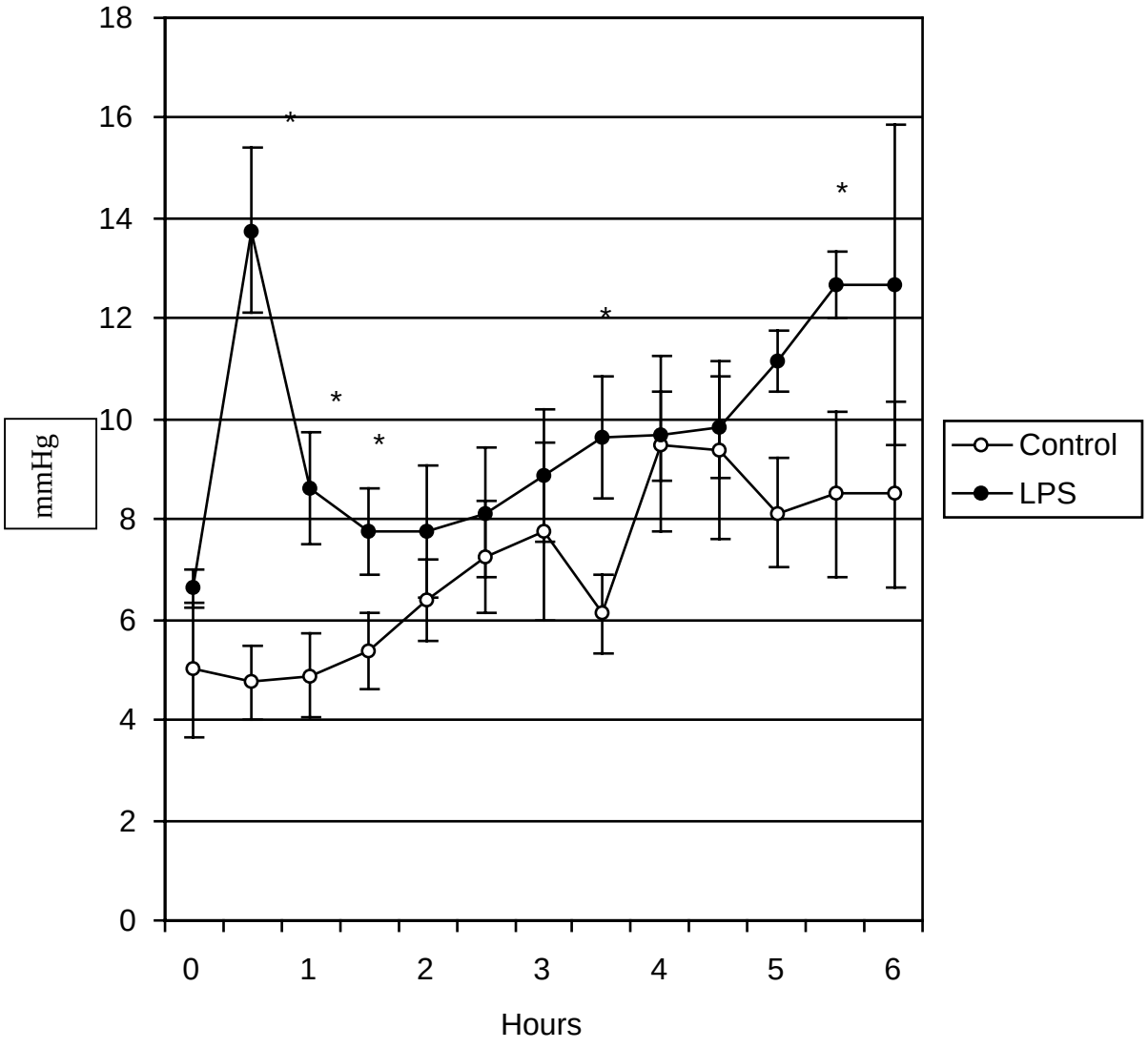


Figure 5. Central venous pressure

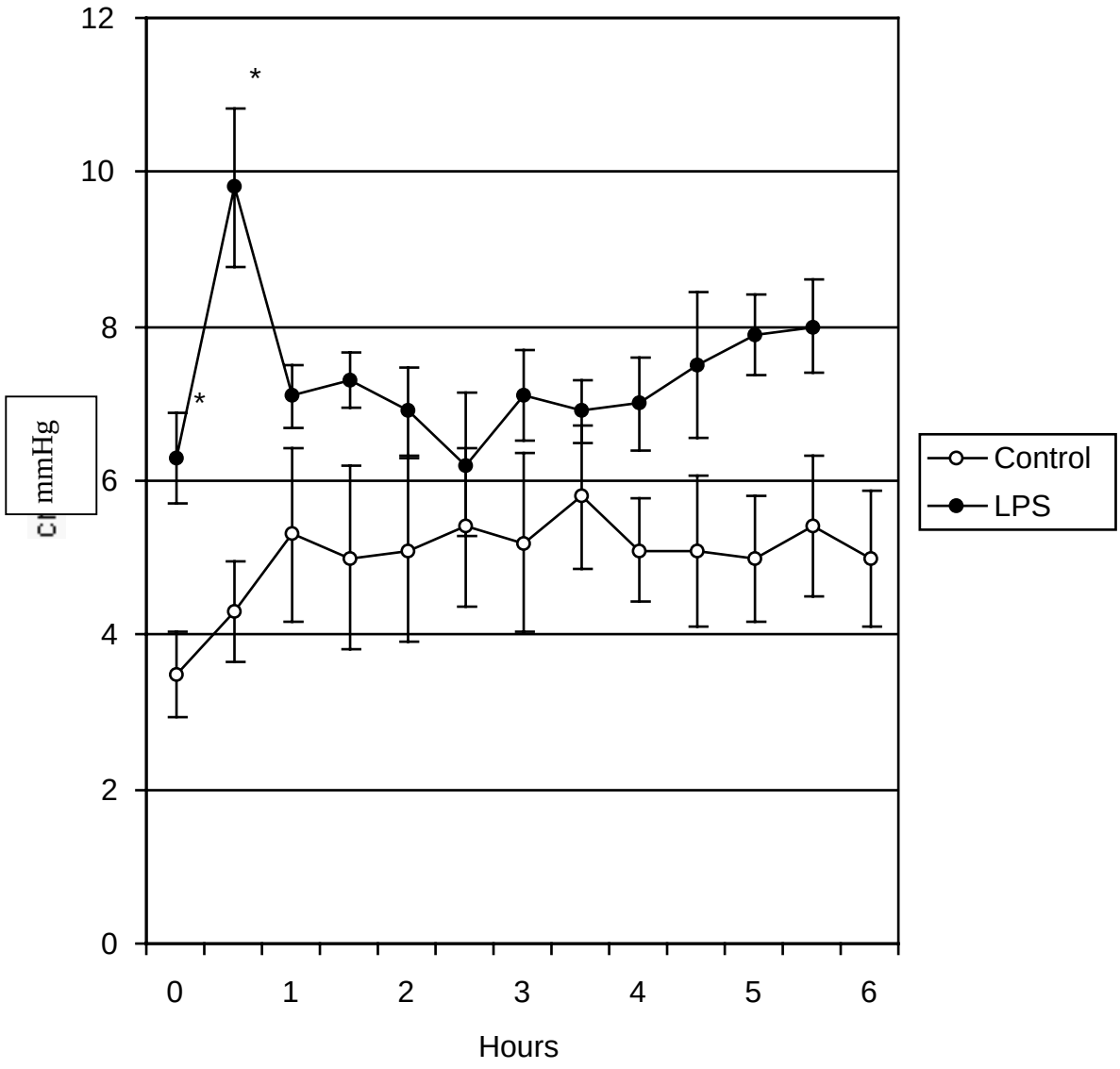


Figure 6. Heart rate

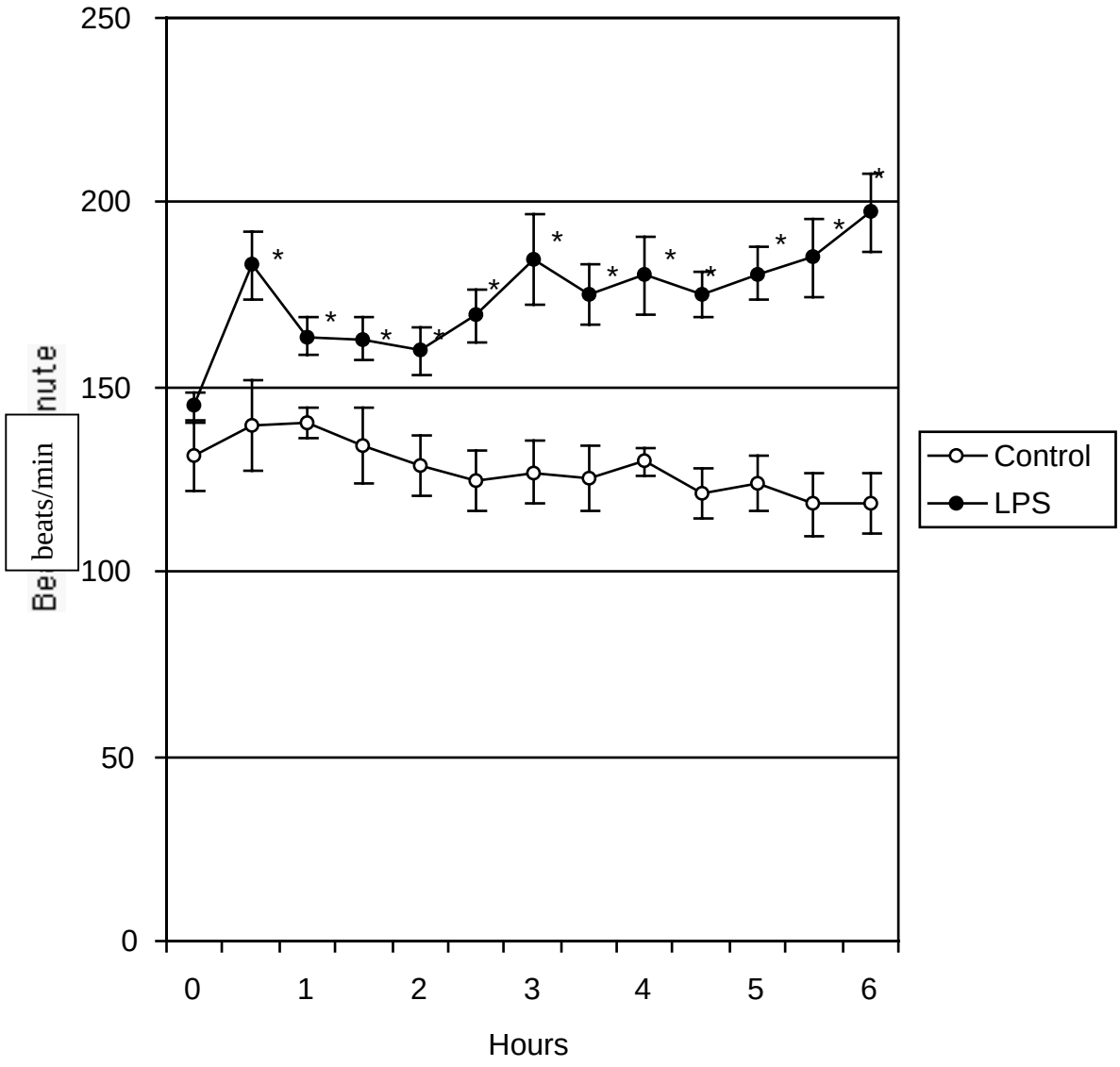


Figure 7. Cardiac output

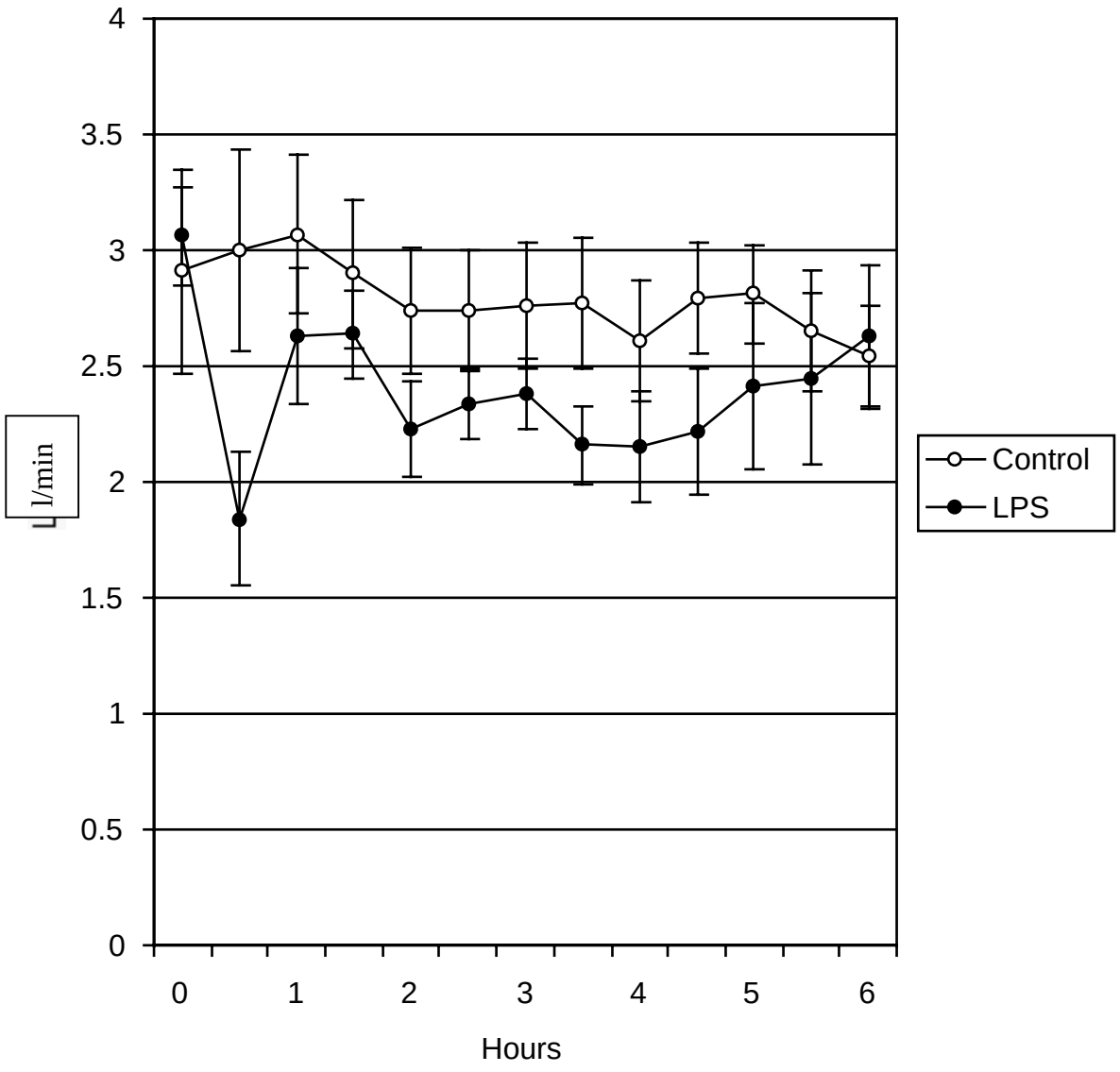


Figure 8. Cardiac index

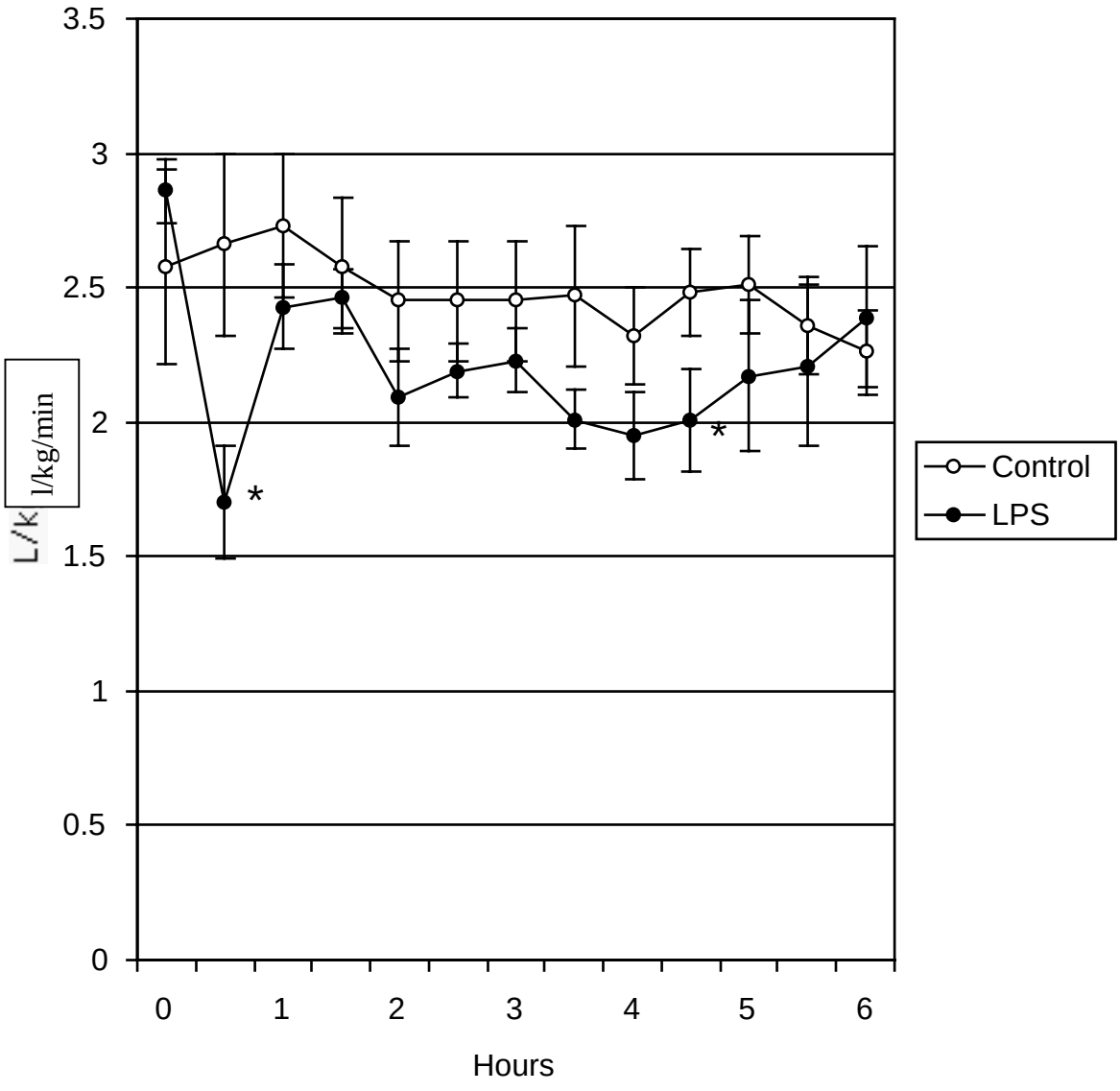


