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Oxidant-related metabolism in the respiratory muscles in response to increased respiratory loads

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The Ohio State University, 1993
OXIDANT-RELATED METABOLISM IN THE RESPIRATORY MUSCLES
IN RESPONSE TO INCREASED RESPIRATORY LOADS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Gisella R. Borzone, M.D.

The Ohio State University
1993

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Department of Medical Biochemistry
To My Family.

To My Husband Pablo

To My Children Juan Pablo and Roberto Ignacio
ACKNOWLEDGMENTS

To my husband Pablo, I offer sincere thanks for his encouragement, optimism and contagious enthusiasm during these years of intense study and research. To our children, Juan Pablo and Roberto Ignacio, many thanks for being the most wonderful children and for being always happy.

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CHAPTER I
GENERAL INTRODUCTION.

This dissertation attempts to characterize the main antioxidant defense systems in the major muscles responsible for breathing. It also evaluates the response of these antioxidant defense systems to increased contractile activity in vivo. In this way, it attempts to identify indirectly, a possible role for free radical species in the respiratory muscle dysfunction often observed with high levels of contractile activity. In addition, it attempts to directly identify the presence of free radical species in the diaphragm following a fatiguing protocol that results in respiratory pump failure in vivo. It does so with the aid of electron paramagnetic resonance (ESR) spectroscopy.

When skeletal muscles contract forcefully over long periods of time, they fatigue. Fatigue can be defined as a reversible loss in force production with continued or repeated contractions (16,25). The respiratory muscles, like any other skeletal muscle can also become fatigued, resulting in a decreased ability to sustain a particular pattern of pressure generation to maintain adequate alveolar ventilation. This happens when they are forced to develop large pressures in response to increased respiratory stimulation or chronic mechanical impairment of
the lungs and/or thorax.

It is recognized that respiratory muscle dysfunction due to excessive respiratory muscle loading may contribute to the development of ventilatory failure in patients with a variety of cardiopulmonary diseases (26). It has been suggested that some of the loss in tension development observed during fatiguing contractions, particularly if the fatiguing stimulus is prolonged, could be due to muscle injury (11,21). In this regard, diverse mechanisms have been postulated to play a role in injury to skeletal muscle under excessive mechanical loads; among them, oxidant stress due to increased formation of free radical species with increased contractile activity has been suggested as one of the mechanisms responsible for the long-lasting component of the fatigue process (2,4,7).

Although the role of muscle injury in respiratory muscle dysfunction of patients with cardiopulmonary diseases has not been established, the possibility of oxidant stress to respiratory muscles has recently been suggested, from evidence (mostly indirect) of free radical generation in skeletal muscle (7,9,11,15,19).

In this regard, it has been postulated that free radicals may be generated in both electrically stimulated and exercising skeletal muscle, and that they may contribute to the dysfunction of its contractile elements. In support of this concept, strenuous exercise in rats has been shown to result in skeletal muscle lipid peroxidation, associated with evidence of free radical generation (7,9,11,15,19). Indirect evidence for the production of free radicals in exercise in humans are also available (13).
It is known that skeletal muscle has many potential sources of free radical generation (9). Mitochondria are possibly the most important source under conditions of exercise or intense stimulation.

With regard to the respiratory muscles, a number of in vitro and in situ studies have recently reported evidence for the production of oxygen-centered free radicals and for the effects of exogenous free radical species in fatigue of the electrically stimulated diaphragm (12,23,24). In addition, the administration of exogenous antioxidants have been shown to attenuate the fatigue process in these preparations (22-24,27).

The research questions experimentally tested were: a) is the production of free radical species increased in the diaphragm when contractile activity is largely increased in vivo? b) is it possible to obtain in the respiratory muscles undergoing increased contractile activity to the point of pump failure, a similar pattern of oxidative stress as that described for in vitro diaphragm preparations and in skeletal muscle with exercise? c) are the antioxidant defense systems of respiratory muscles different that those of other skeletal muscles that could make these muscles more or less vulnerable to oxidant stress? d) what is the response of the antioxidant defense systems in respiratory muscles to conditions of largely increased work of breathing and to respiratory failure? and e) is the energy substrate status of the respiratory muscles affected under conditions of increased activity to the point of pump failure, so as to be responsible for the muscle dysfunction and or affect the cell redox state?
To answer some of these questions, a model of respiratory failure induced by inspiratory resistive breathing was developed in the anesthetized rat. Inspiratory resistive loading is frequently used to experimentally induce increased respiratory muscle activity and eventually, pump failure (1,8,14). Although ventilatory loading using inspiratory resistances is not completely analogous to the derangement produced by disease states (i.e. a resistive device affects the lungs uniformly, whereas diseases seldom produce an uniform pattern of damage), it has been used to simulate the mechanical derangements produced by diseases of the lungs. Ventilatory loading using inspiratory resistances has helped in elucidating the mechanisms involved in the respiratory adjustments to the functional changes produced by diseases. In this study, Sprague-Dawley rats underwent an incremental inspiratory resistive loading protocol that enormously increased the work of breathing. The time course of the changes in pressure development, pattern of ventilation and external work performed by the respiratory pump are described in chapter III. Special emphasis is given to the description of early indicators of impending respiratory failure.

Chapter II corresponds to a background section which deals with relevant aspects of respiratory muscle physiology and structure, mechanisms of muscle fatigue and potential mechanisms of muscle injury. The intracellular antioxidant mechanisms are also reviewed and when pertinent, the relationship to respiratory muscle cells is discussed.

Chapter IV describes the status of the high energy phosphates in the
diaphragm and intercostal muscles following the incremental inspiratory resistive loading protocol. High performance liquid chromatography was used to determine the concentrations of adenine and pyridine nucleotides and of phosphocreatine in muscle acid extracts. One of the postulated mechanisms of skeletal muscle fatigue is the depletion of energy sources in the contracting muscle. Although this seems to be a likely mechanism for the loss of force generation when isolated muscle is stimulated to contract \textit{in vitro}, it is less likely to operate \textit{in vivo} conditions under adequate blood supply. It has been shown that inspiratory resistive loading in the dog is associated with a reduction in the concentration of ATP and phosphocreatine in the diaphragm (5-6).

Although there is a recent interest in investigating the role of free radicals in diaphragm dysfunction, no attempts have been made to study the characteristics of the antioxidant defense systems of the respiratory muscles. Thus, an important part of this dissertation will deal with the characterization of the antioxidant enzyme systems of the two main muscles of respiration, the diaphragm and the intercostals. Only during the last year, data on diaphragm glutathione has become available (3,10).

Chapter V deals with the characterization of the normal antioxidant enzyme systems in respiratory muscles. Large differences in glutathione content and in the specific activity of the antioxidant enzymes, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, were found among diaphragm and intercostal muscles of the rat. The role of blood contamination of the tissue
samples as well as the relationship between oxidative capacity and antioxidant capacity are discussed. In addition, chapter V also deals with the effect of the inspiratory resistive loading protocol on the specific activity of the antioxidant enzymes.

In chapter VI, the response of one of the main cellular antioxidants to the resistive loading protocol is evaluated. The levels of total and oxidized glutathione were determined in both diaphragm and intercostal muscles, in controls and experimental animals. Interestingly, changes in the glutathione redox cycle were expected to represent markers of the presence of oxidative stress in the respiratory muscles (28). The results, however, suggest that the changes observed in the glutathione system may more likely be secondary to some of the systemic effects induced by the resistive loading protocol, rather than to oxidative stress of the respiratory muscles. A critical evaluation of several mechanisms potentially capable of affecting the glutathione cycle under the experimental conditions is presented. Following recent publications reporting changes in liver glutathione with exhaustive exercise in experimental animals (20), the response of liver glutathione to the resistive loading protocol was also evaluated.

Finally, in chapter VII, attempts to directly identify an increase in free radical species generation in the respiratory muscles following intense contractile activity are described. Very few studies in skeletal muscle (11,17,18) and no studies in the diaphragm have been reported which have used electron spin resonance (ESR) spectroscopy to detect free radical species. Diaphragm samples
quickly frozen following the resistive loading protocol were studied. Because there is a lack of information describing the ways of interpreting ESR data in muscle, different ways of analyzing and reporting the data are explored.

The results of the experimental studies are discussed in detail in the chapters in which they are presented. In chapter VIII, a general discussion focusing on the implications of the results is presented.
List of references.


CHAPTER II
BACKGROUND

PART A: RESPIRATORY MUSCLES

1. Functional anatomy of the respiratory muscles.

The respiration of mammals involves a respiratory system composed of two parts:

a) the lungs which serve as the organ that exchanges oxygen and carbon dioxide between the organism and its environment and
b) the thorax, which functions as a pump, moving air in and out of the lungs. Even though the lungs are richly innervated, they do not have an autonomous function and can only fulfill their role by the action of the thorax.

Breathing requires that a high level of coordination be achieved between different muscles with ventilatory functions. These muscles are called respiratory muscles and are conventionally divided into three groups:

a) the diaphragm,

b) the muscles of the rib cage,

c) the abdominal muscles.

Although the diaphragm is the principal respiratory muscle, all the other respiratory muscles must work together in a coordinated manner to rhythmically
displace the chest wall to pump air into and out of the lungs and so keep arterial blood gases within acceptable limits to ensure adequate tissue oxygenation and CO₂ removal (25).

The respiratory system has a remarkable ability to adjust to changes in environmental and metabolic conditions in order to maintain ventilation at appropriate levels and preserve oxygen and carbon dioxide homeostasis. For example, in exercise, ventilation is closely linked to the production of CO₂. The respiratory control mechanisms during exercise adjust the ventilation so as to maintain PaCO₂ more or less constant. The increase in ventilation during exercise is brought about by an increase in tidal volume (Vₜ) and in breathing frequency and by a shortened inspiratory duration (Tᵢ). Progressive increase in Vₜ means that the respiratory muscles have to increase their velocity of contraction and their frequency of contraction (44).

The diaphragm and accessory muscles of respiration are embryologically, morphologically and functionally skeletal muscles (25). However, like the heart they must contract with a regular rhythm throughout the lifespan. They repeatedly perform relatively sustained tension generating and shortening actions, while limb muscles are usually required to generate short bursts of tension and shortening (28). This determines the differences in endurance properties between respiratory and limb muscles. Guenther and Meloney in 1916 introduced their comprehensive study of the diaphragm in this way: "...While most skeletal muscles in the living body contract with varying degrees of intensity and at irregular intervals between
which occur long periods of rest, the diaphragm from the time of birth to that of
death performs day and night a continual succession of brief contractions of a fairly
regular rhythm and fairly uniform in extent, alternating with brief intervals of rest.
Thus, this muscle, together with other respiratory muscles, holds a unique position
among skeletal muscles and suggests a crude analogy with the heart. Like the
heart too, the diaphragm performs during the lifetime of the individual an
incredibly huge amount of work, probably more than any other skeletal muscle.
In view of these facts, it might be expected that a careful study of this important
muscle would reveal physical and chemical peculiarities which would distinguish
it from other muscles" (61).

Another unique characteristic of the respiratory muscles is their resting
position, which is dictated by the elastic recoil forces of the lung and the effect of
gravity on the ribs and on the contents of the thorax and the abdomen (25).

1.1. The diaphragm.

The diaphragm is a thin, flat, musculotendinous structure which divides the
thoracic and abdominal cavities in mammals. In humans, its muscle fibers arise
from three sources: a) a short sternal portion, arising from the xiphoid process,
with no particular functional importance; b) the crural or vertebral portion, which
inserts into the first three lumbar vertebral bodies and aponeurotic arches, with no
direct insertion to the rib cage; and c) the costal portion, which arises from the
inner surfaces and superior aspects of the lower six ribs and interdigitates with
fibers of the transversus abdominis. The costal fibers run craniocaudally, parallel to the long axis of the body, directly apposed to the inner surface of the lower part of the rib cage. All the diaphragmatic fibers converge at the central tendon (Fig. 1).

During inspiration, the diaphragm fibers shorten, causing a descent of its dome. This has three major consequences; a) intrathoracic pressure decreases, air goes into the thorax and lung volume increases, b) abdominal pressure increases, moving the abdominal contents downward; and c) the rib cage is displaced.

After early controversy, it is now well established that the motor supply to the diaphragm is through the phrenic nerves (56). Each phrenic nerve supplies its own hemidiaphragm. Bilateral section of the phrenic nerves causes the loss of all diaphragm respiratory-related activities and the muscle atrophy (11).
Fig. 1. Illustration of the diaphragm at relaxed end-expiration, separating the thorax from the abdomen. (From Rochester et al. 1981).
1.2. Muscles of the rib cage:

This group of muscles comprises the intercostal and the accessory muscles. The main accessory muscles are the scaleni and the sternocleidomastoids, which under some circumstances participate in respiratory acts.

1.2.1. Intercostal muscles.

They comprise two thin muscle layers between the ribs, all innervated by the intercostal nerves. They are identified as "external" or "internal" based on their relationship to the body surface, the external being closer to the skin (Fig. 2). The external intercostal muscles extend from the tubercles of the ribs dorsally, to the costochondral junctions ventrally, with the fibers directed obliquely downward and forward from the rib above to the rib below. The internal intercostals extend from the sternocostal junction to the posterior angles of the ribs, with the fibers running obliquely downward and backward from the upper rib to the rib below. Therefore, there are two muscle layers in the lateral rib cage and only one layer in the ventral and dorsal regions. The internal intercostals have a posterior or interosseous portion and an anterior or intercartilaginous portion, the later constitute the parasternal intercostals which is the only muscle layer between the sternocostal and the costochondral junctions. Parasternal intercostals are active together with the diaphragm during inspiration and have an inspiratory role producing elevation of the ribs and a descent of the sternum (bucket-handle motion) (18,19,51). Their contribution to tidal volume, however, has not been determined. Unlike those of the parasternals, the actions of the external and
internal interosseous intercostal muscles in the mechanics of breathing are unclear, they appear to have both inspiratory and expiratory functions, depending on the lung volume. These muscles also have important non-respiratory functions (20).
Fig. 2: Intercostal muscles

A: External and internal intercostals.

B: Parasternal intercostals.

1.3. Abdominal muscles. Abdominal muscles which make an important contribution to respiration are those forming the ventrolateral wall. These are the external and internal oblique, transversus abdominis and rectus abdominis. Contraction of the abdominal muscles causes an inward displacement of the abdomen and a rise in abdominal pressure; as a result, the diaphragm is displaced into the thorax and lung volume decreases. In doing so, they are the most important muscles of expiration and play important roles in forced expiration, coughing, vomiting, defecation and parturition (20). Although the abdominal muscles are regarded as expiratory, they seem to play an important role in inspiration as well. In hyperpnea of exercise and during breathing against inspiratory mechanical loads, they improve the efficiency of the diaphragm as a pressure generator. When they relax at end expiration, they promote passive descent of the diaphragm dome, so that lung volume increases before the onset of inspiratory muscle contraction (20).
Fig 3: Structure of the diaphragm and intercostal muscles.