Figure 9. Airway pressure

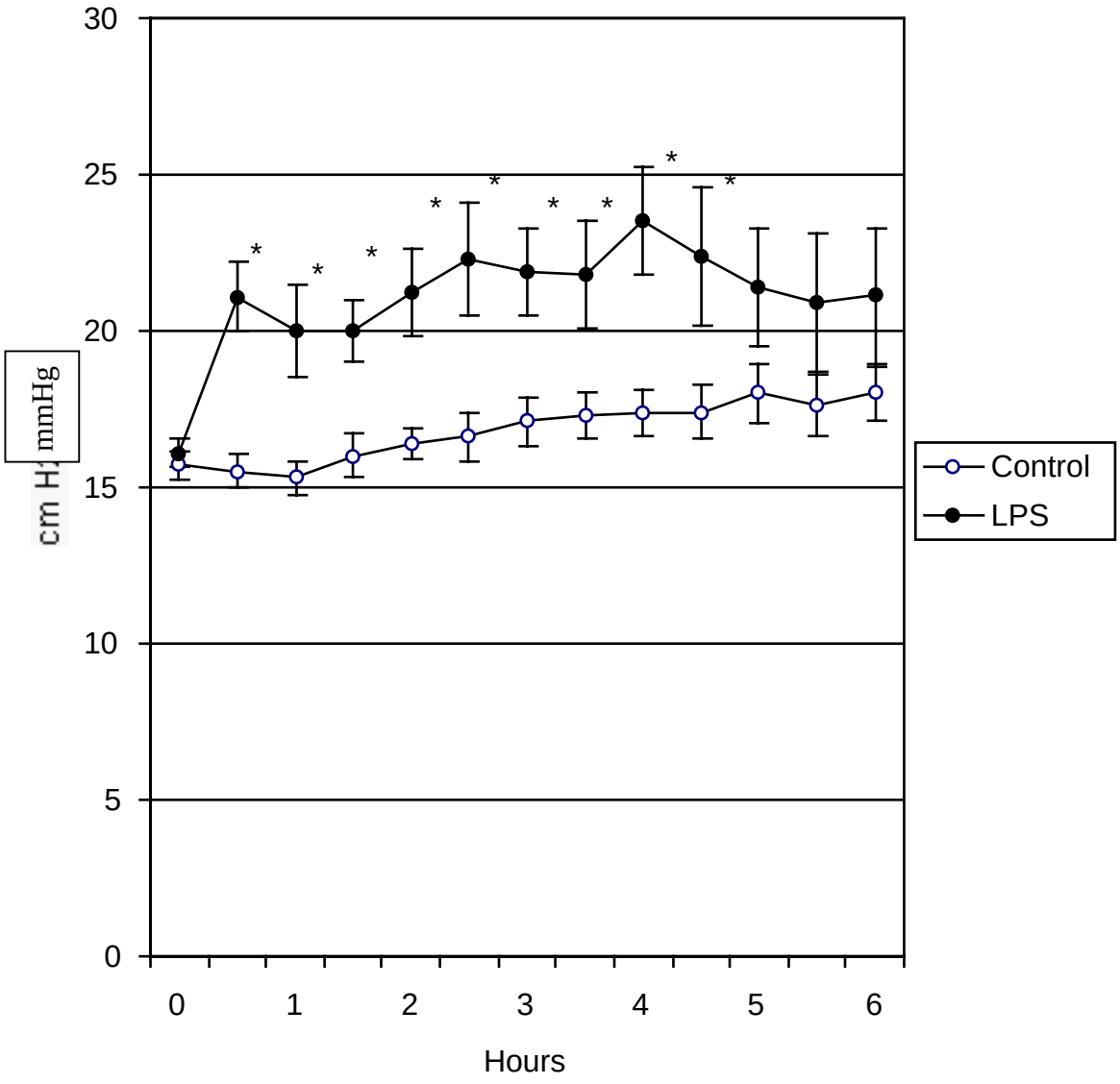


Figure 10. Alveolar to arterial oxygen gradient

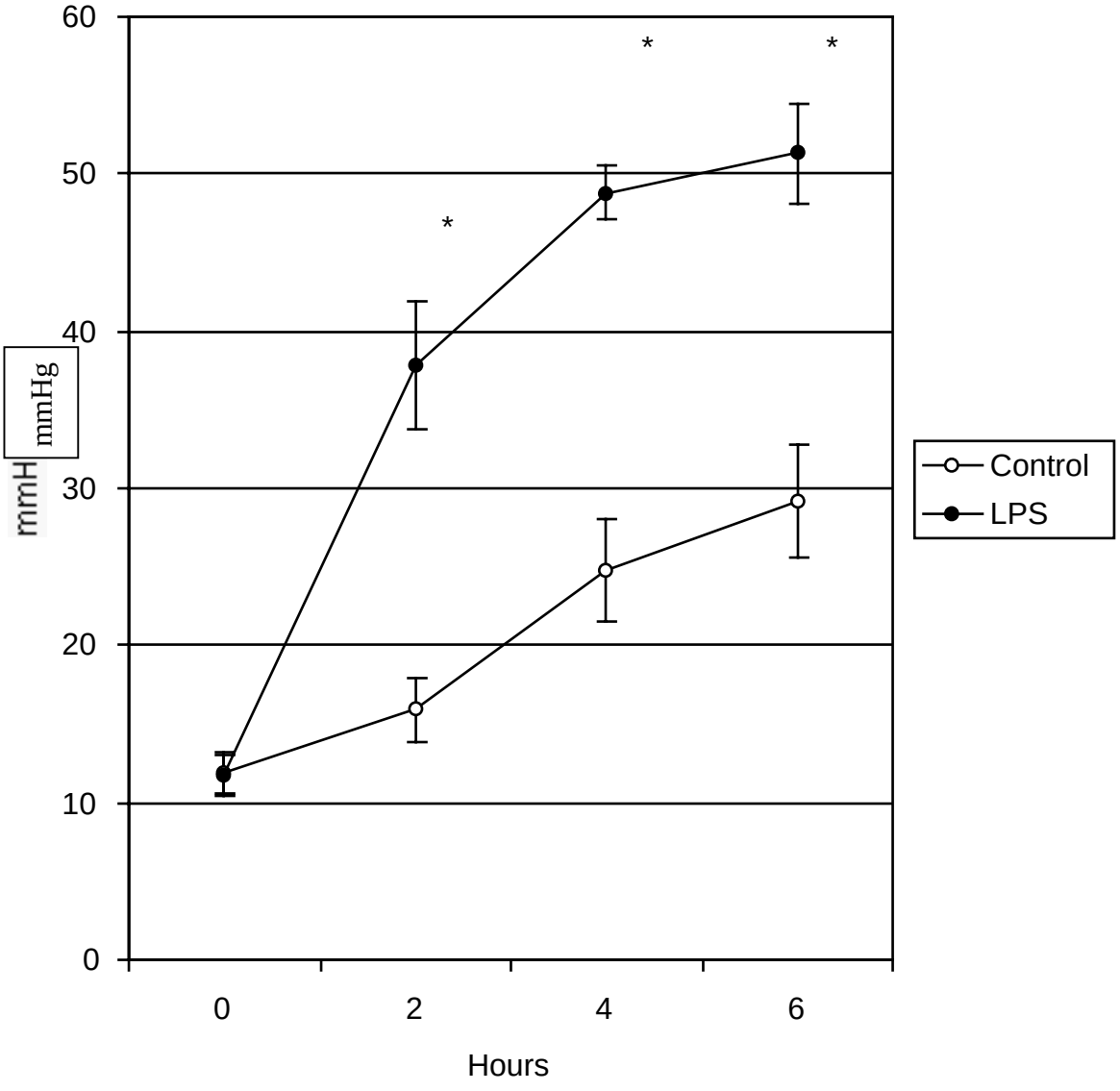


Figure 11. Arterial partial pressure of oxygen and carbon dioxide

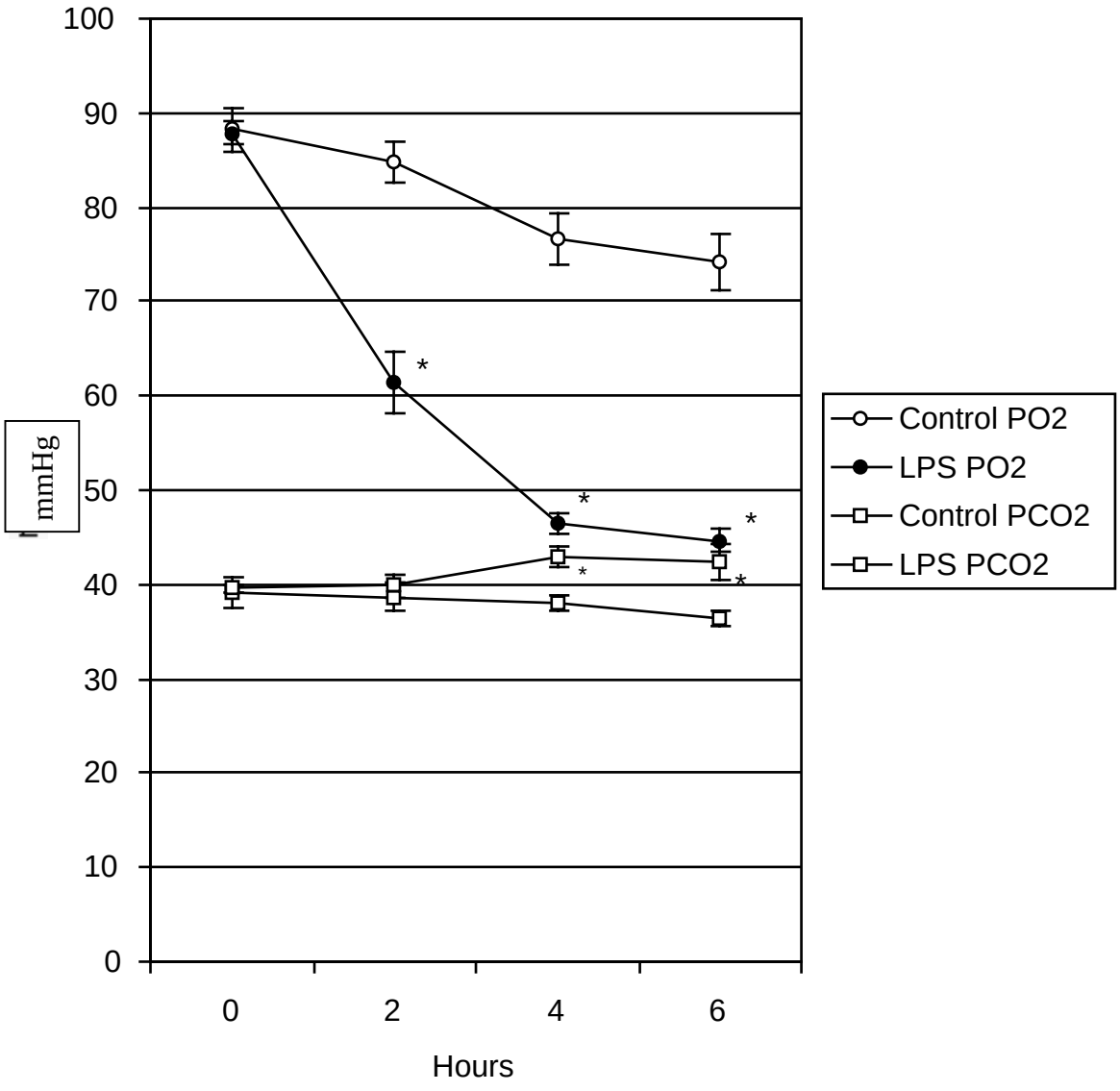


Figure 12. Oxygen saturation