The sarcomere is repeated along the axis of the fibril at distances of 1500 - 2300 nm. (Adapted from Harper. Biochemistry, 1990, p 627-649)
Fig 4: The sarcomere. The length of the fibers of actin and myosin do not change during contraction. The length of the sarcomere decreases during contraction because actin and myosin slide past each other. (Modified from Stryer, L. Biochemistry. 1988, p 921-948)
2.2. Sliding filament hypothesis.

Skeletal muscle shortens by as much as a third of its original length as it contracts. In the 1950s, two groups of investigators (Andrew Huxley and Ralph Niedergerke, and Hugh Huxley and Jean Hanson) independently proposed a sliding filament model for muscle contraction, on the basis of X-ray, light-microscopy and electron-microscopic studies (38-40). The essential features of this model are summarized as follows: a) the length of the thin and thick filaments do not change during muscle contraction, b) the length of the sarcomere decreases during contraction because the two types of filament overlap and slide past each other during contraction (fig. 3), and c) force is generated by processes in which actin, myosin and ATP participate. Muscle is made up of 75% water and more than 20% protein, actin and myosin being the major muscle proteins (60). Actin, a 43,000-MW globular protein comprises 25% of the muscle protein. At physiologic ionic strength and in the presence of magnesium, it polymerizes non-covalently to form an insoluble double helical filament (F-actin) with no catalytic activity. Myosin, contributes 55% of muscle protein, forms the thick filament, has ATP hydrolysis (ATPase) activity and binds to actin. Tropomyosin and the proteins of the troponin system (C, I, T) have important regulatory roles, mediating the regulation of muscle contraction by cytosolic Ca$^{2+}$.

The proposed sequence of events at the molecular level, during muscle contraction, is summarized in fig. 5 and fig. 6. a) The myosin head containing ADP + Pi, which are products of ATP hydrolysis can rotate freely through large
angles in order to locate and bind to actin; b) actin-myosin interaction, favored by the rise in cytosolic Ca$^{2+}$, promotes the release of ADP and Pi from the actin-myosin complex, c) myosin head then changes its angle and pulls the actin filament toward the center of the sarcomere d) a new ATP molecule binds to the myosin-actin complex, decreasing the affinity of myosin for actin and releasing myosin head from actin; e) the ATP is again hydrolyzed by the myosin head, but without releasing ADP and Pi, to re-start the cycle (35,60).

-2.3. Muscle relaxation.

Muscle relaxation occurs when: a) Ca$^{2+}$ in the sarcoplasm falls below $10^{-7}$ M, due to its sequestration in the sarcoplasmic reticulum by an energy-dependent calcium pump, b) Troponin C loses its bound Ca$^{2+}$ and via its interaction with tropomyosin, inhibits further myosin head-actin interaction, and c) in the presence of ATP, the myosin head detaches from F actin (35).
Fig 5: The hydrolysis of ATP is what drives the cyclic association and dissociation of actin and myosin in a series of reactions described in the text. (Modified from Stryer L. Biochemistry, second ed. Freeman, 1981)
Fig 6: Proposed mechanism for the generation of force by interaction between actin and myosin (From Stryer L. Biochemistry, 1988, p 921-948).

3.1. Muscle fiber types.

As in other skeletal muscles, the diaphragm is composed of functional units (motor units), each comprising a motoneurone and the muscle fibers it innervates. The force generated by the diaphragm during different ventilatory and non-ventilatory tasks depends on the number and type of motor units recruited (59).

Classifications of muscle motor units based on mechanical properties alone, allow the distinction of only two types of muscle fibers; fast twitch and slow twitch, based on the presence or absence respectively, of sag during unfused tetanic activation (12). Further classification of motor units was done on the basis of their resistance to fatigue during repetitive activation (13), as seen in Fig. 7. According to this classification, slow twitch motor units are fatigue resistant while fast twitch motor units vary in their fatigue resistance (fast twitch fatigue resistant, fast twitch fatigue intermediate and fast twitch fatigable).

Histochemical and biochemical differences define three groups of muscle fibers (52): a) fast glycolytic fibers (FG), which have relatively short isometric twitch contraction time, high activity of myosin ATPase, a well developed glycolytic enzyme system, a poorly developed mitochondrial oxidative enzyme system and low resistance to fatigue, b) fast oxidative glycolytic fibers (FOG) which also have short isometric twitch contraction time, high activity of myosin ATPase and a well developed glycolytic enzyme system. In contrast to the FG fibers, they have many more mitochondria and oxidative enzymes, and are more resistant to fatigue.
slow oxidative fibers (SO) have longer isometric twitch contraction time, and relatively low specific activity of myosin ATPase; their glycolytic enzyme systems are not well developed and resistance to fatigue is higher than in either of the other two fiber types.

Studies by Sieck et al. (59) have shown, using the activity of the Krebs cycle enzyme, succinate dehydrogenase in individual fiber types, as an index of the overall fiber oxidative capacity, that FG and FOG fibers are not easy to separate. He found values of SDH activity overlapping in both groups of fibers.

Most mammalian muscles contain a mixture of these three types of muscle fibers. For instance, the rat diaphragm is composed of 34% FOG fibers, 27% FG fibers and 39% SO fibers (47). Rat internal intercostals have 15% FOG fibers, 54% FG and 31% SO fibers; whereas the external intercostals have 25% FOG fibers, 47% FG and 28% SO fibers (47). Human diaphragm is composed of 21% FOG, 55% SO, and 24% FG fibers (48).
Fig 7: Motor units. Contractile and fatigue properties of four different types of motor units. FF = fast-twitch fatigable, F Int = fast-twitch intermediate, FR = fast twitch fatigue resistant, S = slow twitch. Muscle unit fibers are distinguished from each other by the profile of histochemical staining. (From Sieck, G.C. Clinics in Chest Medicine 9(2): 195-210, 1988.)
4. Work and energy cost of breathing.

During breathing, the respiratory muscles do work to overcome the elastic, flow-resistive and inertial forces of the lung and the chest wall. The elastic work of breathing is done to overcome the elastic recoil of the lung and chest wall, and the resistive work is done to overcome the resistance of airways and tissues.

The work of breathing can be determined by relating the pressure exerted across the respiratory system to the resulting change in volume, since the product of pressure (P) and volume (V) has the dimensions of work according to the equation:

$$\text{Work} = \int P \, dV$$

(Equation 1)

The relationship between changes in pleural pressure and changes in lung volume during spontaneous breathing can be used to measure work of breathing (Fig. 8). During inspiration, the work done to overcome the elastic forces of the lung is determined from the area of the trapezoid OAECY. The area of the loop ABCEA corresponds to the work done to overcome non-elastic forces during inspiration, and the area of the loop OABCY is the total work of breathing during inspiration.

Expiration during quiet breathing is passive, since the elastic recoil of the lung is sufficient to overcome the expiratory airflow resistance. At high levels of
ventilation and when airway resistance is increased, additional mechanical work during expiration is required to overcome non-elastic forces. Under these circumstances, the pleural pressure exceeds atmospheric pressure and the loop AECDA extends beyond the confines of the trapezoid OAECY.
5. **Response of the respiratory system to mechanical loading.**

Several factors contribute to maintain ventilation during mechanical loading. Factors intrinsic to the respiratory muscles as well as reflex neural mechanisms are known to be involved. The adjustments however, tend to minimize work and may interfere with adequate gas exchange. Thus, at high levels of ventilation and workload, the adjustments to mechanical loads favor a reduction in work.

The force that the muscles develop for a fixed electrical stimulus depends on the length of the muscle (force-length relationship). As the muscles shorten, less force is developed. Force also depends on the velocity of shortening (force-velocity relationship), with less force being developed as the velocity of shortening increases. During loading, both the magnitude and the velocity of shortening of the respiratory muscles tend to decrease, compensating for the mechanical effects of the load. Reduction in shortening of the inspiratory muscles increases the signal from muscle spindles and this translates into increased contractile activity. Afferent information from pulmonary mechanoreceptors also change with loading. Since tidal volume is decreased, inspiratory duration tends to be prolonged. In addition, chemoreceptors, both central and peripheral, are involved in sustaining ventilation.
6. **Respiratory muscle fatigue.**

Muscle fatigue can be defined as an exertion-induced, reversible decrease in muscle strength or in the force exerted by the muscles in response to a given stimulus or load.

Muscle fatigue is distinguished from myopathic weakness by its reversible nature. It is also distinguished from pharmacologic causes of reversible muscle weakness by the fact that it is precipitated by exertion. Fatigue of the respiratory muscle can then be defined as the inability to develop sufficient pressure for adequate alveolar ventilation.

6.1. **Causes of respiratory muscle fatigue:** Respiratory muscle fatigue occurs when respiratory endurance is exceeded, that is when the load against which the muscles must contract requires too great an effort for too long a time (1).

It has been shown that there is a threshold below which diaphragm fatigue does not occur (7). Except under extreme exercise, healthy individuals do not approach this threshold. Patients with chronic obstructive pulmonary disease (COPD) and those with neuromuscular diseases, however, may be very near the threshold at rest, and with minimal exercise they easily go above it (1). The main problem with disease states is the excessive respiratory workload. In many cases, additional factors such as malnutrition, electrolyte disorders, acid-base disturbances and an unfavorable resting length of the muscles, can limit respiratory muscle endurance.
6.2. Types of fatigue: Three general types of fatigue have been described, reflecting the fact that all elements of the command chain for muscle contraction can contribute to fatigue under some circumstances:

6.2.1. Central fatigue.

6.2.2. Transmission fatigue.

6.2.3. Contractile fatigue.

There is not yet a consensus as to what the relative contribution of these types, to the fatigue process in vivo.

6.2.1. Central fatigue can be defined as a reversible decline in central neural respiratory drive that develops after intense contractile activity of the muscles. One of the possible causes of central fatigue is reflex inhibition of respiratory motor neurons in response to chemical or proprioceptive information from contracting muscles. The phrenic nerves carry many sensory fibers, most of which are inhibitory to respiratory neurons (14). Afferents from the intercostal muscles can also inhibit motor neurons.

Inhibitory effects originating in the brainstem may represent another mechanism of central fatigue. It has been suggested that respiratory drive is the result of an integration of competing influences: excitatory influences resulting from chemical drive and inhibitory influences caused by the work of breathing (53). When the work of breathing is excessive, the inhibitory influences may predominate, causing central fatigue despite the resulting further increase in chemical drive.
Cortical influences may also play a role in central fatigue; the effect of encouragement on performance of non-respiratory skeletal muscles is well known.

Endogenous opioids have also been postulated to play a role in central respiratory muscle fatigue. Scardella et al. (57) have shown in goats, that loaded breathing increases endogenous opioid production. Endogenous opioids are able to decrease ventilatory drive in a similar way as exogenous opioids. This causes hypoventilation.

6.2.2. Transmission fatigue is defined as a reversible, exercise-induced impairment in the transmission of neural impulses through nerves or across the neuromuscular junction. Three possible sites of blockade have been suggested in transmission fatigue, based on in vitro studies on the phrenic nerve-diaphragm preparation (1):

1. Impulses may fail to be conducted at nerve branching points.
2. There may be insufficient neurotransmitter release or reuptake at the neuromuscular junction.
3. The muscle membrane may become less excitable.

Although there is evidence obtained in vitro, for all these possible mechanisms and neurotransmission fatigue has been shown to occur in an animal model in vivo (2), it is not yet clear how relevant this type of fatigue is to conditions in vivo.
6.2.3. **Contractile fatigue** is defined as a reversible impairment in the contractile response of the muscles to normal impulses that is not caused by drugs or alterations in the length-tension or force-velocity relationships of the muscles. (1).

Two types of contractile fatigue have been identified (1,26), a transient type, known as high frequency fatigue and a long-lasting type, known as low frequency fatigue. High frequency fatigue is manifested by a reduced response to frequencies of stimulation in the range of 50-100 Hz, and recovers within minutes. This type of fatigue may just be a type of transmission fatigue (1). Low frequency fatigue is manifested by persistent reduction in the responses to low frequencies of stimulation (10-20 Hz), even after the response to high frequency stimulation has returned to normal. It may last for 12 - 24 hours or more. The different patterns of recovery suggests at least two pathophysiological mechanisms involved.

High frequency fatigue may be caused by accumulation of toxic metabolites, by-products of contraction, such as H\(^+\), by altered calcium and electrolyte concentrations or by a decrease in ATP content at critical sites in the muscles. With every depolarization of the muscles, Ca\(^{+2}\) is released from the sarcoplasmic reticulum into the sarcoplasm, where it binds to troponin C, relieving the contraction-inhibitory effect of troponin and tropomyosin and allowing actin to react with myosin. For relaxation to occur, the excessive sarcoplasmic Ca\(^{+2}\) must first be removed. If the depolarization rate is very high, fatigue could result because of inadequate sarcoplasmic reticulum levels of calcium, due to insufficient
time for sarcoplasmic reticulum calcium reuptake or, because the increase in T-
tubule calcium concentration may interfere with the propagation of the action
potential.

The concentrations of Ca$^{2+}$, Na$^+$, K$^+$, and Cl$^-$ at various muscle
compartments are very important. As the action potential propagates along the
sarcolemma and into the T tubules, sodium diffuses into the cell and subsequently,
potassium diffuses out. Both must be pumped back before another action
potential can occur. If pumps are not able to keep up with the rate of
depolarization, fatigue can develop. In addition, during the action potential,
chloride enters the cell promoting repolarization. It has been postulated (1) that
a decrease in sarcolemma chloride conductance induced by acidosis can delay
repolarization and contribute to fatigue.

There is no good explanation for the long lasting type of fatigue. Some type
of minor injury has been postulated to be responsible for this dysfunction. Some
investigators have recently hypothesized that free radicals produced at higher rates
than at resting conditions could be involved as mediators in this type of fatigue.
Both free radicals generated during intense contractile activity and the products
of free radical reactions could be responsible. This long lasting type of fatigue has
been interpreted by some investigators as damage to the muscle, which can no
longer contract at the same level of activity. It can also be interpreted as a
mechanism to protect the muscle against increased stimuli for contraction.
7. Mechanisms of exercise-induced muscle fiber injury

When dealing with decreased muscle force, it is difficult to clearly differentiate whether fatigue or injury are involved. Exercise for which a skeletal muscle is not adequately conditioned, can result in functional and/or structural damage to the fibers (4). Muscle pain together with the loss in force generation are common phenomena following strenuous or unaccustomed exercise in man. Two types of muscle pain occur following different forms of exercise, one occurring during or immediately after high intensity exercise, and the other, having a delayed onset and starting several hours after exercise (42).

The specific event that serves to initiate exercise-induced muscle fiber injury is not known. Some of the postulated mechanisms can be summarized as follows:

7.1. Mechanical disruption of the sarcolemma. It is postulated that stress imposed on the sarcolemma by sarcomere length inhomogeneities occurring with eccentric contractions is what causes the damage to the muscle cell membrane (4).

7.2. Depletion of muscle energy stores. This phenomenon is known to occur in isolated muscle subjected to hypoxic contractile activity (45). During exercise in vivo, mitochondrial respiration is elevated to match ATP synthesis to ATP hydrolysis. This match is adequate during low to moderate intensity exercise, so that muscle fibers maintain ATP concentrations near resting levels. Research in this area has not been able to show a depletion of muscle energy stores when whole muscle is studied after intense exercise in vivo. It has been postulated that energy stores may be depleted in specific groups of fibers or in areas of the muscle.
cell where a particular concentration of ATP is critical (near ionic pumps for example), producing functional alterations, but not being reflected in studies of high energy phosphates in whole muscle (4).

7.3. Muscle damage by free radicals produced with increased activity.

One of the consequences of the elevated metabolic rate during exercise is an increased production of free radicals (43). This may result primarily from increased reducing equivalent flow through the mitochondrial electron transport system. Under most conditions, the reactive oxygen species formed are controlled by the cell antioxidant systems, but in some circumstances these protective mechanisms can be overwhelmed. Uncontrolled free radical production can result in damage to the cells through the oxidation of phospholipids, DNA, carbohydrates and proteins. Membrane lipid peroxidation may disrupt the normal permeability barrier provided by the sarcolemma, permitting abnormal diffusion of molecules down their respective concentration gradients (Ca^{2+} goes in, intracellular enzymes go out). It has been postulated that Ca^{2+} ATPase is susceptible to oxidation and its inactivation causes alteration of Ca^{2+} homeostasis (4).

Many researchers have attempted to examine the possibility that free radicals may be involved in the damage to muscle which accompanies exercise, but firm acceptance or rejection of the hypothesis has not been possible because of a lack of suitable techniques to study free radicals in muscle (5,8,17,32,42).
7.4. Failure of intracellular calcium homeostasis.

This mechanism has been related to muscle damage in various muscle disorders and with excessive contractile activity of muscle \textit{in vitro} (41). When Ca$^{2+}$ is allowed to enter the cell, overwhelming the Ca$^{2+}$ buffering systems, various Ca$^{2+}$-activated degradation mechanisms start to operate to injure the cell. Significant sustained elevation of intracellular Ca$^{2+}$ is believed to be toxic to muscle cells through activation of proteolytic enzymes, mitochondria overloading and activation of phospholipases (4,24).

"There seems to be little doubt that whatever the initial event, the next sequential step in the injury process is an elevation in intracellular Ca$^{2+}$ concentration. The importance of maintaining free cytosolic Ca$^{2+}$ within narrow limits is indicated by the number of mechanisms the cell has for transporting Ca$^{2+}$ out of the cytosolic compartment. If the mechanism for muscle injury is disruption of the sarcolemma, increased free Ca$^{2+}$ is due to diffusion due to the large gradient. Another mechanism for elevating free cytosolic Ca$^{2+}$ could be the dysfunction of the sarcoplasmic reticulum. Sarcoplasmic reticulum failure to re-sequester Ca$^{2+}$ has been shown with high intensity exercise, and it is possible that this process could be mediated through free radical species (4 )."
PART B: OXIDANTS AND ANTIOXIDANTS.

1. Production and metabolism of reactive oxygen species.

A free radical is defined as any species capable of independent existance that contains one or more unpaired electrons. The term reactive oxygen species, on the other hand, refers not only to oxygen-centered free radicals, but also to some non-radical derivatives of oxygen, such as hydrogen peroxide, singlet oxygen, hypochlorous acid and ozone.

It is well established that free radicals and other reactive oxygen species are continuously produced in vivo. Thus, organisms have evolved both antioxidant defense systems and repair systems that prevent the accumulation of oxidatively-damaged molecules (34).

When molecular oxygen (O₂) accepts one electron from a reducing agent, the primary product generated is the superoxide anion (O₂⁻). Although O₂⁻ is relatively unreactive, several of its derivative compounds are capable of oxidizing organic molecules. In aqueous solutions, O₂⁻ is in equilibrium with its protonated form, HO₂⁻, and although high concentrations of HO₂⁻ are favored at acid pH, the major species at neutral pH is O₂⁻ (27). When these two species (O₂⁻ and HO₂⁻) approach equal molar concentrations, spontaneous dismutation occurs, and H₂O₂ is generated. H₂O₂ can also be generated by the enzyme catalyzed dismutation of O₂⁻. Both O₂⁻ and HO₂⁻ are relatively long-lived in biologic systems; H₂O₂ is able to cross membranes directly (27), whereas O₂⁻ crosses cell membranes via anion channels (7,27).
One of the most potent reactive metabolites of oxygen is the hydroxyl radical (OH\(^-\)). It is formed when \( \text{H}_2\text{O}_2 \) is directly reduced by \( \text{O}_2^- \). Additionally, it can be generated by metal-catalyzed transport of one electron from \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \).

2. **Biological sources of oxygen-derived free radicals.**

1. **Auto-oxidation of low molecular weight species.** Oxygen is univalently reduced by cell components, such as thiols, catecholamines and flavins.

2. **Soluble enzymes and proteins.** Proteins can be the source of free radicals through their own enzymatic activity or by autooxidation of protein-bound cofactors or metals (22). Examples of enzymes that produce oxygen-centered free radicals are: mitochondrial NADH dehydrogenase, cytochrome b, cytochrome P450 reductase and xanthine oxidase.

3. **Mitochondria.** About 2% of the electron flow during normal respiration, univalently reduces \( \text{O}_2 \), yielding \( \text{O}_2^- \) (15). Figs. 9 and 10.

4. **Peroxisomes.** Contain a high level of oxidases which do not form \( \text{O}_2^- \), but divally reduce oxygen to form \( \text{H}_2\text{O}_2 \) (22).

5. **Endoplasmic reticulum and nuclear membrane.** Both contain cytochrome P450 and cytochrome b, which can oxidize unsaturated fatty acids and reduce oxygen (22).

6. **Plasma membrane.** Some intermediates of lipid peroxidation are free radicals and can participate in chain reactions. In addition, plasma membranes
have associated enzymes such as lipoxygenase and cyclooxygenase, which have mechanisms of action involving free radical intermediate formation (22).

7. Polymorphonuclear cells and macrophages. During the inflammatory response, phagocytes produce $\text{O}_2^-$, $\text{H}_2\text{O}_2$, $\text{OH}^-$ and hypochlorous acid (22).
Fig. 9. Mitochondrial electron transport chain. * = postulated sites of free radical species production. (Modified from Mathew C.K., and Van Holde K.E., Biochemistry. The Benjamin/Cummings Co. 1990, p. 518)
Fig. 10. Enzymatic antioxidant defense systems for the mitochondrial production of $O_2^-$ and $H_2O_2$. Superoxide anion is produced at the level of the electron transport chain. Mn SOD catalyzes its conversion to $H_2O_2$. Selenium-dependent glutathione peroxidase (GPx) catalyzes the conversion of $H_2O_2$ to $H_2O$. From: Boveris A., Cadenas E. Production of superoxide radicals and hydrogen peroxide in mitochondria. In: Superoxide Dismutase, edited by L.W. Oberley. Boca Raton, CRC Press Inc., 1982, vol II, p 27)
3. Reactions of free radicals in biological systems.

Much is now known about the reactivity of free radicals with biological molecules, and about the mechanisms of those reactions.

Reactivity of the different species is an important factor to consider. Most free radicals are by nature, very reactive, whereas some are not so reactive. The OH\(^{•}\) is one of the most reactive free radicals known. It reacts with most organic molecules at near diffusion-controlled rates; thus, it reacts at or near its site of production. \(\text{O}_2\)\(^{•}\), on the other hand, is much less reactive, and it is believed that it probably does not cause significant damage to biomolecules, per se (16). Radicals such as OH\(^{•}\) and \(\text{ROO}^{•}\) are capable of causing damage to all the major classes of biological molecules: i.e. lipids, proteins, carbohydrates and nucleic acids.

3.1. Lipid peroxidation. It is the most extensively studied of the free radical reactions in biological systems. Lipid peroxidation is defined as the oxidative destruction of lipids, due to fatty acid oxidation. Although cholesterol can also be oxidized, the most susceptible targets are polyunsaturated fatty acids. Once initiated, lipid peroxidation is a self-perpetuating chain reaction.

Any free radical with sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid (LH) can initiate a chain reaction in lipids. The resulting carbon-centered radical (L\(^{•}\)) reacts rapidly with molecular oxygen to form a peroxy radical (\(\text{LO}_2^{•}\)), which itself can abstract an hydrogen atom from an unsaturated fatty acid, leaving a carbon-centered radical and a lipid hydroperoxide (LOOH) (16).
Initiation:

\[ LH + R^- \rightarrow L^- + RH \]  
(Equation 2)

Propagation:

\[ L^- + O_2 \rightarrow LO_2 \]  
(Equation 3)

\[ LH + LO_2 \rightarrow LOOH + L^- \]  
(Equation 4)

Termination:

\[ L^- + L^- \rightarrow LL \]  
(Equation 5)

\[ LO_2^- + LO_2^- \rightarrow LOOL + O_2 \]  
(Equation 6)

\[ LO_2^- + L^- \rightarrow LOOL \]  
(Equation 7)

The free radical chain reaction described in the equations above, propagates until two free radicals destroy each other to terminate the chain (equations 5, 6 and 7). Lipid peroxidation is stimulated by iron ions, but there are some doubts about the effects of iron as initiator of the process (33).
Lipid hydroperoxides (LOOH) are not very stable compounds and, particularly in the presence of transition metals ions, break down to alkoxyl and peroxyl radicals, all capable of initiating new chain reactions.

The direct effects of free radical reactions in cell membranes include changes in the biophysical properties of the membranes. Fluidity is decreased due to the loss of polyunsaturated fatty acids, electrical resistance decreases, protein mobility in the membranes is decreased and phospholipid exchange between halves of the bilayer is increased (54). Destruction of membranes leads to inactivation of membrane-bound enzymes and loss of compartmentalization that is essential to cell metabolism. Indirect effects of lipid peroxidation are also important. Lipid peroxidation of cell membranes yields a variety of aldehydes, some of them with potent biological activity. Some of them react with thiols, inhibiting a wide range of enzyme processes, including calcium pump activity in the sarcoplasmic reticulum. Of all the effects of lipid peroxidation, the worst effect is the loss of homeostasis due to destruction of membranes and influence on ion pumps. The loss of the ability to pump calcium ions out of the cytosol leads to activation of proteases, phospholipases and endonucleases, causing DNA fragmentation, disruption of cell signaling and distortion of the cytoskeleton (16).

3.2. Protein damage by free radicals. Damage to proteins is different than the damage to lipids. First, there are few different substrates for lipid peroxidation whereas there are many different proteins, and second, oxidation of
proteins is much less likely to lead to chain reactions. In consequence damage to proteins can be specific and it is not possible to generalize about mechanisms. In the case of proteins, the concept of "site-specific" damage is important. If damage occurs at the active site of an enzyme, the effects can be enormous. In contrast, random radical attack at non-specific sites of a mixture of large protein molecules may serve to dilute the impact of the damage (16).

The modification of aminoacid residues includes deamination, carbonyl formation, oxidation of methionine to its S-oxide, oxidation of cysteine to cystine and oxidation of tyrosine. Modification of aminoacid residues affect the general properties of proteins. There are changes in charge, hydrophobicity and conformation. These changes are believed to lead to aggregation, loss of function and increased proteolytic turnover (16).

An interesting example of protein inactivation by free radicals is that of the antioxidant enzymes. Catalase and glutathione peroxidase can be inactivated by $O_2^-$. (29). The enzyme lactate dehydrogenase can also be affected by $O_2^-$. Superoxide anion can also react with the iron of hemoglobin, rendering it unable to carry oxygen (30).

3.3. DNA damage by free radicals. Most of the work on free radicals and DNA damage has been done in the field of radiation. The most important reaction seems to be based on hydrogen abstraction to form carbon-centered radicals, followed by oxygen addition to form peroxyl radicals. In cells exposed to an excessive oxidant stress, DNA damage can be extensive and lead to cell death
in a short period of time. In this case the most likely mechanism may be the generation of strand breaks, with subsequent stimulation of poly(ADP ribose) synthetase, which uses NAD⁺ as a substrate. It is postulated that the cell can then be depleted of NAD(P)H and ATP, with serious consequences for the control of calcium levels and maintenance of glutathione (9,58).

4. Antioxidant defense systems.

Numerous investigations during the last 15 years have tested the hypothesis that oxidant-induced cellular damage underlies the pathogenesis of many disease states. Although these investigations have not proven clearly the hypothesis that oxidants play a role in human disease, they do demonstrate that reactive oxygen metabolites and other forms of free radicals must be removed rapidly from tissue before they cause cellular dysfunction and eventual cell death (37).

The multiplicity of the antioxidant systems available in tissues and their overlapping specific activities suggest that tight control of redox balance is critical for the maintenance of normal cell function. Alterations in the balance between oxidants and antioxidants, either through an increase in oxidant stress or through a compromise in the level of antioxidant resources, can initiate a series of pathophysiologic events that culminate in cellular dysfunction (37).

Because of the diverse sources of free radicals during normal cellular function, their molecular reactivity and the number of cellular functions that can be disrupted by oxidative mechanisms, all metabolically active cells have a
sophisticated and complex network of antioxidant defense mechanisms. Antioxidant mechanisms can be defined as any cell process that:

1. prevents the formation of free radicals,
2. converts oxidants to less toxic species,
3. compartmentalizes reactive species away from vital cellular structures, or
4. repairs molecular injury induced by free radicals.

Individual antioxidant systems may function alone or cooperatively through one or more of the above described defense mechanisms.

*The relevance of the known general cell antioxidant mechanisms to cell function in muscle is not yet understood. A review of the cellular antioxidant mechanisms in general, will allow a better understanding of their possible role in respiratory muscles.

4.1. Prevention and compartmentalization of free radical formation.

The prevention of excess free radical formation is a vital first step for cell survival, since potentially toxic oxygen metabolites are continuously produced through normal cellular respiratory processes. Considerable free radical formation seems to occur during normal cellular oxidative phosphorylation (15). Within the mitochondria, the majority of the oxygen metabolized in the respiratory chain is reduced to water without significant formation of intermediate free radicals. This is achieved by a one-step, 4-electron reduction of oxygen to water by cytochrome oxidase, without the release of intermediate oxygen metabolites. It is estimated that at least two percent of the total mitochondrial oxygen consumption goes to the
formation of superoxide anion from the interaction of various mitochondrial components with molecular oxygen, linking the production of oxygen-centered free radicals to cellular metabolism (15). The use of electron transport inhibitors has allowed the identification of possible sites of oxygen-centered free radical generation in the mitochondria, as flavoproteins, coenzyme Q and cytochrome b (Fig.9). A large body of research has supported the semiquinone form of coenzyme Q as the primary compound responsible for interaction with molecular oxygen, forming superoxide anion during both normal and increased electron transport chain activity (10).