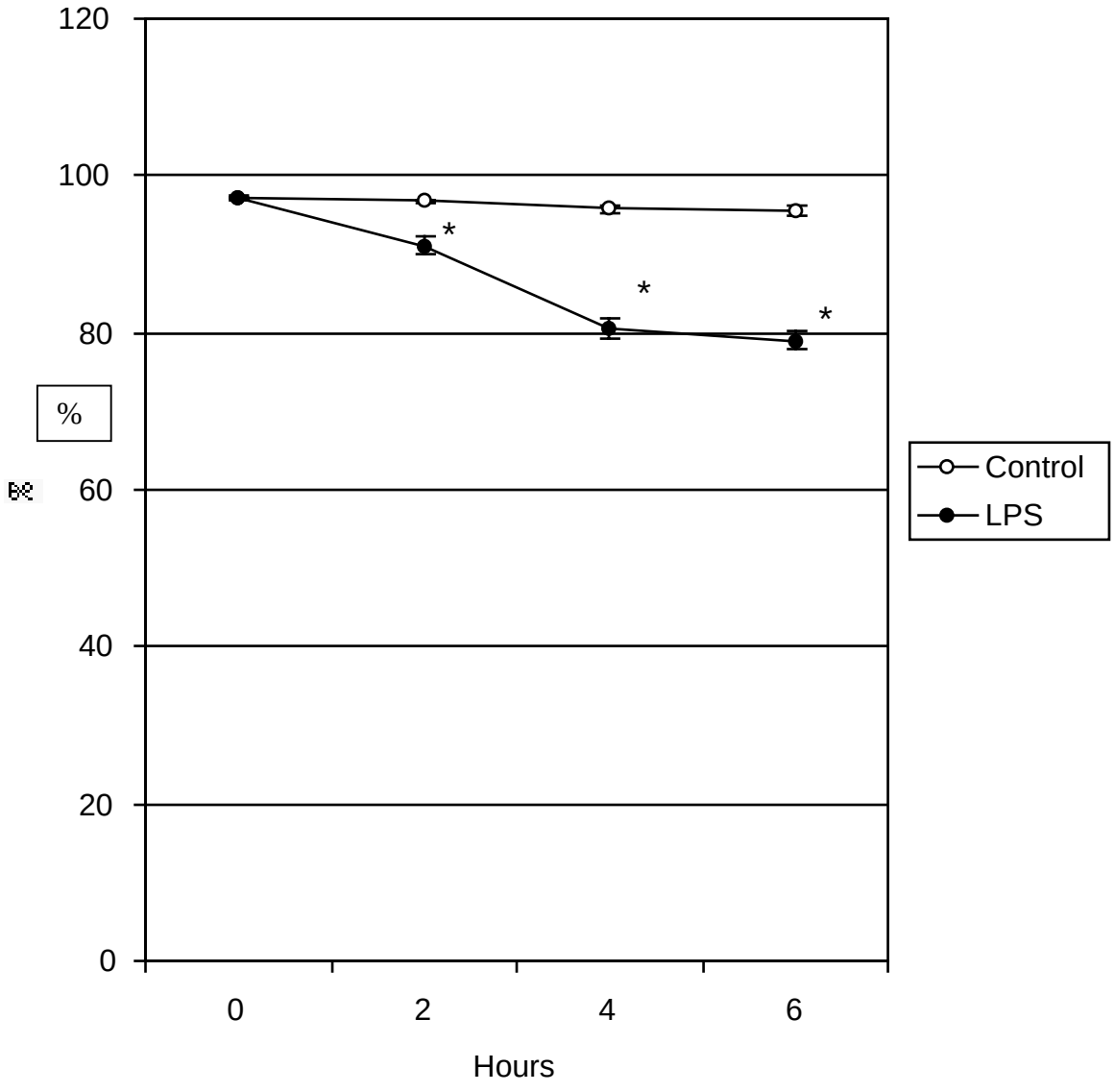


Figure 13. Hematocrit

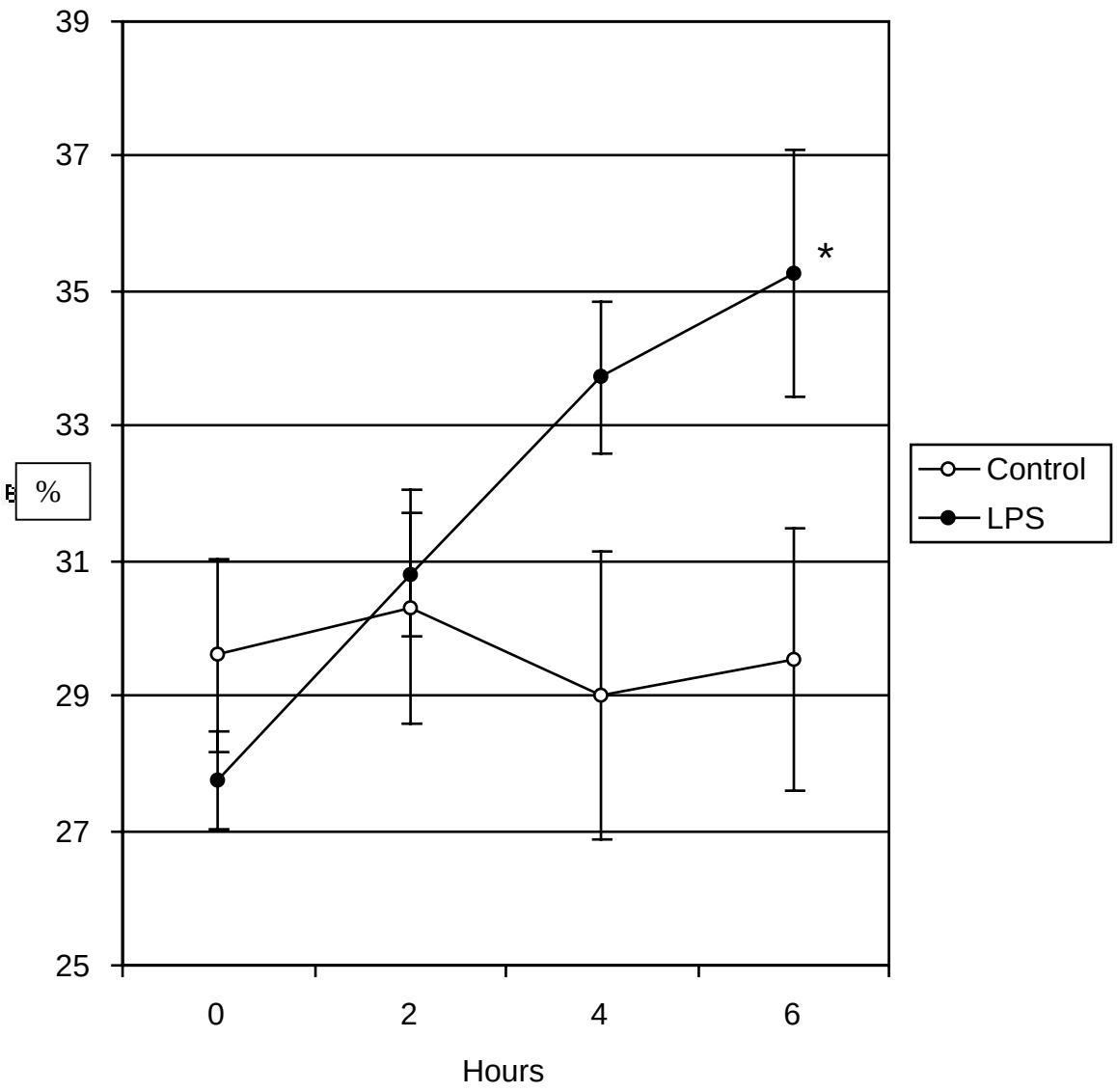


Figure 14. White blood cell counts

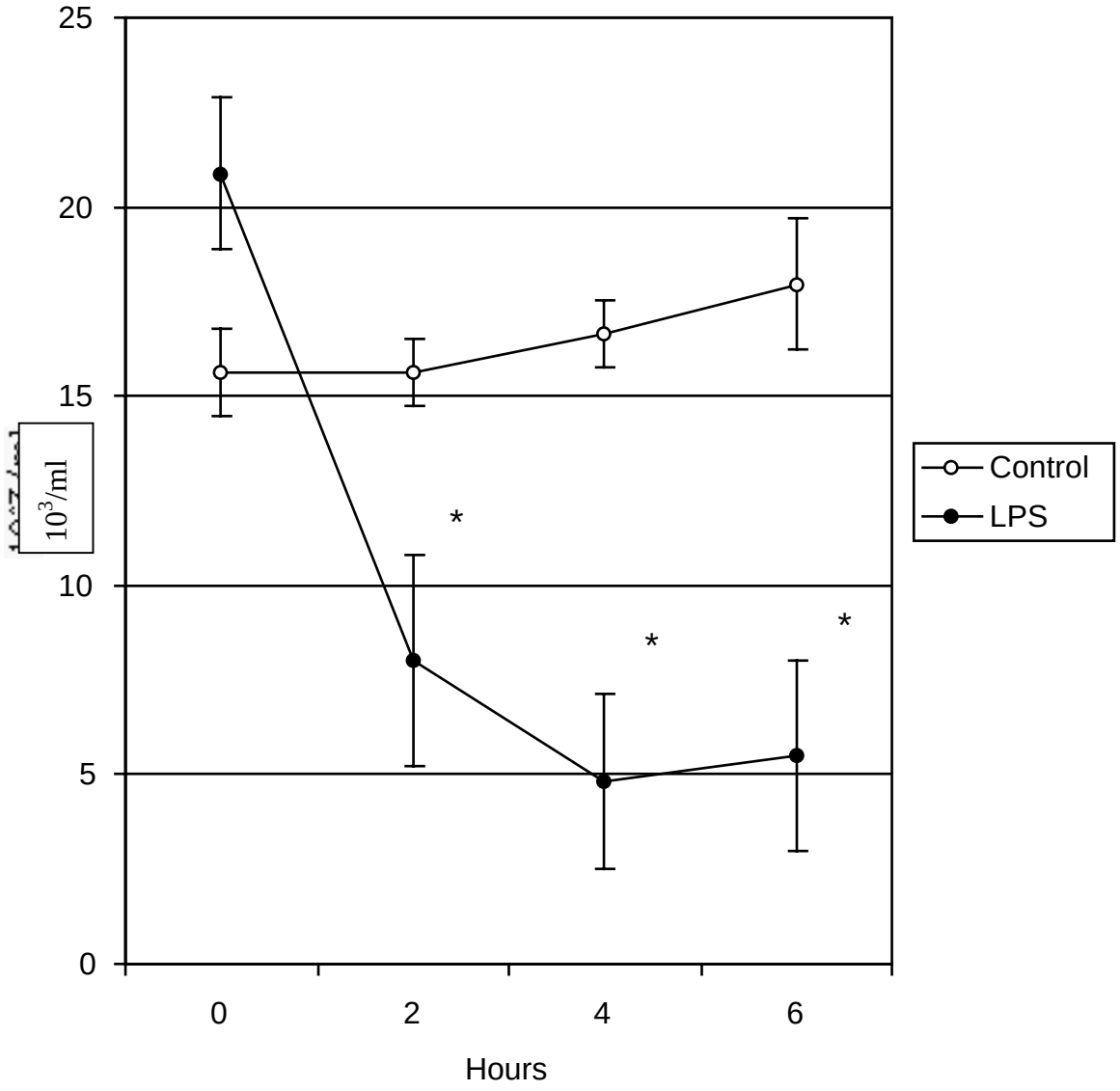


Figure 15. Neutrophil (segmented) counts

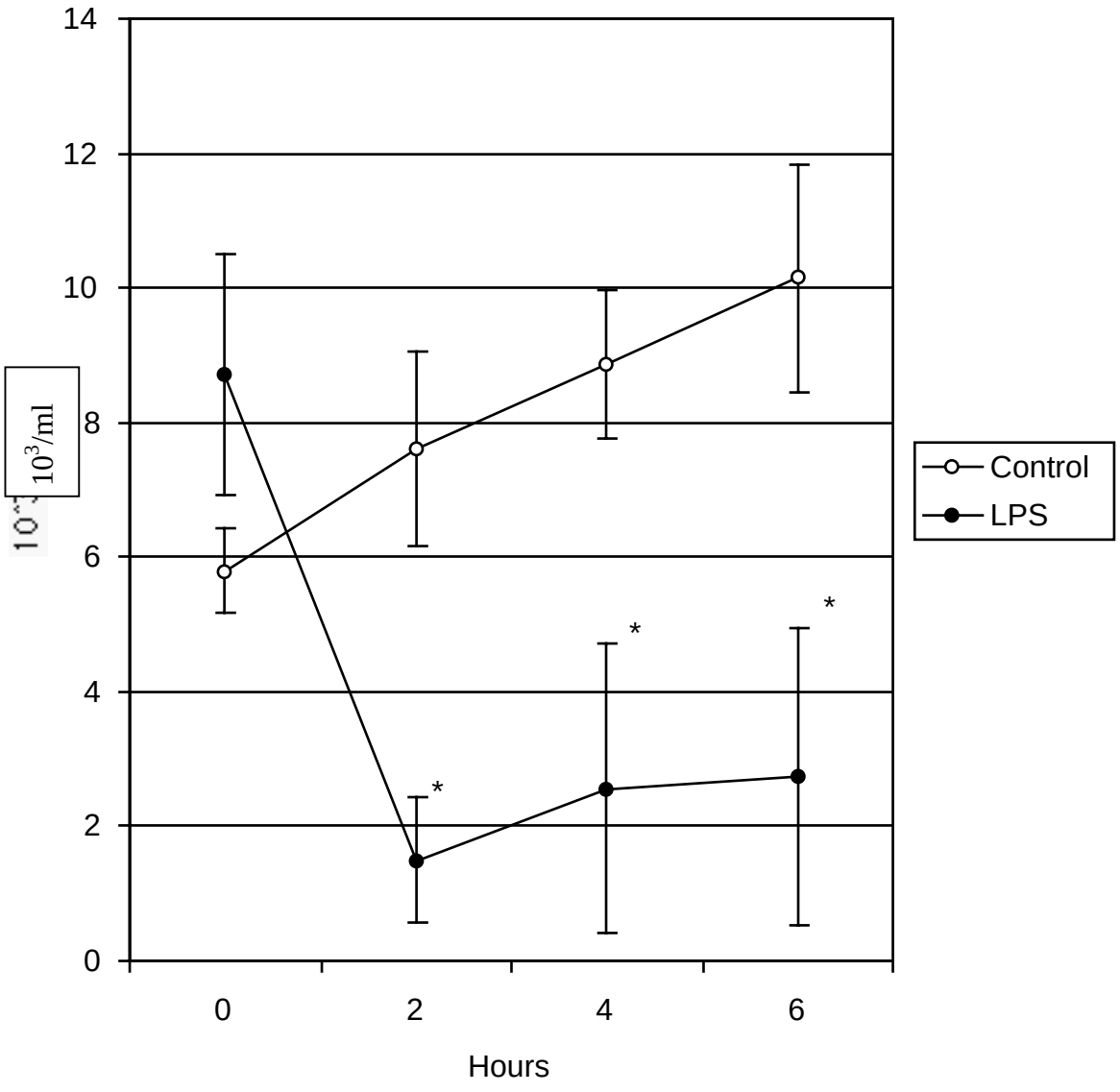


Figure 16. Neutrophil (band) counts

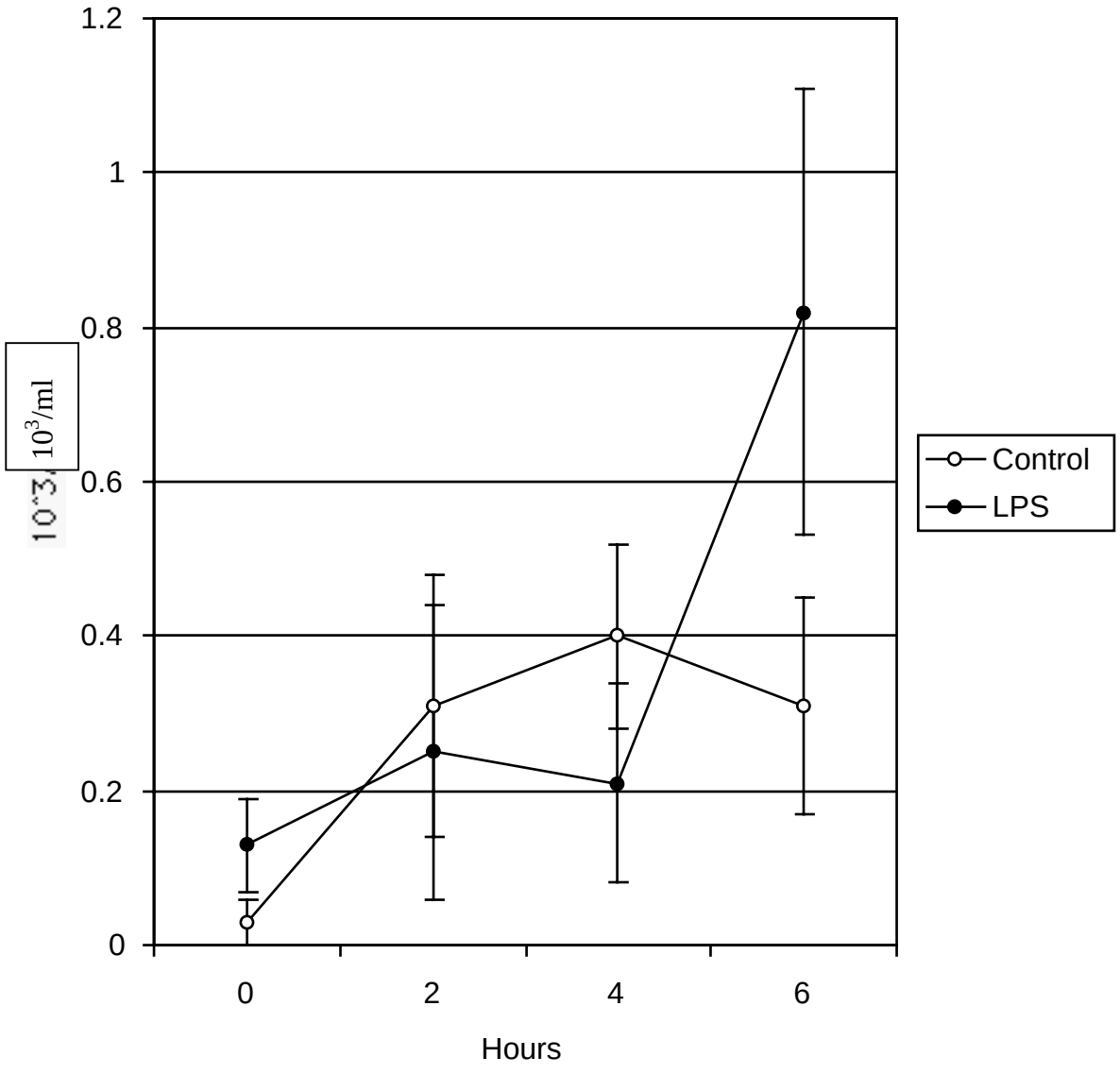


Figure 17. Lymphocyte counts

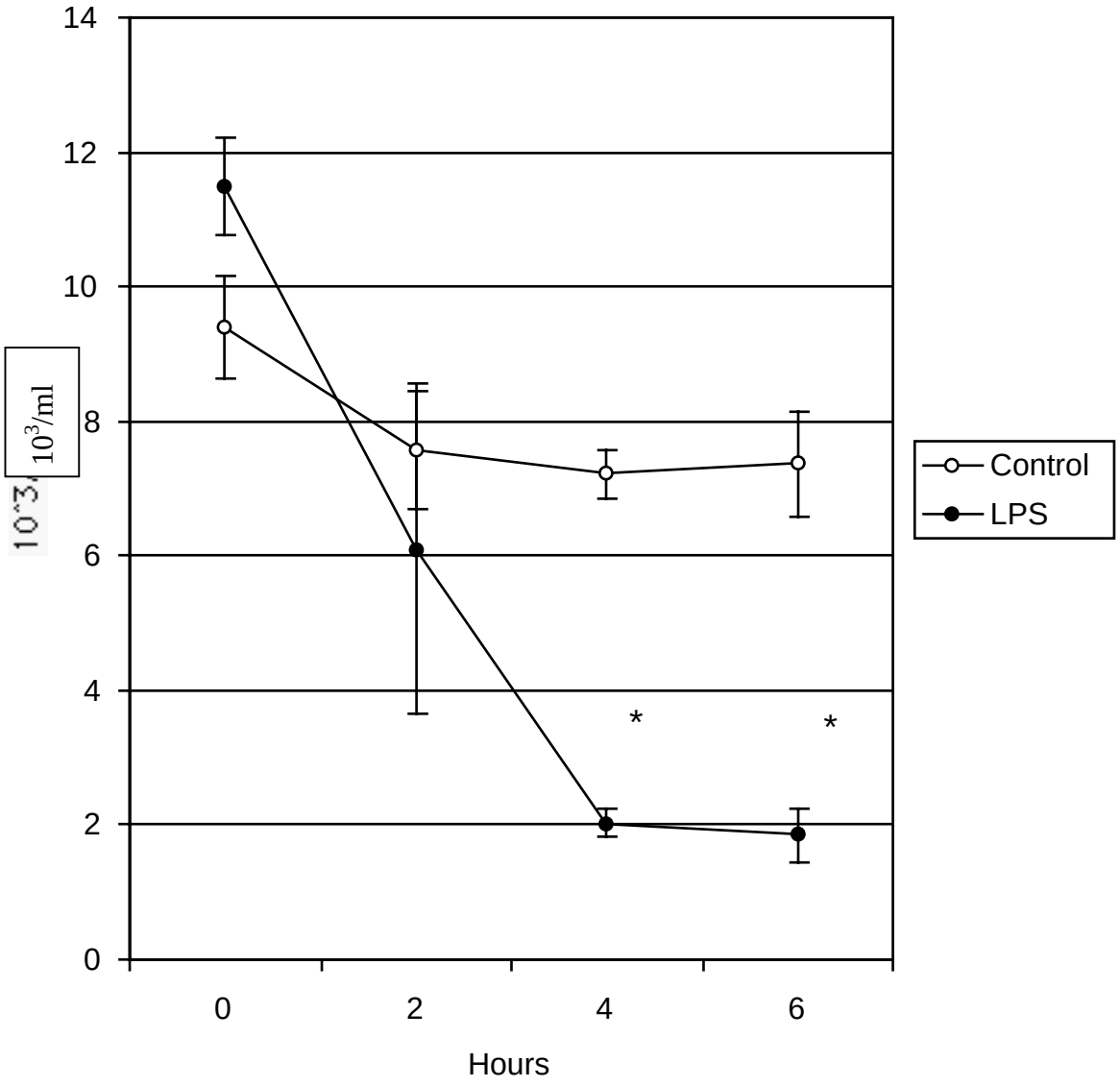


Figure 18. Plasma protein concentration

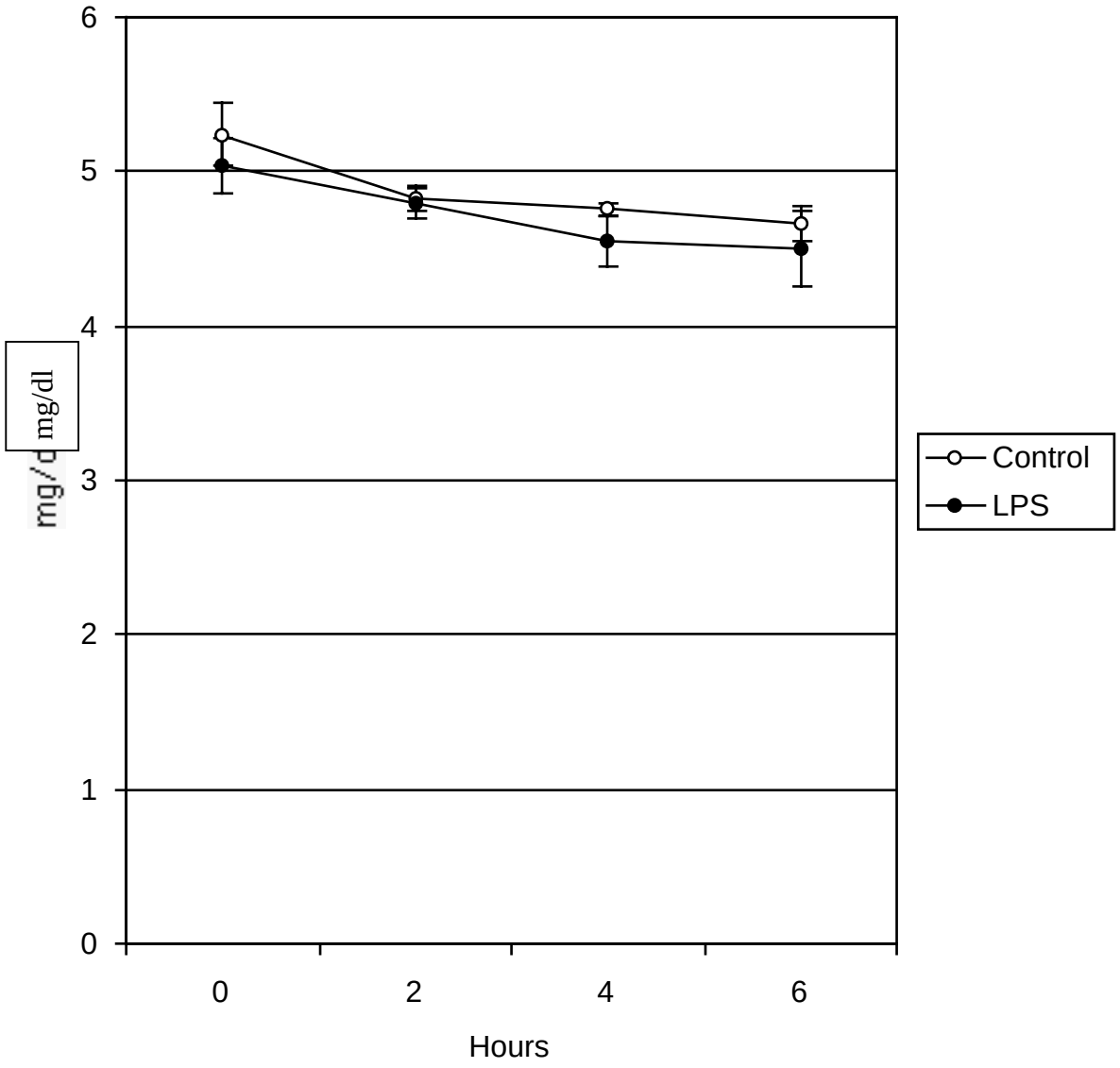


Figure 19. In vitro contraction of bronchi to substance P expressed as a percentage of the maximum contraction to KCl.