Mitochondrial cytochrome oxidase serves as a sink, utilizing more than 95% of cellular oxygen and handling it in a way that avoids significant free radical formation. Superoxide production depends upon the redox state of the electron carriers. In fact, it is when electron carriers in the respiratory chain are reduced that superoxide production is greatest.

Prevention of OH· formation through limitation of transition metal availability is another important cell antioxidant strategy. Transition metals like iron play a major role in the generation of oxidant-induced tissue injury, through several mechanisms: a) facilitation of lipid peroxidation, b) participation in the Haber-Weiss reaction, that generates OH· from O₂⁻ and H₂O₂ and c) acceleration of the non-enzymatic oxidation of molecules like epinephrine. In healthy organisms, iron is compartmentalized and kept away from the sites of free radical reactions. The availability of free extracellular iron to react with O₂⁻ and H₂O₂ is
avoided through several mechanisms. Hemoglobin, which gives up iron readily, is stored within erythrocytes, that are rich in antioxidant defenses. Free hemoglobin is rapidly bound by haptoglobin and hemopexin (37). The remaining extracellular iron is bound to transferrin and lactoferrin, which are two iron-binding glycoproteins that serve as transport vehicles for iron in circulation. Within the cell, most iron is unavailable for participation in free radical reactions because it is bound to ferritin, phosphate esters, organic acids and membrane lipids.

4.2. Free radical scavengers.

The main antioxidant systems available to cells under oxidant stress are free radical scavengers. These agents are present in varying concentrations in the intracellular and extracellular spaces and function by either eliminating oxidants or preventing their conversion to more toxic species.

4.2.1. Antioxidant enzyme systems.

Catalase, superoxide dismutase (SOD) and the enzymes of the glutathione redox cycle are the primary intracellular antioxidant defense mechanisms used by the cells to cope with oxidant stress. They are able to eliminate $\cdot O_2^-$ and hydroperoxides that can produce cell damage and by decreasing the concentration of free radicals needed to initiate substrate oxidation, they prevent free radical chain reactions. They have specific subcellular locations, varied organ distribution and different requirement for specific metals at their catalytic sites (37).

Catalase. The enzyme is a 240,000D tetrameric hemoprotein that catalyzes the reaction:
The enzyme is made in the cytosol as heme free monomers. Monomers are imported into peroxisomes, where they assemble into tetramers in the presence of heme. Catalase is able to reduce \( H_2O_2 \) and methyl or ethyl hydroperoxides but does not metabolize large molecular peroxides such as the lipid hydroperoxides formed by lipid peroxidation. Catalase is highly compartmentalized; it is primarily located in peroxisomes, which contain many of the enzymes that generate \( H_2O_2 \). (21). Different cell types exhibit different catalase activity, with liver cells and erythrocytes having the highest activity (36).

Fig. 11 illustrates some of the characteristics of the structure of catalase.

Enzymes of the glutathione redox cycle.

Glutathione peroxidase (GPx). Is an 85,000D tetrameric protein containing four atoms of selenium, which are essential for catalytic activity. It complements catalase in the reduction of \( H_2O_2 \), and in addition, it participates in the reaction to eliminate other toxic hydroperoxides (37). GPx has an absolute requirement for glutathione as co-substrate and catalyzes the reaction that forms oxidized glutathione (GSSG).
\[ \text{ROOH} + 2 \text{GSH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \]

(Equation 9)

Glutathione reductase (GR). Oxidized glutathione can be reduced back to GSH in the reaction catalyzed by GR (37). The enzyme is a dimeric protein with molecular weight 105,000 and which also catalyzes the reduction of other low molecular weight disulfides, but not mixed disulfides in the reaction:

\[ \text{RSSR} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{RSH} + \text{NADP}^+ \]

(Equation 10)

The reducing equivalents in the form of NADPH are supplied through the activity of glucose 6-phosphate dehydrogenase (G6PD), in the hexose monophosphate shunt.

Cellular distribution of the two enzymes of the glutathione redox cycle is tightly coupled (37). The two enzymes exist mainly in the cytosol, although the mitochondria also have some glutathione redox activity.

As with catalase, cells with the highest activity of GPx and GR are erythrocytes and liver cells. Intermediate levels of activity are found in lung and the heart (49). Although it is true that different cell lines may have different peroxidase systems, experimental and clinical data suggest that the glutathione redox cycle is the most important antioxidant peroxidase system in mammalian species. Studies of human inborn errors of metabolism have shown that catalase
deficiency is secondary in importance to defects in the glutathione redox cycle in removing \( \text{H}_2\text{O}_2 \) produced in vivo at normal physiologic rates (37). This is supported by the fact that with \( \text{H}_2\text{O}_2 \) as substrate, the \( K_m \) value for GPx is lower than for catalase, suggesting that GPx is the preferential pathway for the degradation of low concentrations of \( \text{H}_2\text{O}_2 \) present in intact cells. Fig. 14 illustrates some characteristics of the structure of Se-dependent GPx.

Superoxide dismutase (SOD). Cellular total SOD activity is represented in the cell by a group of metalloenzymes with various prosthetic groups. These enzymes catalyze the reaction:

\[
2 \, \text{O}_2^- + 2 \, \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\]

(Equation 11)

The prevalent enzyme is cupro-zinc (CuZn) SOD, which is a stable dimeric protein with molecular weight 32,000 D. It has copper at the catalytic site and zinc provides stability to the protein structure. CuZn SOD is found mainly in the cytosol but it may also be found in the nucleus. The two-step reaction catalyzed by CuZn SOD is:

\[
\text{SOD-Cu}^{2+} + \text{O}_2^- \rightarrow \text{SOD-Cu}^{1+} + \text{O}_2
\]

\[
\text{SOD-Cu}^{1+} + \text{O}_2^- + 2 \, \text{H}^+ \rightarrow \text{SOD-Cu}^{2+} + \text{H}_2\text{O}_2
\]

(Equations 12)
Liver, brain and testes have the highest activity of the enzyme, while erythrocytes, lung and pancreas have the lowest activity (36).

Manganesum (Mn) SOD is a tetrameric protein that exists mainly in the mitochondria, where it eliminates $O_2^-$ formed during electron transport. Its aminoacid composition and structure are very different than those of the CuZn SOD, indicating that the two enzymes evolved independently (46).

SOD enzymes are very efficient, with a rate constant of $2 \times 10^9/(M.s)$, that is $10^4$ times faster than the spontaneous rate of superoxide anion dismutation (31).

Figs. 13 and 14 show some characteristics of the structure of both CuZn SOD and Mn SOD
Fig. 11. Active site of one of the monomers of the enzyme catalase
Fig. 12. Active site of bovine glutathione peroxidase, with the aminoacids involved in the mechanism of catalysis. (From: Glutathione Centennial. Molecular Perspectives and Clinical Implications, edited by N. Taniguchi, T. Higashi, Y. Sakamoto, A. Meister. Academic Press Inc. 1989, p 111)
Fig. 13. A and B: Polypeptide backbone structure of the subunit of bovine erythrocyte Cu-Zn SOD.

A. Schematic drawing of the tri-dimensional structure.

B. Two dimensional topological relationship of the 8 strands of beta sheet structure. Circles represent residues which contribute ligands to Zn and Xs represent residues that contribute ligands to copper.

Fig. 14. Tri-dimentional structure of the Mn SOD monomer, and details of the active site.
4.2.2. Non-enzyme antioxidant systems.

The antioxidant enzymes efficiently participate in metabolizing most of the cell-derived $O_2^-$ and $H_2O_2$ in different cell compartments, preventing most of the formation of toxic $OH^-$. There are also non-enzymatic mechanisms to protect against the $OH^-$ that is formed. Once formed, $OH^-$ reacts within 3 to 5 molecular diameters of its site of formation and does not diffuse enough to be metabolized at the active site of an enzyme (37). Most biomolecules react very readily with $OH^-$. Therefore, an efficient $OH^-$ scavenger in vivo must: a) react at almost diffusion-controlled rate with $OH^-$, b) exist at higher concentrations than other molecular targets for $OH^-$, c) be at the site of $OH^-$ generation, and d) generate non-toxic secondary products after reacting with $OH^-$. 

**Vitamin E.** Vitamin E or $\alpha$ tocopherol ($\alpha$-TH) is a lipid soluble antioxidant that represents the principal defense against oxidant-induced membrane injury in humans (37). It converts lipid peroxyl radicals in the reaction:

$$\alpha-TH + RO_2^- \rightarrow ROH + \alpha T^.$$  

(Equation 13)

It can also interact with $O_2^-$. It breaks peroxidation chain reactions, because the vitamine E radical formed in reduction reactions is stable and does not further react with lipid substrates. The vitamine E radical can be reduced back in a reaction involving ascorbate. Despite the fact that vitamin E is the major lipid soluble chain-breaking antioxidant in plasma, it contributes very little to
serum total antioxidant capacity. It is believed that plasma proteins play the most important role in plasma.

Ascorbate. Because of its water solubility it is available both intra and extracellularly. It can function as a scavenger or it may have pro-oxidant effects depending on the availability of iron. A major antioxidant role is as a contributor in the reduction of the oxidized vitamin E, allowing it to function as a chain-breaking antioxidant (37).

Uric acid. At concentrations normally occurring in human plasma, uric acid has powerful antioxidant properties. It scavenges OH·, singlet oxygen and peroxyl radicals from lipid peroxidation, participating as a sacrificial antioxidant (37). Another mechanism for its antioxidant protection is through its binding to transition metals.

Other non-enzyme antioxidants include cysteine, taurine and albumin.

Tables 1 and 2 summarize the general cell antioxidant mechanisms.
<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>Structure</th>
<th>Cell Site</th>
<th>Catalyzed reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>Tetrameric</td>
<td>Peroxisomes</td>
<td>Decomposition of $\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>MW 240,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>Cu-Zn-SOD</td>
<td>Cytosol (nucleus</td>
<td>Dismutation of $\text{O}_2^{-}$ to $\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>MW 32,000</td>
<td>also)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn-SOD</td>
<td>Mitochondria</td>
<td>Dismutation of $\text{O}_2^{-}$ to $\text{H}_2\text{O}_2$</td>
</tr>
<tr>
<td></td>
<td>MW 39,500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione Peroxidase (GPx)</td>
<td>Tetrameric</td>
<td>Primarily cytosol (mitochondria also)</td>
<td>Reduction of $\text{H}_2\text{O}_2$ and lipid peroxides</td>
</tr>
<tr>
<td></td>
<td>Seleno-protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MW 85,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione Reductase (GR)</td>
<td>Dimer</td>
<td>Primarily cytosol (mitochondria also)</td>
<td>Reduction of low molecular weight disulfides</td>
</tr>
<tr>
<td></td>
<td>MW 105,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE: 2 MAJOR CELL OXIDANT SCAVENGERS: NON-ENZYME SYSTEMS

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Cell Site</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipophilic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Fat-soluble</td>
<td>Lipid membranes, extracellular fluids</td>
<td>Converts O$_2^\cdot$ , OH$^-$ and lipid peroxide radicals to less reactive forms. Breaks lipid peroxidation chain reaction</td>
</tr>
<tr>
<td></td>
<td>vitamin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquinol-10</td>
<td>Fat-soluble</td>
<td>Mitochondria</td>
<td>Brakes oxidation chain reaction</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Water-soluble</td>
<td>Intracellular and extracellular</td>
<td>Directly scavenges O$_2^\cdot$ , OH$^-$ . Neutralizes oxidants from stimulated neutrophils. Contributes to regeneration of vitamin E.</td>
</tr>
<tr>
<td></td>
<td>vitamin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uric Acid</td>
<td>Oxidized purine base</td>
<td>Wide distribution</td>
<td>Scavenges OH$^-$, O$_2^\cdot$ , peroxyl radicals. Prevents oxidation of vitamin C. Binds transition metals. Scavenges OH$^-$</td>
</tr>
<tr>
<td>Glucose</td>
<td>Carbohydrate</td>
<td>Wide distribution</td>
<td>Binds transition metals.</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Aminoacid</td>
<td>Wide distribution</td>
<td>Reduces compounds by donating electrons from SH groups</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Tripeptide</td>
<td>Largely intracellular</td>
<td>Substrate for GPx in GSH redox cycle. Directly reacts with O$_2^\cdot$ , OH$^-$ and organic free radicals</td>
</tr>
<tr>
<td></td>
<td>(γ-Glutamyl-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cysteinyllglycine)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>β Aminoacid</td>
<td>Cells with high rate of oxygen radical generation</td>
<td>Conjugation of xenobiotics, reaction with HOCl</td>
</tr>
<tr>
<td>Plasma Proteins</td>
<td>Albumin</td>
<td>Plasma</td>
<td>Binds transition metals, &quot;Sacrificial Antioxidant&quot;</td>
</tr>
</tbody>
</table>
5. **List of references.**


CHAPTER III

INSPIRATORY RESISTIVE LOADING

1. INTRODUCTION.

Several models of inspiratory resistive loading have been used to study the relative contribution of diverse mechanisms that could take part in the development of respiratory failure under excessive mechanical loads. Evidence supporting both central mechanisms of fatigue and contractile failure have been obtained with severe loads, both in vivo and in vitro (1,3,8-10,12,15). Most of the studies, however, have characterized the response of the respiratory pump to loading in terms of changes in respiratory timing and in peak pressures under fixed resistances, from the time of load imposition until respiratory failure develops.

In this study a model of incremental inspiratory resistive loading is described in the adult rat, which obtains high levels of muscle contractile activity for a prolonged period of time. The model has been used to study biochemical changes in the respiratory muscles associated with increased respiratory muscle activity to the point of pump failure (5). This chapter focuses on the time course of the changes in ventilation, pressure development, and various other physiological measurements during the process of failure. Particular attention is paid to the relationship of these measurements to the external work performed by
the respiratory pump. This information was used to evaluate early indicators of impending respiratory failure under incremental inspiratory resistive loading.

The earliest sign of impending respiratory failure was found to be a rapid decrease in power output which occurred at a time of increasing peak inspiratory pressure development, with no significant changes in respiratory timing. Later changes prior to apnea, included a decrease in peak pressure development, a decrease in respiratory rate and a deterioration in arterial blood pressure.

2. MATERIAL AND METHODS.

Animals and care: Experiments were done in accordance with guidelines established by the Institutional Animal Care and Use Committee at The Ohio State University. The study was carried out in male Sprague-Dawley rats, fed standard stock chow diets, given water ad libitum and weighing 300-450 g. All animals were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg initially plus 2 small supplements of 10 mg/kg). A 2.2 mm internal diameter tracheal cannula was placed through a tracheostomy and connected to a two-way non-rebreathing valve (model 2300, Hans Rudolph, Kansas City, MO). The inspiratory side of the two-way valve was then attached to a variable inspiratory resistance consisting of a fine needle valve. Tracheal pressure (P₁) was continuously monitored by way of a side port in the tracheal cannula, which was connected to one side of a differential pressure transducer (Validyne, Northridge,
CA). Inspiratory flow was measured with an in-line pneumotachograph (model 0648, A. Fleisch), and inspiratory volume was obtained by integration of the flow signal.

A water-filled catheter, which was connected to a pressure transducer (Validyne, Northridge, CA) was inserted through the mouth and placed in the middle third of the esophagus in order to monitor esophageal pressure ($P_e$). An air-filled balloon connected to another pressure transducer was placed in the abdominal cavity, under the left hemidiaphragm, through a small medial incision in the abdominal wall, to measure changes in abdominal pressure ($P_a$).

The right carotid artery was cannulated for continuous blood pressure measurement and periodic blood sample collection for pH and blood gas analysis (model BMS 3, Radiometer, Copenhagen). Arterial blood pressure, along with $P_e$, $P_i$, $P_a$, and inspiratory volume, were recorded on a multi-channel recorder. Rectal temperature was continually monitored with a thermistor and maintained at approximately 38.5°C, using heating pads.

Seventy percent oxygen in the inspired air was provided for the entire period on the load. In our first attempts with the resistive breathing protocol 4 animals breathing air were studied. These animals developed a rapid and severe hypoxemia with resultant hypotension, at about 10 min after loading had started. They remained severely hypotensive, developing bradypnea and a progressive decrease in tracheal pressure for another 5 to 8 min, until apnea. Therefore, seventy percent inspired oxygen was chosen for the experimental group in order
to prevent the extreme hypoxemia and resultant systemic hypotension generally associated with high inspiratory loads (4). This enabled the experimental animals to undergo the resistive breathing protocol for longer than 2.5 hours with higher than normal PaO₂.

**Incremental inspiratory resistive loading:** An estimation of the maximal inspiratory pressure (PImax), generated against an occluded airway, was obtained by occluding the inspiratory side of the two-way valve for 30 sec. or until blood pressure fell to 80 mm Hg. The initial resistance of the respiratory valve was then set to result in a generated peak P₁ of 30% of PImax. After 10 minutes, the resistance was increased by an additional 10% of PImax. This procedure was repeated at 10 min. intervals until either tidal volume fell to 15-30% of its baseline value or there was evidence of circulatory collapse. This yielded an average increase in P₁ of approximately 3.5 cm H₂O every 10 minutes. In this way, animals reached the highest tolerated resistance at about one hour after starting on the load, and remained at that level for the rest of the time, until they developed evidence of pump failure. Pump failure was defined as marked bradypnea associated with a sudden decrease in peak P₁ without any change in inspiratory resistance. This was often related to a rapid decrease in arterial blood pressure.
Measurements. Inspiratory time (Tᵢ), total respiratory cycle time (Tₜₙ) and \( Tᵢ/Tₜₙ \) were obtained from the pressure-time trace. Pressure-volume (P-V) work per breath was calculated by integration of the area under the P-V curves constructed for representative breaths at each level of resistance and expressed as P-V work per minute (Power output). Pressure-time product was calculated for each level of resistance by integration of the area under the pressure-time curve, over \( Tₜₙ \).
Fig. 15: The inspiratory device. System used to increase the resistance to inspiratory flow. Expiration was unobstructed. (Pa = arterial blood pressure, Pt = tracheal pressure, and Pes = esophageal pressure).
Fig. 16: The experimental preparation

The changes in tracheal \( (P_t) \), esophageal \( (P_e) \), abdominal \( (P_a) \) and arterial blood pressures \( (P_a) \) are monitored. They are shown at baseline (pre-load) and at the level of resistance that induces pump failure. Tidal volume \( (V_t) \) and arterial blood gases are also shown in the same way as pressures.
3. RESULTS

3.1. Physiologic changes from initiation of loading to just prior to pump failure.

Figure 15 illustrates the system used to increase the resistance to inspiratory flow. Figure 16 shows the experimental preparation and very schematically, the time course of some of the physiologic parameters that will be discussed.

3.1.1. Changes in pressure development. Peak $P_t$ increased progressively, reaching to a maximum of $52.4 \pm 12.9$ cm H$_2$O. Most animals showed evidence of pump failure at approximately the same level of peak $P_t$. Fig. 17 shows in a reduced scale, a typical record of the changes in $P_t$ over time, in one animal. The group data is shown in Fig. 18.
Fig. 17: A slow, strip chart trace showing a typical example of the progressive increase in peak $P_i$ at the bottom, with the changes in arterial blood pressure at the top.
Fig. 18: Time course of the changes on peak $P_t$ with increasing inspiratory resistance.
$P_a$ followed the same pattern as $P_u$, being approximately 7% higher than $P_t$, due to elastic recoil of the lung and airway resistance. Very small changes in abdominal pressure were observed with increasing inspiratory resistance. $P_{ab}$ went from near 0 cm H$_2$O at end expiration, to a range of 2-10 cm H$_2$O during inspiration.

3.1.2. Changes in breathing frequency, tidal volume ($V_t$) and minute ventilation:

$V_t$ decreased progressively from a pre-load value of 2.34 ± 0.53 ml to 0.62 ± 0.29 ml at the end of the increasing loading protocol (25% of baseline $V_t$, Fig. 19A). The changes in $V_t$ in relationship to the changes in peak $P_t$ are illustrated in Fig. 21. This shows an almost linear decrease in $V_t$ with the increase in $P_t$, up to a point in which $V_t$ starts decreasing at a lower rate with increasing peak $P_t$, suggesting an hyperbolic relationship. Respiratory rate remained constant during the entire resistive loading time (fig 19B). Minute ventilation decreased from 200 ± 73 ml/min to 48 ± 33 ml/min (Fig. 19C). Inspiratory time did not change significantly with increasing loads nor did the $T_i/T_{tot}$ ratio, which remained approximately 0.26.
Fig. 19. The effect of the resistive loading protocol on tidal volume, respiratory rate and minute ventilation.
Fig. 20: Time course of the changes in tidal volume with increasing resistance
3.1.3. Changes in Pressure-time curve: With increasing inspiratory resistance we observed a change in the shape of the pressure-time curve. Time to peak pressure decreased progressively while time at the peak did not change appreciably, translating into an increase in integrated pressure over T₁. At very high levels of resistance, time at the peak decreased markedly, with the result of a non proportional increase in $\bar{P}_{\text{t}}$, and in many cases, an actual decrease in $\bar{P}_{\text{t}}$ with the increase in peak $P_t$.

3.1.4. Changes in Pressure-time product. Changes in integrated tracheal pressure over the total respiratory cycle time ($\bar{P}$) increased linearly with the increase in peak $P_t$, up to a point at which it started to plateau, with the increase in $P_t$ (Fig. 22).

The same relationship was found among peak $P_t$ and $P_{\text{t}}$.

3.1.5. Changes in P-V work of breathing. With increasing resistance, the progressive decrease in $V_t$ associated with the increase in peak $P_t$, translated into an increased P-V work/breath until a point was reached at which peak $P_t$ still continued to increase in response to increasing resistances, while P-V work/breath decreased (Fig. 23).
Fig. 21  Relationship between changes in tidal volume and changes in peak $P_t$, with increasing resistance.
Fig. 22. Changes in integrated inspiratory pressure in relation to changes in peak $P_t$ with increased inspiratory resistance.
Fig. 23 Changes in pressure-volume work/breath with increasing resistances (a to f). Area increases for curves a to e. Area of curve f, however is smaller than that of curve e.
3.1.6. Changes in arterial blood pH and gases.

Pre-load arterial blood gases and pH while animals were anesthetized and breathing air, are shown in table 3. Arterial blood gases and pH at the resistance able of inducing respiratory failure are also shown in table 3. \( \text{PaO}_2 \), \( \text{PaCO}_2 \) and pH were determined at several times while animals were on the load and are shown in figs. 24-26, in relation to the level of \( P_t \) reached with increasing resistance. \( \text{PaO}_2 \) decreased progressively with increasing resistance, but was never below 100 mmHg at the beginning of failure. \( P_t\text{CO}_2 \) increased linearly whereas pH decreased also linearly with the increase in \( P_t \). Although most of the acidosis was due to \( \text{CO}_2 \) retention, there was also a metabolic component, since BE became more negative with loading (from -3 pre-load to -9 at the beginning of failure).
Fig. 24. Arterial pressure of oxygen in relation to the tracheal pressure generated
Fig. 25. Arterial pressure of CO₂ in relation to the generated tracheal pressure.
Fig. 26. Changes in arterial blood pH with increases in tracheal pressure.
<table>
<thead>
<tr>
<th></th>
<th>Baseline (21% O₂)</th>
<th>At Failure (70% O₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PaO₂ (torr)</strong></td>
<td>67 ± 10</td>
<td>183 ± 23</td>
</tr>
<tr>
<td><strong>PaCO₂ (torr)</strong></td>
<td>40 ± 11</td>
<td>107 ± 36</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>7.36 ± 0.06</td>
<td>6.98 ± 0.12</td>
</tr>
</tbody>
</table>
3.2. Physiologic changes upon development of pump failure.

Pump failure, as we defined it, was characterized by a decrease in peak $P_t$, associated with a later decrease in respiratory rate until apnea occurred. Large differences in the rate of decrease in $P_t$ were observed in the animals studied, and it was possible to identify two different groups:

a) one group in which the initial event was a decrease in arterial blood pressure. In this group, the rate of decrease in peak $P_i$ paralleled the rate of decrease in arterial blood pressure (Fig. 27A).

b) a second group, in which arterial blood pressure did not decrease while peak $P_i$ decreased slowly and progressively at the same level of resistance. The rate of decrease in peak $P_i$ in this group was much slower than in the previous group (Fig. 27B). Changes in arterial blood pressure in this group, developed at a later time and were probably secondary to the decrease in peak $P_t$, $V_t$ and minute ventilation.
Fig. 27: The two different patterns in which pump failure developed:

A: The changes in tracheal pressure are preceded by a decline in arterial blood pressure.

B. Tracheal pressure declines slowly and progressively, without an initial change in arterial blood pressure.
4. DISCUSSION.

A significant decrease in the work per breath performed by the respiratory pump despite an increase in peak pressure development with increasing inspiratory resistance, was found to be a good indicator of impending respiratory failure induced by incremental resistive loading in the anesthetized adult rat.

Impairment of respiratory muscle function has been recognized as a possible mechanism contributing to respiratory failure (11). Animal models, both awake and anesthetized have been used to study the underlying mechanisms that lead to failure to maintain a certain pre-determined pressure, even under conditions known to stimulate respiratory drive (1-3,8-10,12,15). Whether or not anesthesia has been used, evidence for both, peripheral and central mechanisms of fatigue have been documented and it has not been possible to separate the relative contribution of each type of mechanism. In addition, several studies have attempted to identify early changes that could predict the development of respiratory failure under constant loads. Changes in diaphragm EMG, as well as increases in Tn have been reported to correspond to early indicators of impending respiratory failure (3,12,14).

The model described in this study differs from most of the previous models in the fact that progressive increases in the level of resistance over time, associated with the use of supplemental oxygen (70%) have allowed the animals to remain at high levels of resistance for prolonged periods of time (2.5 - 3.5 hours). The
need for supplemental oxygen had been previously described by Bazzy et al. (4), for the awake sheep model of inspiratory resistive loading, to prevent the hypoxemia and resultant hypotension that develop with loading.

In a pilot study in our laboratory, animals breathing air were only able to remain on the resistance for 12 to 15 minutes. Under those conditions, animals developed severe hypoxemia with resultant hypotension at about 10 min. after the beginning of loading. They remained severely hypotensive, developing bradypnea and a progressive decrease in tracheal pressure for another 5 to 8 min. until complete apnea occurred. Thus the model breathing air is limited by the hemodynamic changes, presumably associated with the rapid onset of hypoxemia. For example, the animals on air only generated a peak tracheal pressure of 42 ± 9 cm H₂O prior to failure, whereas the animals on oxygen reached 52.7 ± 8.5 cm H₂O and essentially sustained this for prolonged periods.

Hypercapnic acidosis associated to a small degree of metabolic acidosis was observed even with low levels of resistance. PaCO₂ increased and pH decreased in a linear fashion with loading, over a wide range of resistances and up to the point at which failure, as we defined it, started. Although we did not measure PaCO₂ and pH at the very end of the experiment, when respiratory failure was completely established, we did not observe an exponential increase in PaCO₂ as that described with other models. This may have been due to the changes in the relationship between peak Pₜ and Vᵢ, seen at high levels of resistance. Vᵢ decreased almost linearly with the increase in resistance, for a wide range of
resistances, but at high levels of resistance $V_t$ remained relatively constant with large changes in $P_t$.

Increases in $T_i$ in response to loading have been reported in both awake and anesthetized animals. This increase is related to both the severity of the load and the magnitude of the reduction in $V_t$ induced by the load. Interestingly, animals in this study did not show an increase in $T_i$, which remained at approximately 26% of $T_{tot}$ for the entire range of resistances studied. It has been shown in rabbits, that 100% inspired oxygen during loading reduces the duration of inspiration (6). It is possible that 70% oxygen in this study may have had a role preventing the increase in $T_i$.

With increase in resistance, there was a progressive decrease in $V_t$ and a progressive increase in peak $P_t$, which translated in an increase in $P$ and in $P$-$V$ work/breath, indicating that over a wide range of resistances the relationship between power output of the respiratory pump and load is constant. At high levels of resistance, however, the increase in $P_t$ and the decrease in $V_t$ did not translate into an increase in $P$ or in $P$-$V$ work, and in most cases $P$-$V$ work decreased while peak $P_t$ continued to increase with the increase in load. In attempting to establish the relative contribution of changes in volume and pressure to the decrease in $P$-$V$ work, we found that both may contribute, since the changes in shape of the pressure-time curve, although substantial, do not explain all of the effect.

Changes in shape of the pressure-time curve may represent mechanisms related with differential recruitment of different motor units in the same type of
muscle, or changes in pattern of muscle recruitment (diaphragm vs. intercostal).