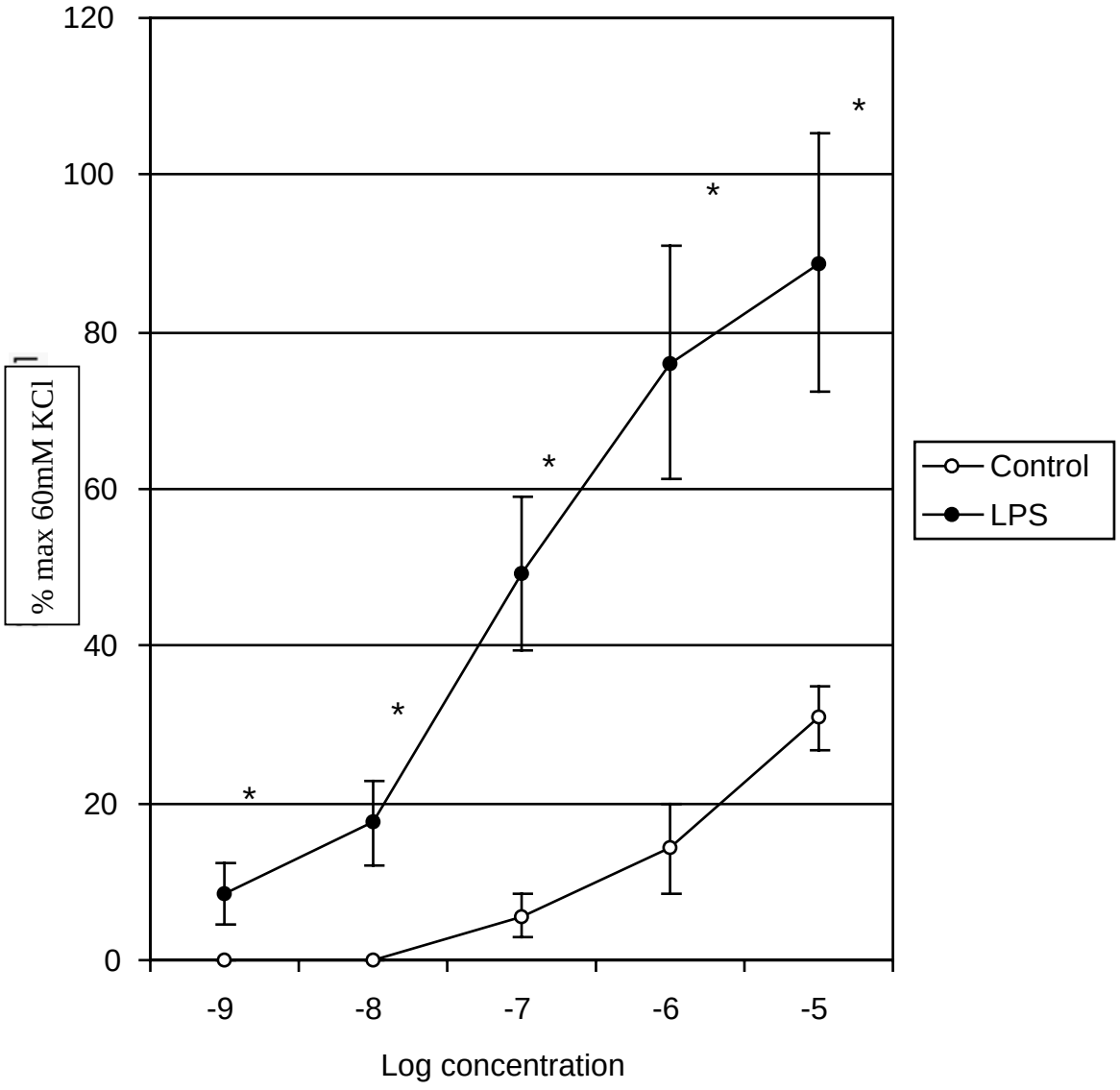


Figure 20. In vitro contraction of bronchi to substance P expressed as a percentage of the maximum contraction to carbacol.

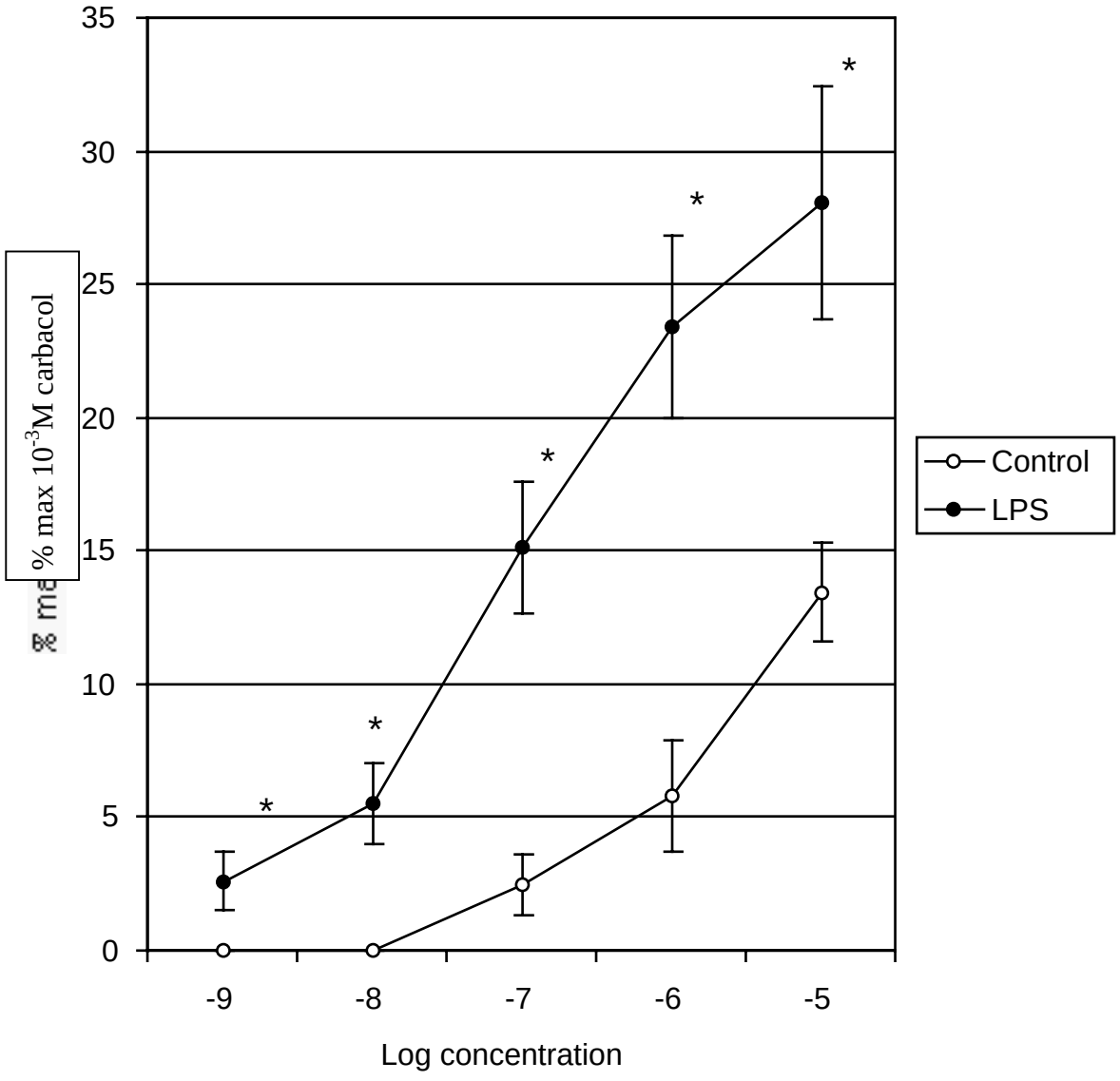


Figure 21. In vitro contraction of bronchi to bradykinin expressed as a percentage of the maximum contraction to KCl.