Interestingly, the changes observed in P and in P-V work occur prior to the changes we have used to define pump failure, that is a decrease in pressure generation followed by decrease in respiratory rate, until apnea. That the decrease in P-V work/breath occurs prior to changes in rate is best illustrated by the fact that power output (work/min) decreased after P-V work per breath decreased.

In summary, in this anesthetized rat model of incremental inspiratory resistive loading, the chain of events that precedes overt respiratory pump failure, can be summarized as follows: At high levels of resistance (enough to generate at least 30-35 cm H₂O of peak P_{i}), there is a change in the shape of the pressure-time curve, that translates into a reduction in P_{exp}. This is able to reduce P-V work per breath, even when peak P_{i} is still able to increase in response to loads.

In conclusion; the analysis of the work rate of the respiratory pump provides a new way of evaluating the response of the respiratory pump to incremental inspiratory resistive loading.
5. LIST OF REFERENCES


CHAPTER IV

RESPIRATORY MUSCLE HIGH ENERGY PHOSPHATES

1. INTRODUCTION.

Respiratory muscle fatigue due to an imbalance between energy substrate supply and energy demands in respiratory muscles has been suggested as one of the mechanisms leading to ventilatory failure in patients with cardiopulmonary diseases (26). Studies of diaphragm circulation show that the diaphragm receives its arterial supply from several sources and has a rich anastomotic system, which is unique among skeletal muscles (11). This would suggest that blood flow and the availability of substrates for energy supply to the diaphragm would be protected at high mechanical loads. However, alterations in diaphragm glycogen and lactate, along with changes in diaphragm function, have been shown under a variety of conditions (2-4, 14, 17), suggesting that biochemical alterations in the respiratory muscles may lead to a reduction in contractile force and ventilatory failure.

Unlike non-respiratory skeletal muscles, for which there is good evidence that increased activity is associated with biochemical alterations leading to contractile fatigue (18, 28), very little work has been done to determine the relationship between increased work of breathing and biochemical alterations of
the respiratory muscles, such as changes in high energy phosphate compounds (2-3).

The working hypothesis is that some of the biochemical changes found in the diaphragm under loading conditions with different models may have been exaggerated by an alteration in the delicate balance between demands for and adequate supply of blood flow at high levels of contractile activity. The purpose of this study is to determine, in a model of severe and prolonged incremental inspiratory resistive loading if there are alterations in the levels of high energy phosphates in the respiratory muscles under conditions of severe load with maintenance of arterial blood pressure and blood oxygenation, at the point of impending pump failure in spontaneously breathing rats.

2. MATERIALS AND METHODS.

Animals and care: Experiments were done in accordance with guidelines established by the Institutional Animal Care and Use Committee at The Ohio State University. The study was carried out in male Sprague-Dawley rats, fed standard stock chow diets, given water ad libitum and weighing 300-400 g. All animals were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg initially plus 2 small supplements of 10 mg/kg). A 2.2 mm internal diameter tracheal cannula was placed through a tracheostomy and connected to a two-way non-rebreathing valve (model 2300, Hans Rudolph, Kansas City, MO). The
animals were randomly assigned to one of the following two groups:

1) the experimental group (n=5) which underwent incremental inspiratory resistive loading while breathing 70% oxygen for 2.5 - 3 hours;
2) the control group (n=5) which was anesthetized while unloaded and breathing room air.

Seventy percent inspired oxygen was chosen for the experimental group in order to prevent the extreme hypoxemia and resultant systemic hypotension, generally associated with high inspiratory loads (7). This enabled the experimental animals to undergo the resistive breathing protocol for longer than 3 hours, with higher than normal PaO₂.

Measurements: Tracheal pressure (Pₜ) was continuously monitored by way of a side port in the tracheal cannula, which was connected to one side of a differential pressure transducer (Validyne, Northridge, CA). Inspiratory flow was measured with an in-line pneumotachograph (model 0648, A. Fleisch), and inspiratory volume was obtained by integration of the flow signal.

A water-filled catheter, which was connected to a pressure transducer (Validyne, Northridge, CA) was inserted through the mouth and placed in the middle third of the esophagus in order to monitor esophageal pressure (Pₑ).

The right carotid artery was cannulated for continuous blood pressure measurement. Arterial blood pressure, along with Pₑ, Pₜ and inspiratory volume, were recorded on a four channel recorder. Rectal temperature was continually monitored with a thermistor and maintained at approximately 38.5°C.
C, using heating pads.

Pressure-volume (P-V) work of breathing was later calculated from the area under the pressure-volume curves constructed using representative breaths at each level of resistance, to establish if a relationship exists between the level of respiratory muscle contractile activity and the concentration of high energy phosphates.

**Incremental inspiratory resistive loading:** An estimation of the maximal inspiratory pressure (PImax), generated against an occluded airway, was obtained by occluding the inspiratory side of the two-way valve for 30 sec. or until blood pressure fell to 80 mm Hg. The inspiratory side of the two-way valve was then attached to a variable inspiratory resistance consisting of a fine needle valve. The resistance was set initially to result in a generated P, of 30% of PImax. After 10 minutes, the resistance was increased by an additional 10% of PImax. This procedure was repeated at 10 min. intervals until either tidal volume fell to approximately 25% of its baseline value or there was evidence of circulatory collapse. This yielded an average increase in P, of approximately 3.5 cm H2O every 10 minutes. They reached the highest tolerated resistance in about one hour and remained at that level, for other 2 - 2.5 hours until either blood pressure decreased below 100 mm Hg or there was a decrease in respiratory rate associated to a decrease in peak Pt, without an increase in resistance. Both of these are in our experience, the initial manifestations of pump failure in this model.

**Sample collection:** Costal diaphragm and parasternal intercostal samples were quickly obtained by freeze-clamping with liquid nitrogen, using metallic
clamps. The samples were kept under liquid nitrogen until they were ready to be assayed.

**Sample preparation:** Perchloric acid extracts were prepared from samples kept at liquid nitrogen temperature, using a modification of the procedures used for *in vitro* $^{31}$P NMR (6). Samples were ground to a fine powder using a porcelain mortar and pestle chilled with liquid nitrogen. Three ml 0.6 N perchloric acid were added to the mortar containing liquid nitrogen and approximately 0.2 - 0.4 g of muscle sample. Tissue powder was then removed from the mortar and added to a polyalomer centrifuge tube containing 2 ml of 0.6 N perchloric acid at room temperature. The mixture was allowed to warm by constant shaking to allow coating of tissue particles with perchloric acid, until a paste consistency was obtained (approximately -14°C). Samples were then centrifuged at 500 x g and the aqueous acid soluble cellular components separated. The pellet was used for protein determination, by the method of Lowry (21). Neutralization of the acid extracts was quickly done using Freon-trioctylamine (19): 0.6 ml of acid extract was mixed with 1 ml Freon-trioctylamine (6.5 ml trioctylamine diluted to 25 ml with freon), centrifuged at 500 x g and the aqueous layer placed in glass tubes to be kept at -70 °C until HPLC analysis.

**Analysis of purine and pyridine nucleotides:** The neutralized muscle extracts were analyzed by anion exchange HPLC, based on the method of Harmsen et al. (15). Aliquots of 250 µl of neutralized extract were placed on a Whatman Partisil 10-SAX column and eluted at 2 ml/min. Elution
began with 100% buffer A (0.01 M H₃PO₄, pH 2.65) for 3 min. It was followed by 30 min with 66% buffer B (0.75 M K₂PO₄, pH 4.5) and ended after 14 min of 100% buffer A. Peaks were identified by comparison of their retention times with those of authentic standards and quantitation was based on peak area using the same standards. All samples were run in duplicate.

**Total creatine, free creatine and phosphocreatine determination:** Creatine concentration was determined in the neutralized extracts, before and after acid hydrolysis for 9 min. at 65°C, by the method of Eggleton (13). The phosphocreatine (PCr) concentration was then determined by subtracting the concentration of creatine found prior to the hydrolysis (free creatine) from that found after hydrolysis (total creatine).

PCr was also determined by HPLC at 220 nm (1).

The concentration of PCr and of purine and pyrimidine nucleotides was expressed in two different ways: a) per mg protein and b) per total creatine. Because the determinations of protein were carried out in the pellet obtained after the neutralization step, there is the possibility for losses of protein that could account for the slightly larger variability of data when expressed per mg protein. The determination of creatine is done in the same neutralized acid extract that is used for the determination of nucleotides and PCr, and the expression of the data per total creatine gives a much smaller scatter.

**Statistical analysis:** Results are expressed as means ± 1 SD. The Mann Whitney U test was used to compare results among control and experimental
groups. Linear regression analysis was used to express differences between the two methods used for the determination of phosphocreatine (27). A p value $\leq 0.05$ was considered statistically significant.

3. RESULTS.

3.1. Diaphragm.

Table 4 shows the concentration of PCr in control and experimental diaphragms, by the two methods of analysis, expressed per mg protein as well as per total creatine. The HPLC method gave approximately 25% higher values than the spectrophotometric method (Fig. 28). This may be due in part to the presence in the extract, of other compounds that absorb at 220 nm.

Control rat diaphragm quickly freeze-clamped with the animal on a mechanical ventilator and with maintenance of arterial blood pressure contains $131 \pm 33$ nmol PCr/mg protein or $0.64 \pm 0.09$ nmol/nmol total creatine, when determined by HPLC and $91 \pm 23$ nmol PCr/mg protein or $0.44 \pm 0.067$ nmol/nmol total creatine, when determined spectrophotometrically.

Large variability in the diaphragm PCr concentration was found with the resistive loading protocol (Table 5). We were not, however, able to show a relationship between physiologic parameters indicating intensity of respiratory muscle contractile activity and the PCr concentration, perhaps due to small sample size. Differences in PCr concentration between control and experimental diaphragms that were statistically significant, are shown in table 4.
The anion exchange HPLC used in this study also allowed a clear separation of the major nucleotides found in rat respiratory muscles. The energy charge was 0.92 ± 0.008 for the control diaphragm and 0.91 ± 0.012 for the experimental diaphragm. Table 6 shows the concentration of the adenine nucleotides and IMP, expressed per mg protein, as well as the energy charge and the ATP to ADP ratio in control and experimental diaphragms. No significant differences were found in any of these parameters, when data was expressed per mg protein. Table 7 shows the concentration of adenine nucleotides and IMP, expressed per total creatine, in control and experimental diaphragms. A significant decrease in total adenine nucleotides (AN) (p < 0.01) associated with a decrease in ATP (p < 0.01) was found when data was expressed per total creatine. Differences in the ATP ADP ratio however, did not reach statistical significance. The concentration of IMP, a product of the deamination of AMP, was found to be higher in the experimental diaphragm.

Table 5 summarizes the results for the five individual experimental diaphragms, together with the mean for the control group. Linear regression analysis was used to correlate the level of ATP, total AN and IMP (not shown) with the largest change in tracheal pressure generated during loading, the highest power output and the total amount of P-V work. No significant correlations were found.

The NADH/NAD ratio was not affected by the resistive loading protocol. Table 8 summarizes the results on pyridine nucleotides and table 9 shows the data
for individual experiments.

Fig. 29 shows a typical chromatograph of the diaphragm sample at 255 nm. Fig. 30 shows the chromatograph of a solution containing 0.9 mM PCr standard, and fig. 30 shows a typical chromatograph of a diaphragm sample, at 220 nm.

3.2. Parasternal intercostal muscles.

Table 10 shows the concentration of PCr in control and experimental parasternal intercostal muscles by the two methods of analysis, and expressed by mg protein as well as per total creatine. A significant decrease in PCr concentration was observed with the experimental protocol (p < 0.05).

As in the case of the diaphragm, the energy charge was not affected by the resistive breathing protocol (0.92 in both control and experimental groups). The concentrations of adenine nucleotides and IMP, expressed per mg protein are shown in table 11. The same data expressed per mg protein is shown in table 12. Unlike the diaphragm, total AN concentration was not decreased in these muscles. A significant increase in ADP concentration, which translated into a reduction in the ATP to ADP ratio was the only finding. The increase in IMP was smaller than in the diaphragm and not statistically significant. Unlike the diaphragm, the intercostal muscles showed a significant increase in NADH with the resistive loading protocol (Table 13).

Interestingly, the concentration of both NAD and NADH in control intercostals, was found to be significantly lower than in control diaphragm. This
is probably related to the differences in mitochondrial density and oxidative capacity between the two types of muscle (12,16).
Fig. 28: Phosphocreatine determination by HPLC and using a spectrophotometric method. Continuous line represents the identity line, and dotted line corresponds to the regression line for the data points.
Fig. 29: A typical chromatogram of a diaphragm sample, at 255 nm.
Fig. 30: The upper trace corresponds to the chromatogram of a solution containing 0.9 mM PCr, at 220 nm, the lower figure corresponds a typical chromatogram of a diaphragm sample, at 220 nm.
TABLE 4: PHOSPHOCREATINE LEVEL IN DIAPHRAGM OF CONTROL AND EXPERIMENTAL ANIMALS DETERMINED BY TWO DIFFERENT METHODS

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>Spectrophotometric</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCr</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. mg prot⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 5)</td>
<td>131.00 ± 33.00</td>
<td>91.00 ± 23.00</td>
</tr>
<tr>
<td>Experimentals (n = 5)</td>
<td>96.00 ± 37.00</td>
<td>73.00 ± 28.00</td>
</tr>
<tr>
<td><strong>PCr</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. nmol total creatine⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 5)</td>
<td>0.64 ± 0.09</td>
<td>0.443 ± 0.067</td>
</tr>
<tr>
<td>Experimentals (n = 5)</td>
<td>0.39 ± 0.18</td>
<td>0.312 ± 0.110</td>
</tr>
<tr>
<td><strong>PCr / Cr_free</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n= 5)</td>
<td>0.82 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Experimentals (n= 5)</td>
<td>0.48 ± 0.24</td>
<td></td>
</tr>
</tbody>
</table>

( * ) p = 0.05
TABLE 5: PHOSPHOCREATINE, ADENINE NUCLEOTIDES, AND IMP IN DIAPHRAGM OF INDIVIDUAL EXPERIMENTAL ANIMALS COMPARED TO THE AVERAGE OF FIVE CONTROL ANIMALS

<table>
<thead>
<tr>
<th></th>
<th>PCr</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>Σ AN</th>
<th>IMP</th>
<th>ATP/ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average of Controls (n = 5)</td>
<td>0.64 ± 0.09</td>
<td>0.236 ± 0.014</td>
<td>0.042 ± 0.014</td>
<td>0.0017 ± 0.0006</td>
<td>0.281 ± 0.018</td>
<td>0.0004 ± 0.0005</td>
<td>5.83 ± 0.52</td>
</tr>
<tr>
<td>Experimental 1</td>
<td>0.20</td>
<td>0.190</td>
<td>0.044</td>
<td>0.00084</td>
<td>0.236</td>
<td>0.0081</td>
<td>4.34</td>
</tr>
<tr>
<td>Experimental 2</td>
<td>0.55</td>
<td>0.220</td>
<td>0.033</td>
<td>0.00092</td>
<td>0.254</td>
<td>0.0020</td>
<td>6.57</td>
</tr>
<tr>
<td>Experimental 3</td>
<td>0.37</td>
<td>0.160</td>
<td>0.036</td>
<td>0.00140</td>
<td>0.196</td>
<td>0.0170</td>
<td>4.50</td>
</tr>
<tr>
<td>Experimental 4</td>
<td>0.28</td>
<td>0.175</td>
<td>0.037</td>
<td>0.00430</td>
<td>0.213</td>
<td>0.0019</td>
<td>4.96</td>
</tr>
<tr>
<td>Experimental 5</td>
<td>0.62</td>
<td>0.200</td>
<td>0.035</td>
<td>0.00280</td>
<td>0.234</td>
<td>0.0006</td>
<td>5.83</td>
</tr>
</tbody>
</table>

*Results are all in nmol per nmol of total creatine*
<table>
<thead>
<tr>
<th></th>
<th>Controls (n=5)</th>
<th>Experimentals (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATP</strong> (nmol·mg prot⁻¹)</td>
<td>48.8 ± 9.0</td>
<td>43.9 ± 7.1</td>
</tr>
<tr>
<td><strong>ADP</strong> (nmol·mg prot⁻¹)</td>
<td>8.43 ± 1.47</td>
<td>8.54 ± 1.36</td>
</tr>
<tr>
<td><strong>AMP</strong> (nmol·mg prot⁻¹)</td>
<td>0.304 ± 0.09</td>
<td>0.332 ± 0.18</td>
</tr>
<tr>
<td><strong>Total AN</strong> (nmol·mg prot⁻¹)</td>
<td>57.6 ± 9.8</td>
<td>52.8 ± 8.1</td>
</tr>
<tr>
<td><strong>IMP</strong> (nmol·mg prot⁻¹)</td>
<td>0.086 ± 0.1</td>
<td>1.49 ± 1.2</td>
</tr>
<tr>
<td><strong>Total AN + IMP</strong> (nmol·mg prot⁻¹)</td>
<td>57.6 ± 9.9</td>
<td>53.9 ± 7.2</td>
</tr>
</tbody>
</table>

**ATP** = adenosine 5'-triphosphate  
**ADP** = adenosine 5'-diphosphate  
**AMP** = adenosine 5'-monophosphate  
**AN** = adenine nucleotides  
**IMP** = inosine 5'-monophosphate
TABLE 7: ADENINE NUCLEOTIDES AND IMP IN THE DIAPHRAGM OF CONTROL AND INSPIRATORY RESISTIVE LOADED ANIMALS: EXPRESSED PER TOTAL CREATINE

<table>
<thead>
<tr>
<th></th>
<th>Controls (n= 5)</th>
<th>Experimental (n= 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP / Total Creatine</td>
<td>** 0.23600 ± 0.014</td>
<td>0.18900 ± 0.02</td>
</tr>
<tr>
<td>ADP / Total Creatine</td>
<td>0.04160 ± 0.00512</td>
<td>0.03700 ± 0.0042</td>
</tr>
<tr>
<td>AMP / Total Creatine</td>
<td>0.00167 ± 0.00062</td>
<td>0.00146 ± 0.00066</td>
</tr>
<tr>
<td>Total AN / Total Creatine</td>
<td>** 0.28100 ± 0.018</td>
<td>0.22700 ± 0.022</td>
</tr>
<tr>
<td>IMP / Total Creatine</td>
<td>** 0.00040 ± 0.0005</td>
<td>0.00680 ± 0.006</td>
</tr>
<tr>
<td>(Total AN + IMP) / Total Creatine</td>
<td>** 0.28200 ± 0.017</td>
<td>0.23120 ± 0.024</td>
</tr>
<tr>
<td>ATP / ADP</td>
<td>5.83000 ± 0.52</td>
<td>5.24000 ± 0.94</td>
</tr>
<tr>
<td>Energy Charge</td>
<td>0.92000 ± 0.008</td>
<td>0.91000 ± 0.012</td>
</tr>
</tbody>
</table>

All results are in nmol

ATP= adenosine 5'-triphosphate
ADP= adenosine 5'-diphosphate
AMP= adenosine 5'-monophosphate
AN= adenine nucleotides
IMP= inosine 5'-monophosphate

( ** ) p < 0.01
<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 5)</th>
<th>Experimentals (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. mg prot⁻¹)</td>
<td>6.1700 ± 0.94</td>
<td>5.7400 ± 0.89</td>
</tr>
<tr>
<td><strong>NADH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. mg prot⁻¹)</td>
<td>1.2100 ± 0.20</td>
<td>1.1000 ± 0.39</td>
</tr>
<tr>
<td><strong>NAD / Total Creatine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. nmol⁻¹)</td>
<td>0.0300 ± 0.0050</td>
<td>0.0240 ± 0.0020</td>
</tr>
<tr>
<td><strong>NADH / Total Creatinine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. nmol⁻¹)</td>
<td>0.0064 ± 0.0018</td>
<td>0.0048 ± 0.0018</td>
</tr>
<tr>
<td><strong>NADH / NAD</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol. nmol⁻¹)</td>
<td>0.2050 ± 0.0600</td>
<td>0.1980 ± 0.0860</td>
</tr>
</tbody>
</table>
TABLE 9: NAD AND NADH IN THE DIAPHRAGM OF EXPERIMENTAL ANIMALS COMPARED TO AVERAGE OF CONTROL GROUP

<table>
<thead>
<tr>
<th></th>
<th>NAD</th>
<th>NADH</th>
<th>NADH / NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average of Control Group</td>
<td>0.030</td>
<td>0.0064</td>
<td>0.205</td>
</tr>
<tr>
<td></td>
<td>± 0.005</td>
<td>± 0.0018</td>
<td>± 0.060</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>0.023</td>
<td>0.0079</td>
<td>0.345</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.027</td>
<td>0.0033</td>
<td>0.120</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>0.023</td>
<td>0.0039</td>
<td>0.170</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>0.028</td>
<td>0.0045</td>
<td>0.190</td>
</tr>
<tr>
<td>Experiment 5</td>
<td>0.031</td>
<td>0.0043</td>
<td>0.165</td>
</tr>
</tbody>
</table>

All results are in nmol. nmol total creatine

1
<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>Spectrophotometric</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCr</strong> (nmol mg prot⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 4)</td>
<td>185.0 ± 48.00</td>
<td>143.00 ± 34.00</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Experimentals (n = 4)</td>
<td>104.00 ± 34.00</td>
<td>100.00 ± 13.00</td>
</tr>
<tr>
<td><strong>PCr</strong> (nmol nmol total creatine⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 4)</td>
<td>0.62 ± 0.10</td>
<td>0.450 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Experimentals (n = 4)</td>
<td>0.40 ± 0.09</td>
<td>0.400 ± 0.06</td>
</tr>
<tr>
<td><strong>PCr / Cr_free</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 4)</td>
<td>0.92 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>Experimentals (n = 4)</td>
<td>0.69 ± 0.16</td>
<td></td>
</tr>
</tbody>
</table>

(* ) p = 0.05
TABLE 11: ADENINE NUCLEOTIDES AND IMP IN THE PARASTERNAL INTERCOSTALS OF CONTROL AND INSPIRATORY RESISTIVE LOADED ANIMALS: EXPRESSED PER MG PROTEIN

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=4)</th>
<th>Experimentals (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (nmol mg prot⁻¹)</td>
<td>57.9 ± 10.0</td>
<td>49.8 ± 5.4</td>
</tr>
<tr>
<td>ADP (nmol mg prot⁻¹)</td>
<td>8.40 ± 1.10</td>
<td>8.53 ± 1.30</td>
</tr>
<tr>
<td>AMP (nmol mg prot⁻¹)</td>
<td>0.20 ± 0.16</td>
<td>0.45 ± 0.53</td>
</tr>
<tr>
<td>Total AN (nmol mg prot⁻¹)</td>
<td>66.7 ± 11.0</td>
<td>58.7 ± 5.2</td>
</tr>
<tr>
<td>IMP (nmol mg prot⁻¹)</td>
<td>0.209 ± 0.24</td>
<td>0.61 ± 0.5</td>
</tr>
<tr>
<td>Total AN + IMP (nmol mg prot⁻¹)</td>
<td>66.9 ± 10.8</td>
<td>59.0 ± 5.0</td>
</tr>
</tbody>
</table>

ATP = adenoside 5'-triphosphate
ADP = adenosine 5'-diphosphate
AMP = adenosine 5'-monophosphate
AN = adenine nucleotides
IMP = inosine 5'-monophosphate
TABLE 12: ADENINE NUCLEOTIDES AND IMP IN THE PARASTERNAL INTERCOSTALS OF CONTROL AND INSPIRATORY RESISTIVE LOADED ANIMALS: EXPRESSED PER TOTAL CREATINE

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 4)</th>
<th>Experimentals (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP / Total Creatine</td>
<td>0.20000 ± 0.009</td>
<td>0.19000 ± 0.014</td>
</tr>
<tr>
<td>ADP / Total Creatine</td>
<td>* 0.02800 ± 0.0009</td>
<td>0.03300 ± 0.004</td>
</tr>
<tr>
<td>AMP / Total Creatine</td>
<td>0.00070 ± 0.0007</td>
<td>0.00179 ± 0.002</td>
</tr>
<tr>
<td>Total AN / Total Creatine</td>
<td>0.22000 ± 0.008</td>
<td>0.22600 ± 0.014</td>
</tr>
<tr>
<td>IMP / Total Creatine</td>
<td>0.00070 ± 0.0008</td>
<td>0.00240 ± 0.0015</td>
</tr>
<tr>
<td>(Total AN + IMP) / Total Creatine</td>
<td>0.22300 ± 0.007</td>
<td>0.22900 ± 0.014</td>
</tr>
<tr>
<td>ATP / ADP</td>
<td>* 7.00000 ± 0.38</td>
<td>5.90000 ± 0.98</td>
</tr>
<tr>
<td>Energy Charge</td>
<td>0.92000 ± 0.032</td>
<td>0.92000 ± 0.016</td>
</tr>
</tbody>
</table>

All results are in nmol

ATP = adenosine 5'-triphosphate
ADP = adenosine 5'-diphosphate
AMP = adenosine 5'-monophosphate
AN = adenine nucleotides
IMP = inosine 5'-monophosphate

(*) p < 0.05
### TABLE 13: PYRIDINE NUCLEOTIDES IN THE PARASTERNAL INTERCOSTAL MUSCLES OF CONTROL AND INSPIRATORY RESISTIVE LOADED ANIMALS

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Experimentals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((n = 4))</td>
<td>((n = 4))</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Experimentals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAD</strong> ((\text{nmol. mg prot}^{-1}))</td>
<td>5.3500 ± 0.80</td>
<td>5.1000 ± 0.50</td>
</tr>
<tr>
<td><strong>NADH</strong> ((\text{nmol. mg prot}^{-1}))</td>
<td>0.5900 ± 0.12</td>
<td>0.6700 ± 0.15</td>
</tr>
<tr>
<td><strong>NAD / Total Creatine</strong> ((\text{nmol. mmol}^{-1}))</td>
<td>0.0180 ± 0.0005</td>
<td>0.0195 ± 0.001</td>
</tr>
<tr>
<td><strong>NADH / Total Creatinine</strong> ((\text{nmol. mmol}^{-1}))</td>
<td>* 0.0019 ± 0.0004</td>
<td>0.0025 ± 0.0003</td>
</tr>
<tr>
<td><strong>NADH / NAD</strong> ((\text{nmol. mmol}^{-1}))</td>
<td>0.1100 ± 0.03</td>
<td>0.1300 ± 0.02</td>
</tr>
</tbody>
</table>

\((*) \ p < 0.05\)
4. DISCUSSION.

This study examined the status of the high energy phosphate compounds in rat respiratory muscles, following a prolonged protocol of severe inspiratory resistive loading in vivo, with preservation of arterial blood pressure and oxygenation. Very small changes in phosphocreatine and in the adenine nucleotide pool were found in both diaphragm and intercostal muscles, suggesting that the development of pump failure as we defined it, is not associated with significant changes in the concentration of the high energy phosphate compounds.

Inspiratory resistive loading is frequently used to experimentally induce increased inspiratory muscle activity in vivo and eventually, respiratory failure (1,7,14). It is not known what mechanisms are primarily responsible for the failure to sustain a certain level of contractile activity. Central mechanisms as well as a limitation of energy supply to meet energy demands, a variety of other mechanisms at the level of the muscle cell and limitations of the neural impulse transmission have all been postulated to play a role of variable magnitude depending on the model studied (1,7,14).

Alterations in diaphragm function associated with changes in muscle glycogen and lactate levels as well as in ATP and phosphocreatine concentrations (2,3,14), have been demonstrated in animal models with inspiratory loading and phrenic nerve pacing, suggesting that biochemical alterations in the diaphragm may lead to reduction in contractile force and eventually, to respiratory failure. In addition, a decrease in ATP and PCr concentration has been reported in the
intercostal muscles of patients with chronic obstructive pulmonary disease, associated with morphological changes in the muscles fibers (10).

It is known that the respiratory muscles use high energy phosphate compounds to fuel the processes that mediate muscle contraction and relaxation as well as to energize ionic pumps (25). Because cellular stores of these compounds are small, maintenance of intracellular concentrations adequate to sustain high levels of contractile activity depends on continuous regeneration of ATP (25). The diaphragm is a highly oxidative muscle, highly dependent on the level of muscle blood flow, which determines the rate of energy substrate delivery to the muscle and the rate at which metabolites, byproducts of contraction are removed (12,16). Although the compressive effect of tension development to increase diaphragm vascular resistance may play a role in limiting blood flow at very high levels of tension development (5,8), most studies have shown that for a wide range of workloads, the diaphragm, and to a lesser extent the intercostal muscles, have a large capacity to increase blood flow to meet their metabolic demands. Both diaphragm and intercostal muscles in the dog are able to exponentially increase muscle blood flow with linear increases in work (24,25). Whether similar changes in blood flow are operative in the rat model of inspiratory resistive loading is unknown.