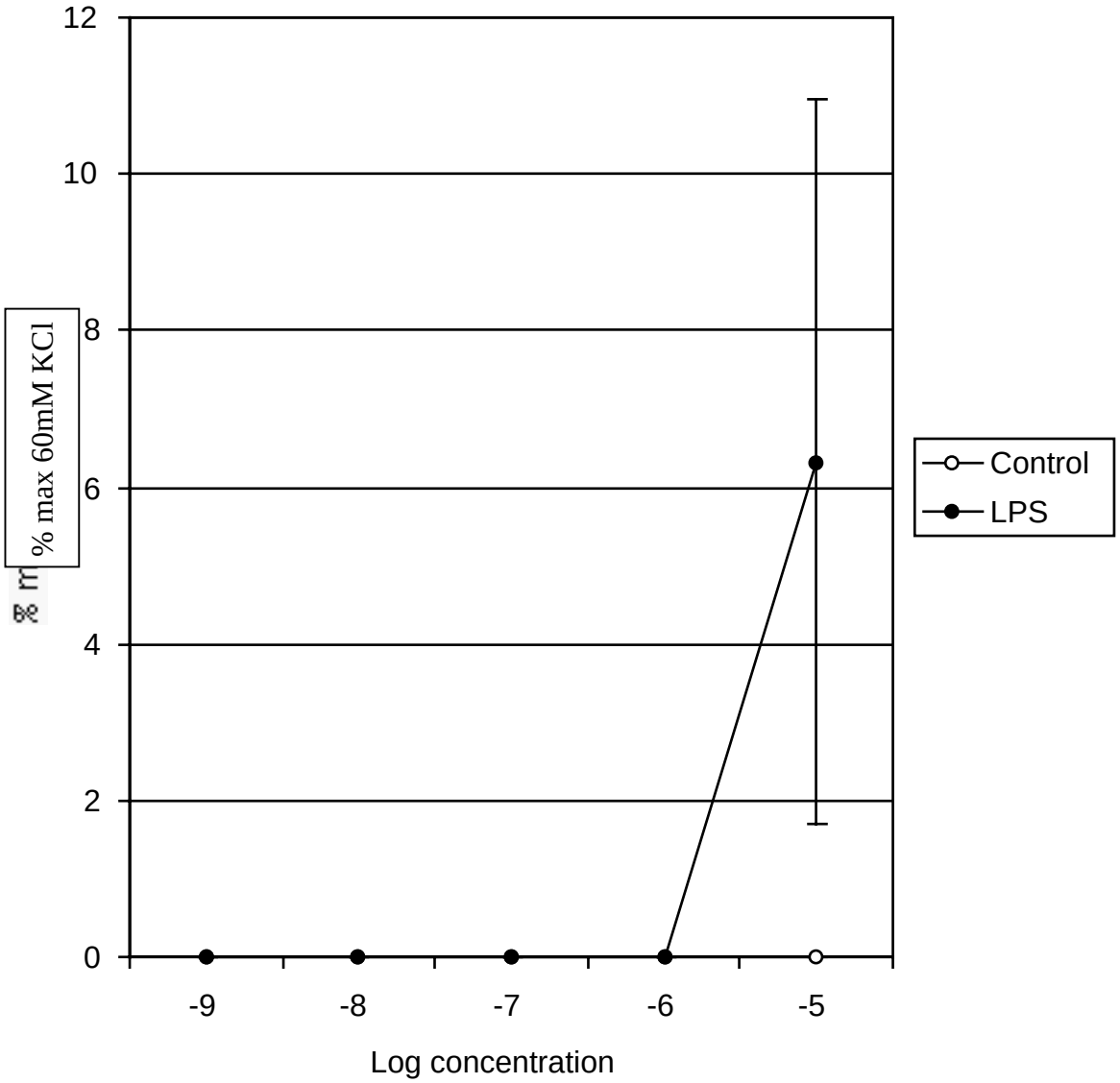


Figure 22. In vitro contraction of bronchi to bradykinin expressed as a percentage of the maximum contraction to carbacol.

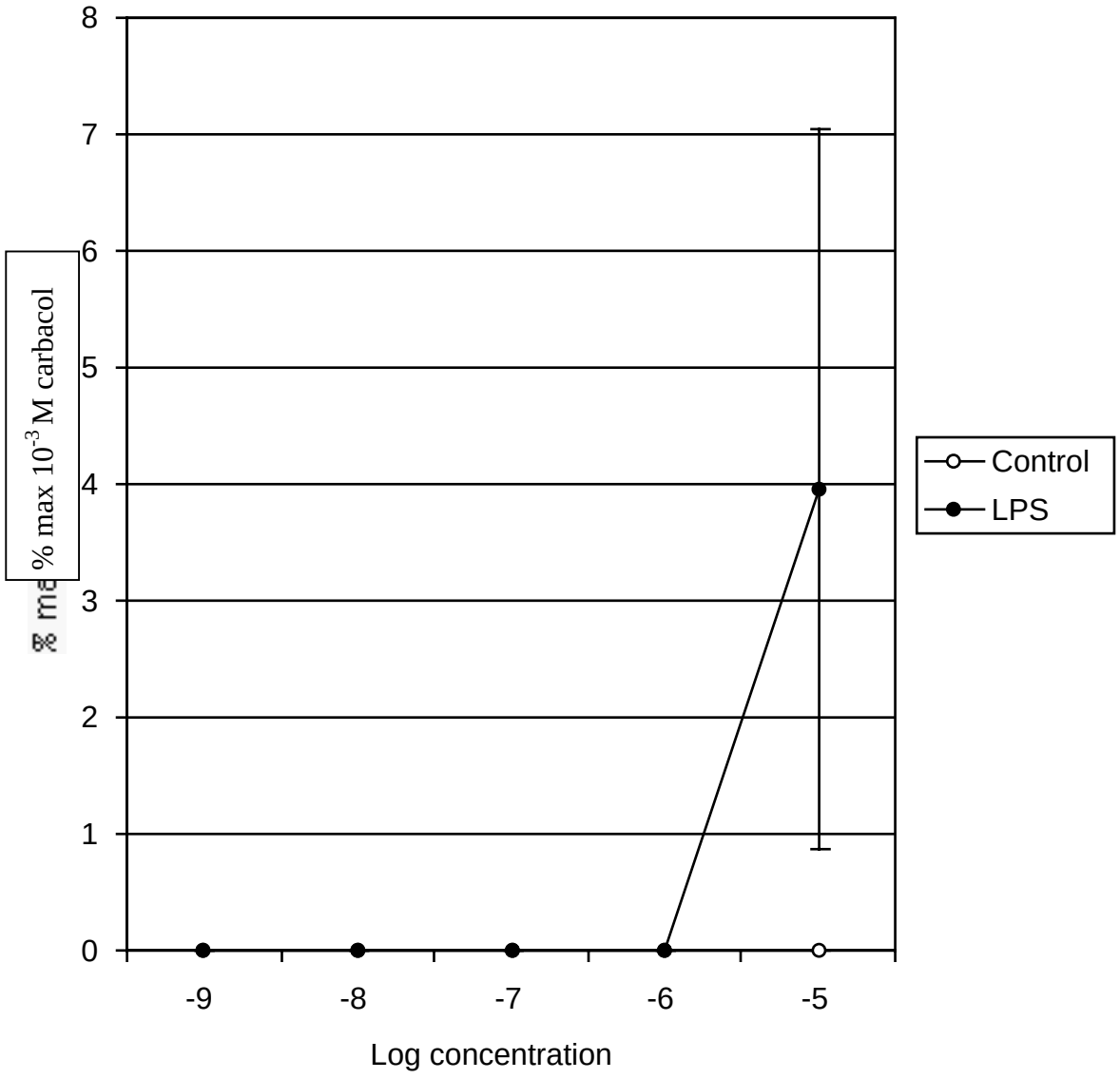


Figure 23. In vitro contraction of bronchi to carbacol expressed as a percentage of the maximum contraction to KCl.

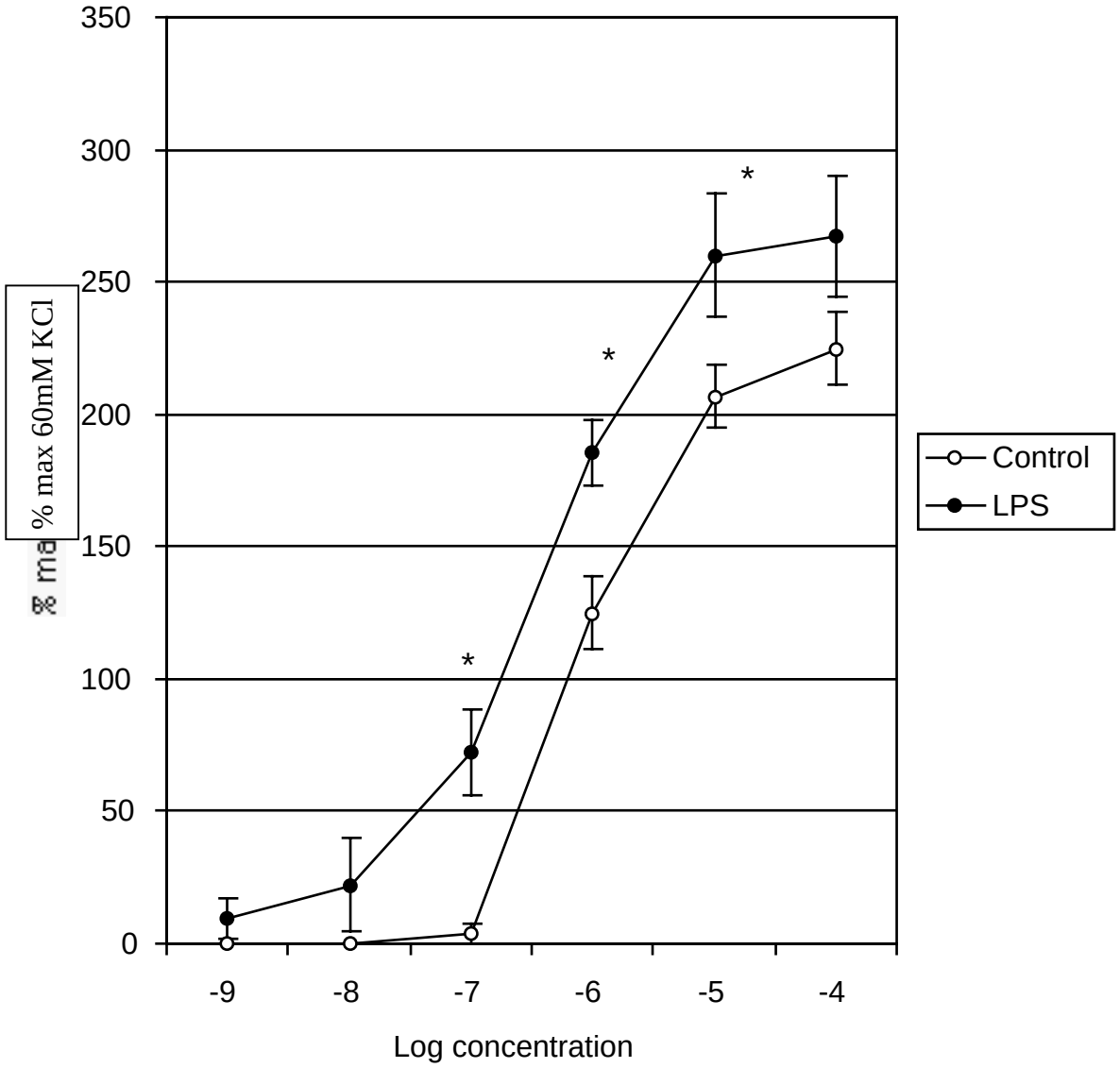


Figure 24. In vitro contraction of bronchi to carbacol expressed as a percentage of the maximum contraction to carbacol.

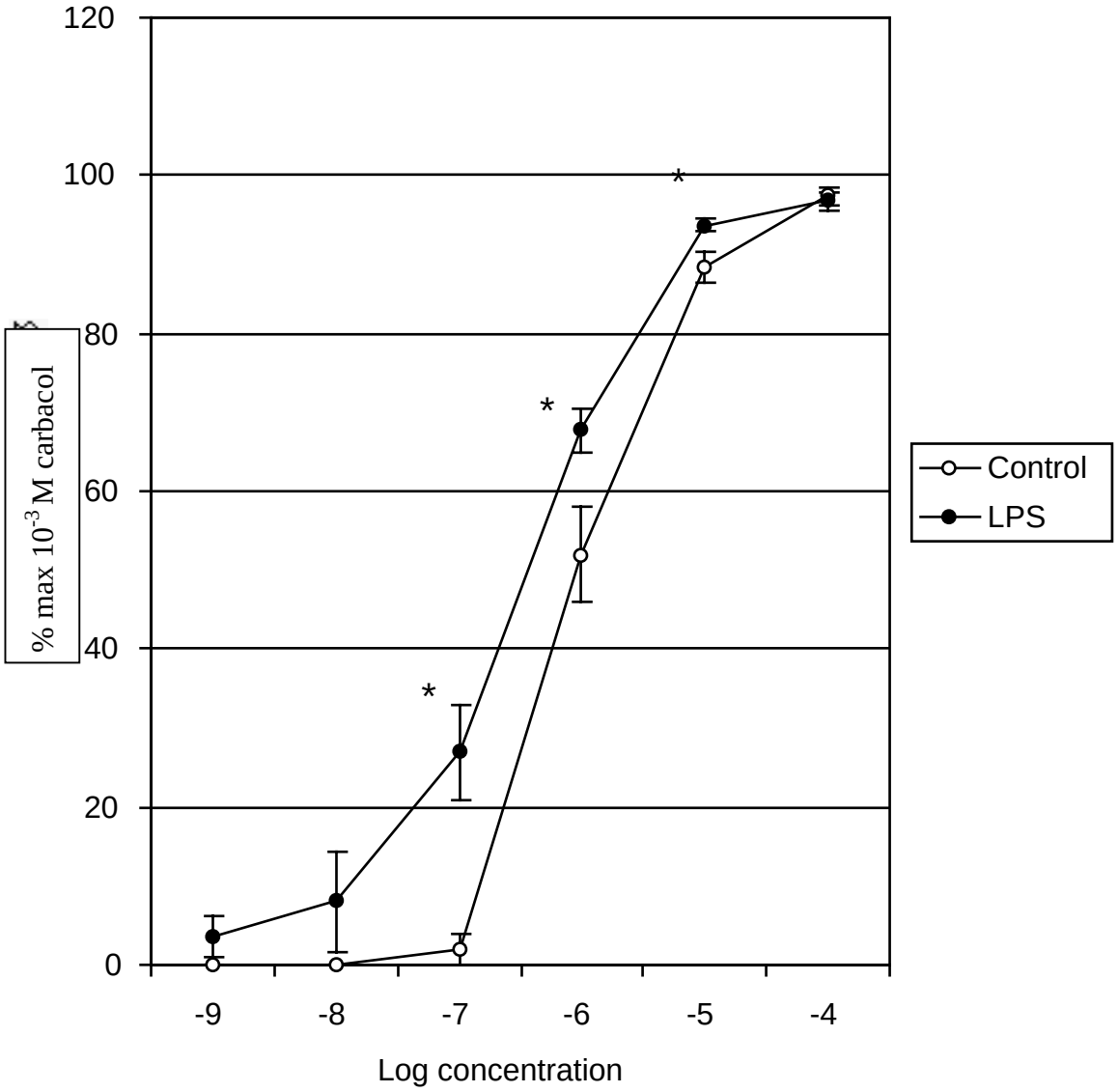


Figure 25. In vitro contraction of bronchi to electrical field stimulation expressed as a percentage of the maximum contraction to KCl.

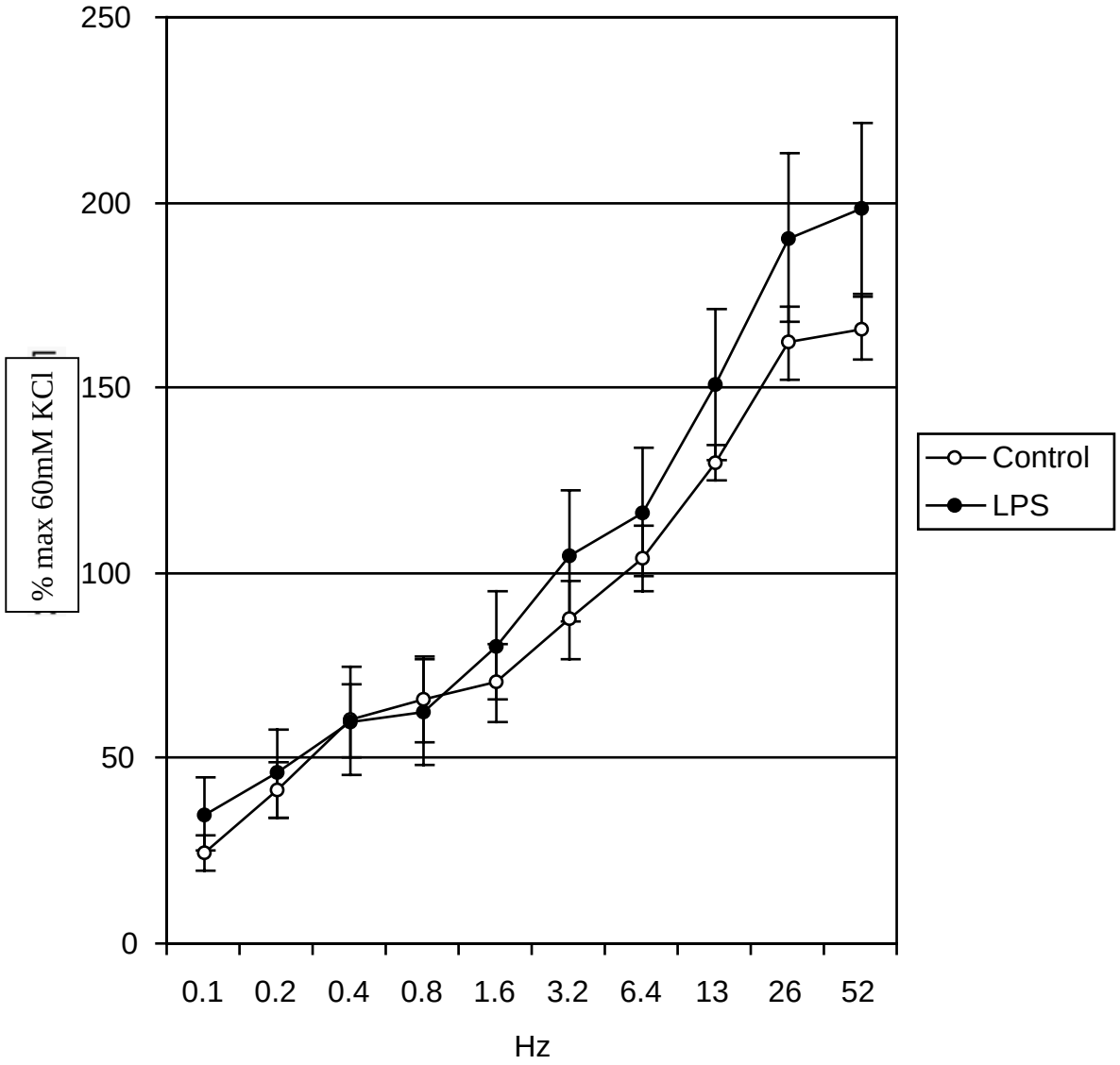


Figure 26. In vitro contraction of bronchi to electrical field stimulation expressed as a percentage of the maximum contraction to carbacol.

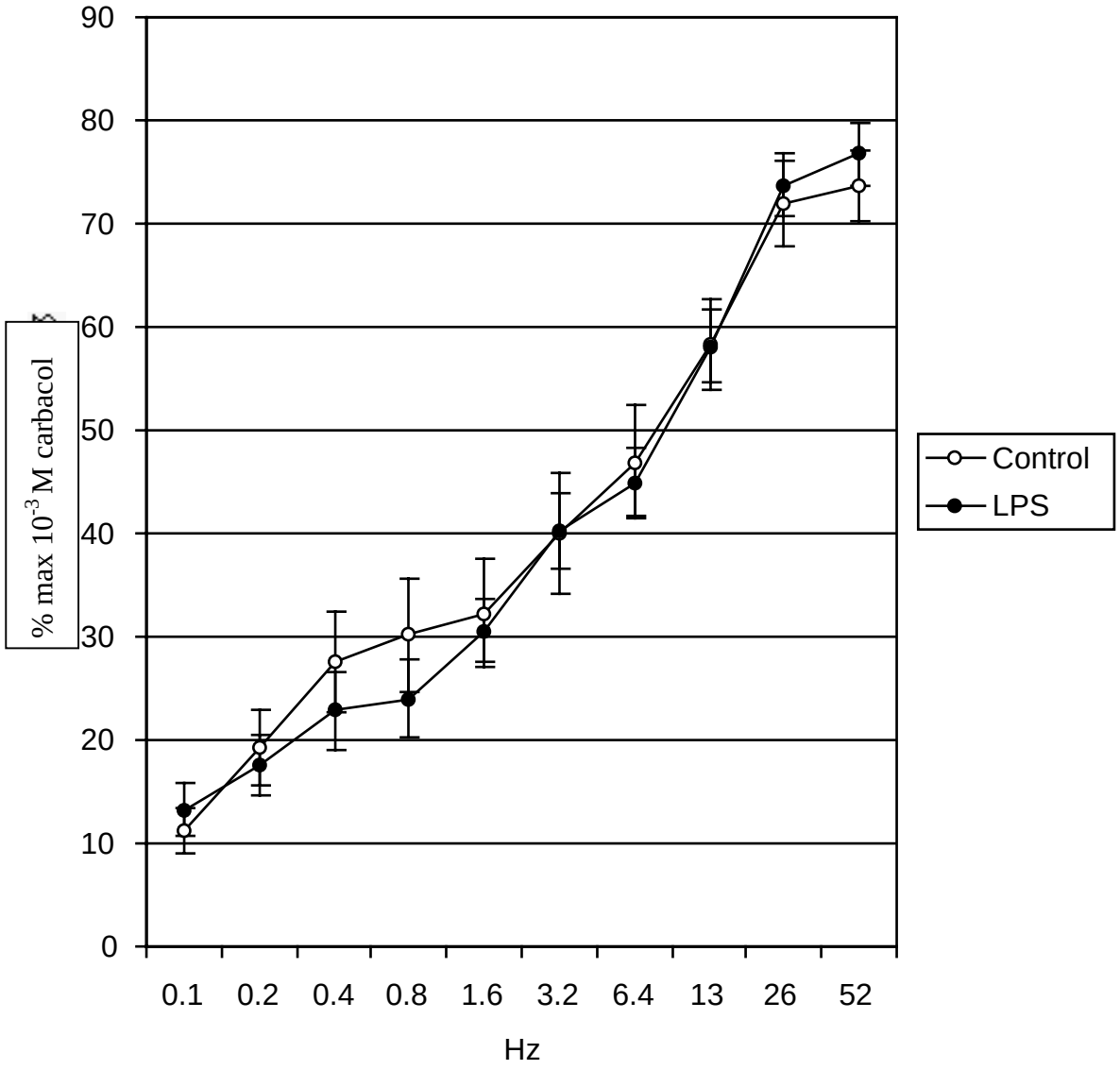


Figure 27. In vitro contraction of bronchi to electrical field stimulation, in the presence of thiorphan and captopril, expressed as a percentage of the maximum contraction to KCl.

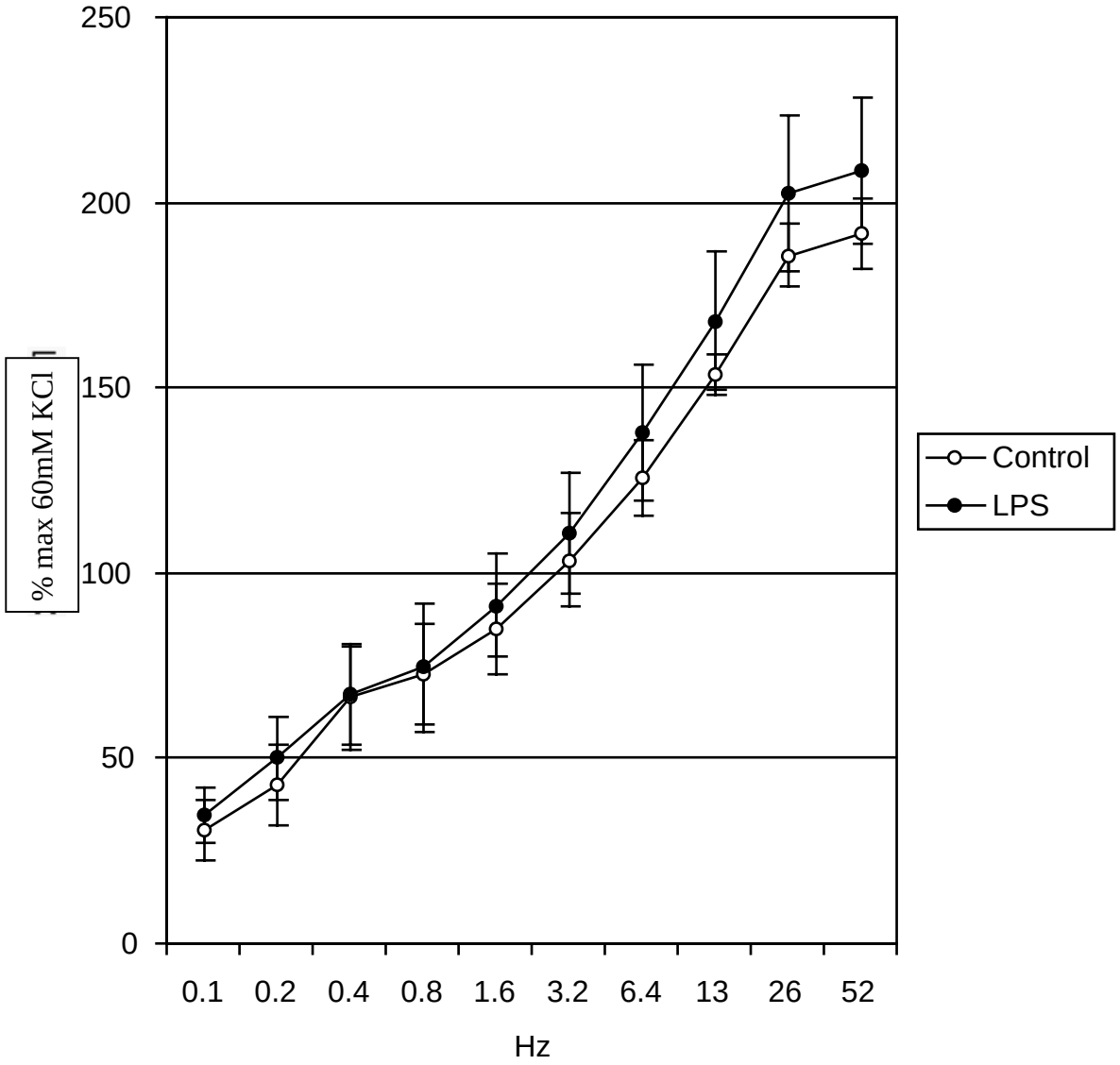
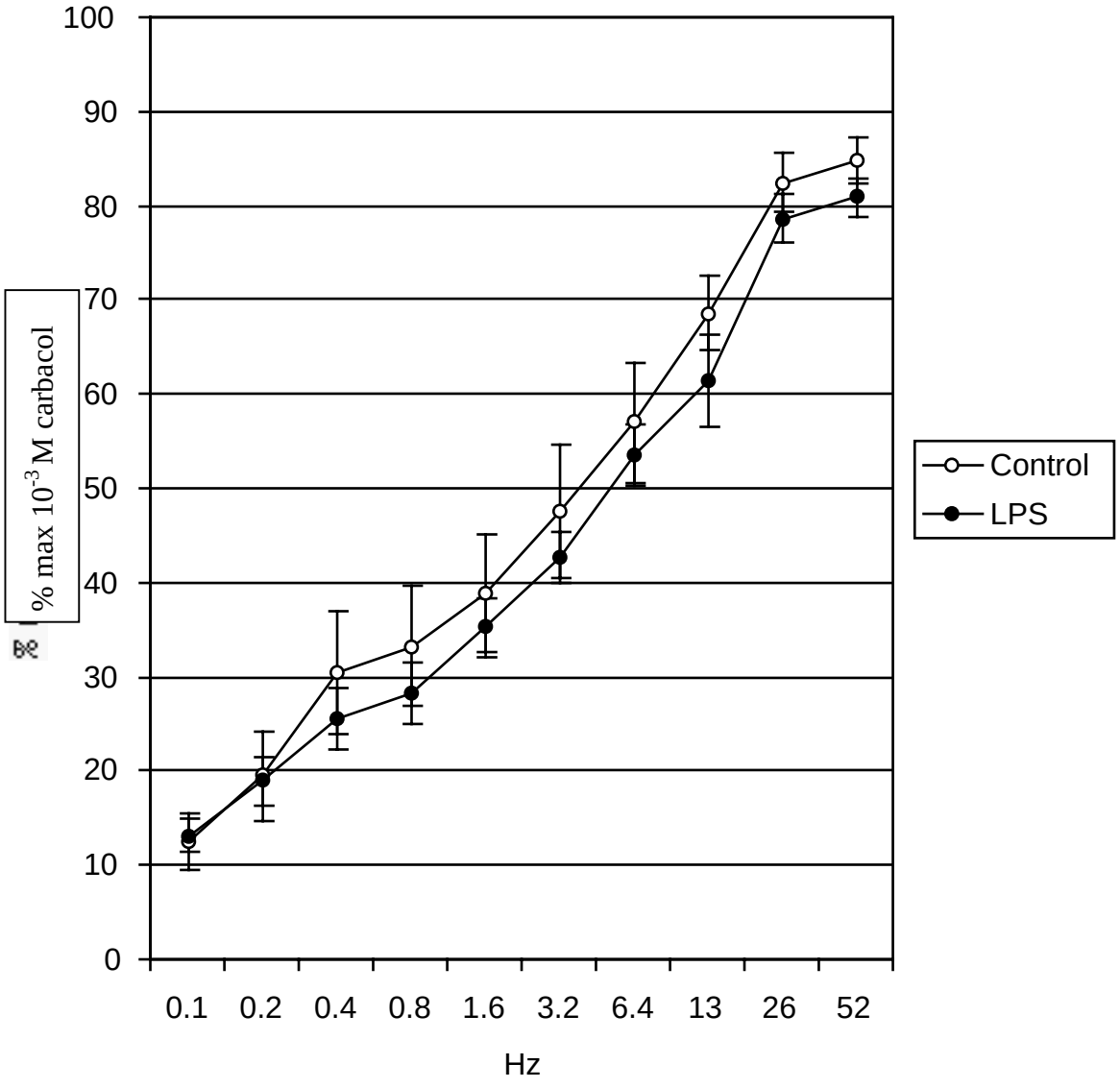


Figure 28. In vitro contraction of bronchi to electrical field stimulation, in the presence of thiorphan and captopril, expressed as a percentage of the maximum contraction to carbacol.



Discussion

The changes observed during endotoxemia are the result of dynamic interactions between the complex body systems of the animal and the Gram negative bacterial cell wall product, lipopolysaccharide. The complexity of these interactions is enhanced by the cumulative effects of the changes produced. For example, increases in mean pulmonary arterial pressure and in pulmonary arterial wedge are the result of increased pulmonary vascular resistance, which is due to a vasoconstrictive response caused by inflammatory mediators released in response to endotoxin. These changes in turn lead to increases in pulmonary capillary hydrostatic pressure, edema formation, increased vascular shunting and ventilation perfusion mismatches. As the disease progresses, the diffusion distances for oxygen and carbon dioxide increase, resulting in an decreased partial pressure of oxygen and oxygen saturation, and an increased partial pressure of carbon dioxide and alveolar to arterial oxygen gradient. Increased vascular shunting, decreased ventilation and ventilation perfusion mismatching may contribute to these changes as well. The decreased delivery of oxygen to the tissues, resulting from the decreased oxygenation of the blood, may then contribute to the multiple organ failure that is characteristic of endotoxemia.

The increased pulmonary vascular resistance and pulmonary hypertension can cause an increased afterload in the right side of the heart, which causes an increased myocardial workload, decreased stroke volume and a decreased cardiac output. The decreased partial pressure of oxygen and oxygen saturation can lead to a decrease in cardiac function, due to the importance of oxygen dependant cellular respiration for proper myocardial activity. This is in addition to the direct myocardial depressant effects of endotoxin (Schnider 1986, Teule 1985). These changes in afterload and contractility coupled with an increase in heart rate, which causes a decreased preload, stroke volume and filling time, lead to a decrease in cardiac output and cardiac index. The increased heart rate may be due to a combination of catecholamine release and a baroreceptor reflex response to the systemic hypotension. The decreased output by the heart leads to a poor

perfusion of organs, contributing to the decreased delivery of oxygen and other essential nutrients to, and the decreased removal of toxic waste products from, the body's major organs. These changes may lead to irreversible damage to essential organ systems. The reduction in cardiac output also leads to a pooling of blood in the major veins, causing an increase in central venous pressure. This increase in CVP causes an increase in the hydrostatic pressure in the vessels and contributes to edema formation.

The systemic arterial pressure shows a biphasic response, initially increasing, then decreasing in response to endotoxin administration. The initial systemic hypertension corresponds to an increase in total peripheral resistance, which is probably due to the vasoconstrictive response to endotoxin. This increase in vascular resistance may also be partially caused by the activation of the renin-angiotensin-aldosterone system, due to reduced renal perfusion. This vasoconstriction initially overcomes the decreased cardiac output to produce the early rise in systemic arterial pressure. During the early phase of endotoxemia, vasoconstriction may cause shunting of blood, which helps maintain adequate perfusion to vital organs, such as the heart, brain (and possibly the kidneys), in spite of the decreased cardiac output. After the initial hypertensive phase a loss of the vasoconstrictive response occurs, causing systemic hypotension. This may be due to a combination of vasodilation, leading to a decreased total peripheral resistance, and the decreased cardiac output. The decreased cardiac output, vasodilation and systemic hypotension again lead to a decreased perfusion of the vital organs, which over time can cause irreversible loss of function in those organs.

Increased airway pressure occurs early in the course of events following endotoxin infusion. Active bronchoconstriction, caused by the release of an array of inflammatory mediators from inflammatory cells causes smooth muscle contraction which in turn leads to a decrease in airway diameter. By Poiseuille's law a decrease in airway diameter by half would lead to a sixteen-fold increase in airway resistance, by making it extremely difficult for the animal to inflate its lungs, leading to an increased inspiratory effort and decreased ventilation. Using positive pressure ventilation this increased resistance manifests itself as increased airway pressure. The ventilator delivers a constant volume of air, so it must produce a higher pressure to move the same volume of air through the

smaller diameter airways. Decreased ventilation would lead to ventilation perfusion mismatches and reduced oxygenation of the blood, contributing to the decreased partial pressure of oxygen, decreased oxygen saturation and the increased partial pressure of carbon dioxide. Bronchoconstriction is the cause of these changes in the early stage of endotoxemia, but as fluid accumulates within the lung parenchyma and airways, the subsequent edema may contribute further to increased airway pressure/resistance as well as decreased dynamic compliance.

The white blood cell count dropped dramatically with administration of endotoxin. The two major circulating leukocyte types, segmented neutrophils and lymphocytes, both decreased but for different reasons. The number of circulating segmented neutrophils decreased following their extravasation into the tissues as part of the inflammatory response. The reasons for this extravasation of neutrophils are many-fold including vascular endothelial damage, the potent chemotactic effects of lipopolysaccharide itself and the chemotactic mediators released due to endotoxin. The neutrophils probably enter a variety of organs, but have been observed on histological examination of the lungs where (with the help of other inflammatory cells) they play a major role in the pathologic changes that are taking place. Neutrophils release an array of inflammatory mediators, such as eicosanoids and oxygen free-radicals, which promote the inflammatory reaction and cause direct damage to the lungs.

Unlike neutrophils, lymphocytes do not leave the blood to promote inflammation in the tissues. They enter the lymph nodes, where they await antigen-presenting cells so they can undergo blast transformation. If stimulated by a specific antigen they will proliferate, forming a germinal center, allowing the body to mount a more specific defense against the pathogens that are present. This entry into the lymph nodes is due to a combined acute inflammatory and stress response and is thought to be mediated in part through the release of corticosteroids. The corticosteroids then cause the expression of adhesion molecules on the lymphocytes causing them to adhere to the vascular endothelium adjacent to the lymph nodes and allowing them to extravasate into the lymph nodes.

Band neutrophils, which are immature neutrophils, tended to increase after beginning endotoxin administration. This was observed at six hours, but was not statistically significant. It takes from twelve to twenty four hours for the appearance of significant increases in band neutrophils (Jain 1978, 1991). Immature neutrophils are being released early from the bone marrow storage pool due to the effects of interleukins and Granulocyte Macrophage Colony Stimulating factor. The release of band neutrophils is probably an adaptive response to compensate for the increased consumption of neutrophils in the tissues. However, in the case of endotoxemia where neutrophils themselves are the principal cause of the damage, the value of this response is questionable.

Direct endothelial damage by endotoxin cause a multitude of changes in the vascular system. The endothelial cells are responsible for producing antithrombogenic mediators, which prevent platelet aggregation and clot formation. Because of endothelial sloughing, not only are these antithrombogenic mediators lost but the collagen layer beneath the endothelial cells is also exposed. The collagen layer contains many negatively charged surfaces, which promote blood coagulation and the formation of microthrombi throughout the vascular system. The loss of endothelial cells also increases capillary permeability and leads to edema. The formation of microthrombi consumes large quantities of blood coagulation factors, which can lead to disseminated intravascular coagulation.

Hematocrit also progressively rises during endotoxemia. Two possible explanations exist for this increase. First, as mentioned before, there is an increased release of catecholamines (Teule 1984), which can lead to contraction of splenic smooth muscle causing red blood cells stored in the spleen to enter the circulation and increase the hematocrit. The other mechanism by which the hematocrit increases is the loss of plasma from the vasculature due to increased vascular permeability. This loss of plasma usually exceeds loss of red blood cells and thus leaves the red blood cells in the blood more concentrated.

Hyperresponsiveness of bronchi to various contractile agents was observed in vitro. This phenomenon, which has many potential causes, was not unexpected based on

the increased airway pressures observed in vivo. An increase in receptor numbers could cause the hyperresponsiveness by allowing more binding sites for the agents to stimulate the smooth muscle cells. A decrease in degradative enzymes would allow contractile agonists to build up to higher concentrations and remain there for longer times. Higher concentrations of agonists lead to more receptors bound and a greater cellular response. The affinity of the receptors could also be enhanced, making them more sensitive to the presence of the contracting agents. Finally, a change in the levels of other mediators already present, or the levels released due to the administration of our contracting agents, might also produce hyperresponsiveness to contractile agonists. Thus, a synergistic agent, produced due to the presence of lipopolysaccharide, could be present in the tissues. Administration of the contractile agonist would then produce a stronger contractile response due to synergistic activity with the preexisting agent. Alternatively, some contractile agonists act partially or entirely through the release of other chemical transmitters to contract smooth muscle. So if substance P, which may contract smooth muscle partially by production of prostaglandins, were to be added to a bronchus where there was an upregulation of cyclooxygenase enzymes, it might be able to cause the production of larger quantities of prostaglandins and produce a stronger contraction.

Histologically the lungs of endotoxin treated pigs demonstrated signs of acute inflammation. Neutrophilic infiltration caused by the release of chemotactic mediators due to the presence of endotoxin was observed. Hemorrhage was present and was most likely caused by damage to the blood vessels as a result of the vasculitis that endotoxin causes. Additionally, increased blood pressure in the lungs and disseminated intravascular coagulation could also potentiate or lead to pulmonary hemorrhage. The congestion present was likely due to a combination of decreased cardiac output, increased vascular tone and obstruction of blood vessels by thrombus formation.

The results of these experiments were similar to those seen by other investigators where overlap occurred and similar to the changes seen in adult respiratory distress syndrome in humans. The pathological changes are consistent with an acute pneumonia and the contraction studies demonstrated that the third generation bronchi were hyperresponsive to various bronchoconstrictive agents, as was predicted.

Conclusions

From the results obtained during these experiments there are many possible directions that further research in this area could pursue. Our next step is to pre-treat the pigs with capsaicin to reduce the levels of nonadrenergic noncholinergic mediators available for release by capsaicin sensitive unmyleinated nerves. In so doing we hope to evaluate the role of neurogenic inflammation in the pathogenesis of endotoxic shock. The changes caused by reduction of the nonadrenergic noncholinergic component of the inflammatory response will again be evaluated by a combined in vivo, in vitro, and histopathologic approach.

Other possible experiments involve the blocking of specific neurokinin receptors or other receptors of mediators released from the nonadrenergic noncholonergic system. The purpose of these experiments would be to determine which endotoxin related changes were attenuated, and thus to have indirect information regarding the role of the nonadrenergic noncholinergic system.

In vitro we observed no significant differences in the contractile response to electric field stimulation between the endotoxin treated group and the controls. This is surprising because of the increased response to substance P and carbachol observed in the same contractile studies. The nonadrenergic noncholinergic system, acting through the same receptors as substance P, and the cholinergic autonomic nervous system, acting through the same receptors as carbachol, are believed to be the major systems activated by electric field stimulation. Thus since both of these systems show a degree of hyperresponsiveness in the endotoxin treated group, one would expect to see an increase to electric field as well. A possible reason why this was not observed is that electric field stimulation is not specific and may activate the adrenergic autonomic nervous system as well. If there was simultaneously increased adrenergic activity, its relaxing effects might mask the hyperresponsiveness of the other two systems. Thus experiments conducted in the presence of a beta-adrenergic blocker would be helpful in determining whether or not

an increased release of adrenergic mediators is masking the bronchial hyperresponsiveness, caused to endotoxin, that would be expected in electric field stimulated bronchi.

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