Although the first studies of diaphragm high energy phosphates focused on the effects of sample handling on the measured concentrations (23), later studies by Busse did not show a change in either ATP or phosphocreatine concentrations
in spontaneously breathing dog undergoing inspiratory resistive loading (9). However, Rochester et al., found a decrease in ATP and phosphocreatine when inspiratory resistive loading was associated with phrenic nerve stimulation (2,3). This decrease in ATP and phosphocreatine was more pronounced when hypoxemia and respiratory acidosis were present. Further studies by the same group suggested that neither hypoxemia nor hypercapnia per se had any effect on ATP and phosphocreatine concentrations unless they were associated with increased contractile activity (2,3). All these studies were done in the dog and in all of them ATP and phosphocreatine concentrations were determined using enzymatic methods. There are no available studies attempting to characterize the phosphocreatine status and adenine and pyridine nucleotide concentrations simultaneously in the diaphragm or in the intercostal muscles with inspiratory resistive loading, in spontaneously breathing animals. Therefore, this study provides, for the first time, a complete evaluation of the status of the high energy phosphate compounds in a model of inspiratory resistive loading in vivo, with maintenance of arterial blood pressure and oxygenation.

The main finding of this study is a significant reduction in the total adenine nucleotide pool of the diaphragm, associated with a decrease in ATP and with no significantly different concentrations of ADP and AMP, suggesting some level of degradation of adenine nucleotides. The reduction in total adenine nucleotides however, far exceeded the increase in IMP (produced by deamination of AMP) observed in the experimental animals. These findings would suggest further
degradation of IMP into various oxypurine intermediates.

In this study we also found a decrease in diaphragm phosphocreatine concentration with the experimental protocol. Data in individual animals show that those with the lowest phosphocreatine are also those with the larger decrease in ATP and increase in IMP.

No significant changes were found in the concentration of either NAD\(^+\) or NADH or in the NADH to NAD\(^+\) ratio, suggesting that the diaphragm was probably not ischemic at the time of sampling.

Regarding the changes in the intercostal muscles, the main finding was a significant decrease in phosphocreatine. The total AN nucleotide pool, however, was not decreased and the increase in IMP was of smaller magnitude than in the diaphragm. An interesting finding was the significant increase in NADH with the resistive loading protocol. This would suggest some degree of reduction in oxygen availability to the intercostal muscles at the time of sampling. Although this may be an effect of the resistive loading protocol, we speculate that it may also be due to the fact that intercostal muscles were sampled after the diaphragm, at a time when arterial blood pressure had decreased below 60 mmHg.

Energy charge was also determined in both diaphragm and intercostal muscles, and found to be not different than in control animals. Energy charge has been postulated to be a critical factor in preserving membrane integrity (22). The finding of a normal energy charge added to the fact that the concentration of total creatine was also normal in the experimental animals, makes the possibility of
membrane damage less likely in this model.

Muscles with different fiber type composition seem to handle high energy phosphates differently (20). Although fiber type composition in the rat diaphragm is different than that in the dog (12), our results are in agreement with those of Busse in the spontaneously breathing dog under inspiratory resistive loading (9).

An interesting study in rabbits has shown that inspiratory resistive loading was not able to decrease diaphragm glycogen levels when the animals were allowed to breathe spontaneously through the loading device, whereas a substantial decrease in diaphragm glycogen was seen when the compensatory mechanisms used by the diaphragm to adjust to loads were overcome by electrical stimulation of the phrenic nerve and binding of the abdomen and lower thorax, which center the work of breathing in the diaphragm, avoiding the participation of the intercostal muscles (14).

In conclusion, despite the severe loading conditions, at the point of respiratory failure, with preservation of arterial blood pressure and oxygenation, the high energy phosphate compounds are relatively well preserved. Therefore, it is unlikely that in this model, the development of pump failure was due to a lack of available energy substrates. It is likely that a series of defense mechanisms are operative in this model that function to avoid the depletion of high energy phosphates in the respiratory muscles. It is possible that equally severe loading protocols may have a larger impact on the high energy phosphate compounds if oxygen supplementation is not provided, with the consequent effect of decreasing
perfusion pressure to the working muscles.

5. **LIST OF REFERENCES.**


CHAPTER V

PART A: ANTIOXIDANT PROPERTIES OF THE RAT DIAPHRAGM ARE DIFFERENT THAN THOSE OF THE INTERCOSTAL MUSCLES

1. INTRODUCTION.

Recent studies have suggested that oxidant stress may be one of the mechanisms contributing to dysfunction of skeletal muscle in exhaustive exercise and of respiratory muscles in particular, under conditions of severe overload (16,22). It has been shown in vitro, that exogenous free radicals can affect diaphragmatic function and that the electrically stimulated diaphragm is capable of producing oxygen centered free radicals (7,25,26). Moreover, it has been postulated that the decrease in total diaphragm glutathione seen with resistive breathing in vivo, is secondary to oxidant stress induced by increased diaphragmatic activity (1). Although the potential sources of free radical species in muscle are numerous, the increased oxygen consumption at the level of the mitochondrial respiratory chain with increased muscle activity has been postulated as the main source of oxygen-centered species, through the univalent reduction of molecular oxygen (5). These highly reactive species can cause a wide range of cell damage, such as lipid peroxidation and protein oxidation. (13)
Mammalian cells possess both non-enzymatic and enzymatic antioxidant defenses to cope with oxygen-derived free radicals. Non-enzymatic defenses scavenge hydroxyl radical (OH\(^-\)) and organic oxy-radicals while enzymatic defenses metabolize superoxide anion (O\(_2^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)). The former includes a variety of compounds such as alpha tocopherol (vitamin E), B carotene and ascorbic acid, whereas the later includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In addition, glutathione reductase (GR) provides glutathione in the reduced form as the substrate for GPx. All these enzymes form an important defense system to ensure that cytotoxic oxygen-derived species are degraded to less harmful compounds so that extensive cell damage does not occur (13).

The diaphragm and intercostal muscles have different oxidative capacities (8,17) and if oxidative stress is related to oxidative capacity as has been postulated (5,14), it is likely that their antioxidant characteristics may also be different.

The aims of this study were: a) to determine the specific activity of the most important antioxidant enzymes in both the diaphragm and parasternal intercostal muscles of normal adult rats, b) to relate these activities to those of limb skeletal muscles with well characterized oxidative capacity, c) to measure the level of glutathione in respiratory muscles and compare it with that of skeletal muscles of known oxidative capacity, and d) to establish if a relationship exists between the levels of glutathione and antioxidant enzymes, and the known oxidative capacity of the various muscles studied.
2. METHODS.

Guidelines established by the Institutional Animal Care and Use Committee at The Ohio State University were followed. Male Sprague-Dawley rats weighing 350-450 g were anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg) and exsanguinated through the vena cava. Samples of diaphragm, parasternal intercostals, soleus and gastrocnemius muscles were taken using freeze clamping with liquid nitrogen, stored at -70°C and assayed for a) total and oxidized glutathione and b) the specific activity of the antioxidant enzymes: glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase. Liver samples were taken and assayed for total and oxidized glutathione and for the specific activity of glutathione peroxidase (GPx) and superoxide dismutase (SOD), in the same way as the muscle samples.

2.1. Glutathione assay: Glutathione determinations were done within 24 hours of sampling. Muscle samples were individually homogenized in 5% sulfosalicylic acid to produce 20% homogenates which were centrifuged at 0°C and 200 x g for 8 min. The resulting supernatant was centrifuged again for 2 min at 12,000 x g, and the DTNB-GSSG reductase recycling assay, modified by Griffith was then used (11). The amount of total glutathione was determined from a standard curve in which the glutathione equivalents were plotted against the rate of change in absorbance at 412 nm. Results are expressed as nmol/g wet weight. For oxidized glutathione (GSSG) derivatization with vinylpyridine was used and
the assay run in the same way as for total glutathione; results are expressed as nmol of GSH equivalents per gram wet weight.

2.2. Antioxidant enzymes: For the enzyme assays, frozen samples were quickly weighed and 10% homogenates prepared in ice cold buffer (potassium phosphate, pH 7.4, supplemented with 30mM KCl), using a Polytron for 40 to 60 sec.

2.2.1. The specific activity of total superoxide dismutase (SOD, EC 1.15.1.1) was determined according to Marklund et al. (19), using the pyrogallol auto-oxidation test. One unit of total SOD was defined as the amount of SOD that results in 50% inhibition of pyrogallol auto-oxidation. Results are expressed as U/mg protein.

2.2.2. Catalase (CAT, EC 1.11.1.6) was determined by measuring the decomposition of H₂O₂ at 24°C, at 240 nm (6). Specific activity was defined as μmol H₂O₂ consumed.min⁻¹.mg protein⁻¹.

2.2.3. Glutathione peroxidase (GPx, EC 1.11.1.9) was assayed at 37°C according to Gunzler et al. (6,12), using tert-butyl hydroperoxide as the substrate. This avoids problems contributed by the presence of catalase in the tissue homogenates. The method is based on the oxidation of glutathione by GPx, coupled to the disappearance of NADPH by GR. One unit of GPx is that which causes the oxidation of 1 μmol of glutathione per minute. Results are expressed as mU/mg protein.
2.2.4. Glutathione reductase (GR, EC 1.6.4.2) was assayed using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance change at 412 nm due to TNB formation was measured (28) at 24°C. This assay allows for measuring activity in tissue homogenates in the presence of other NADPH dependent enzymes. Results are expressed as nmol of TNB produced.min⁻¹.mg protein⁻¹.

Protein concentration was determined by the method of Bradford (4), using bovine albumin as standard. Blood contamination of respiratory muscle samples was determined by measuring hemoglobin concentration (20) in aliquots of the tissue homogenates.

2.3. Statistical analysis: Data are presented as the mean ± 1 SD. One way analysis of variance (ANOVA) was used to study differences between muscles. A p value < 0.05 was considered statistically significant (29). Linear regression analysis was used to establish correlations between antioxidant enzyme specific activities and oxidative capacity, and t-test was used to determine statistical significance of the correlations.

3. RESULTS.

3.1. Total glutathione: The levels of total glutathione in the respiratory muscles and in the soleus and gastrocnemius are shown in Fig. 31. Total GSH was 1,149 ± 142 nmol/g of wet diaphragm and 690 ± 118 nmol/g of wet intercostal (p=0.004), while GSSG (not shown) was 45 ± 14 and 27 ± 12 nmol of GSH
equivalents per gram of diaphragm and intercostal muscle, respectively (p < 0.05). Although the diaphragm showed more GSH equivalents in the form of GSSG than intercostal muscles, percent GSSG in both muscles was the same (3.9% for the diaphragm and 3.8% for intercostals). Total glutathione in soleus was 1,592 ± 459 nmol/g wet weight, (approximately 40% higher than diaphragm total glutathione, p < 0.05) whereas it was 780 ± 107 nmol/g wet weight for the gastrocnemius (approximately 30% lower than diaphragm total glutathione, p < 0.01, and not significantly different from intercostal total glutathione). Compared to the liver, the diaphragm exhibits only one third of the liver glutathione content per gram of wet tissue.

3.2. Antioxidant enzymes:

3.2.1. Enzymes of the glutathione redox cycle:

3.2.1.1. GPx: The specific activity of glutathione peroxidase was almost two times higher in the diaphragm than in the intercostals (p < 0.001), with 119 ± 47 mU/mg protein for the diaphragm and 85 ± 19.5 mU/mg protein for the intercostals. Soleus showed significantly higher activity than the diaphragm (p < 0.01), whereas gastrocnemius showed significantly lower activity (p < 0.01) than the intercostal muscles. Fig. 32 illustrates the differences in GPx activity among muscles. The diaphragm has approximately 20% of the liver GPx.

3.2.1.2. The specific activity of glutathione reductase was determined only in the respiratory muscles, and it was significantly higher in the diaphragm than in the intercostal muscles (p < 0.01), with 5.7 ± 1.4 nmol.min⁻¹.mg protein⁻¹ for the
diaphragm and $3.54 \pm 0.29 \text{nmol.min}^{-1}\text{.mg prot.}^{-1}$ for the intercostals (Fig. 33).

3.2.2. Total SOD (Fig. 34): The diaphragm showed significantly higher activity of total SOD than intercostal muscles ($p<0.01$), with $2.32 \pm 0.78 \text{U/mg protein}$ for the diaphragm and $1.53 \pm 0.28 \text{U/mg protein}$ for the intercostal. The soleus was found to have the same activity as the diaphragm ($2.31 \pm 0.18 \text{U/mg protein}$), and the gastrocnemius, the same activity as the intercostal muscles ($1.59 \pm 0.22 \text{U/mg protein}$).

Total SOD in the diaphragm was found to be 15% of the liver total SOD.

3.2.3. Catalase: Fig. 35 shows a) significant differences in catalase activity between diaphragm and intercostal muscles, with $20 \pm 12 \text{U/mg protein}$ for the diaphragm and $11 \pm 8 \text{U/mg protein}$ for the intercostals, and b) no significant difference in catalase activity between diaphragm and soleus, and between gastrocnemius and intercostal muscles.

3.3. The contribution of antioxidant enzymes present in blood contaminating the respiratory muscle samples was found to be very small ($0.2 \text{mg hemoglobin per ml of 10% homogenate}$ or $2 \text{mg per gram of wet diaphragm}$ and $0.06 \text{mg hemoglobin per ml of 10% intercostal homogenate}$). Based on data on enzyme activity measured simultaneously in both blood and muscle of rabbits (20), it was calculated that the contribution of blood to activity of the antioxidant enzymes in tissue was minimal (less than 5% in the diaphragm).
3.4. A determination was made as to whether a relationship exists between the known muscle oxidative capacity and both the activities of the antioxidant enzymes and the glutathione content. Previously published data on a) succinate dehydrogenase (SDH, a membrane bound mitochondrial oxidative enzyme) (3,17,23) and b) percent of highly oxidative fibers (type I and IIa) (3,17) were chosen and correlated with the antioxidant enzyme activities and the glutathione content.

Total glutathione content was found to be linearly related to both SDH activity \((r = 0.82, p < 0.01)\), and the percent of highly oxidative fibers \((r = 0.81, p < 0.01)\) (Fig. 31). GPx activity (Fig. 32) was also found to have a very significant correlation with both, SDH activity \((r = 0.87, p < 0.01)\) and the percent of highly oxidative fibers \((r = 0.89, p < 0.01)\).

For total SOD activity (Fig. 34) we found a linear relationship with oxidative capacity, although less significant than the relationship for GPx. The correlation coefficient for the relationship between SDH activity and total SOD activity was 0.607 \((p < 0.01)\), whereas the correlation coefficient for the relationship between % highly oxidative fibers and total SOD activity was 0.498 \((p < 0.01)\).

Data on catalase activity showed a linear relationship \((r = 0.5, p < 0.05)\) with SDH activity (Fig. 35). The relationship with % highly oxidative fibers, however, was also significant \((r = , p < 0.05)\).
Fig. 31. Level of total glutathione in respiratory muscles, soleus and gastrocnemius. Solid squares represent values for individual muscle samples. Stars represent significant differences among muscles, obtained by ANOVA. *: p < 0.05; **: p < 0.01; ***: p < 0.001

The relationship between previously published data on SDH activity (3,17,23) and total glutathione content is also illustrated. Solid line corresponds to the regression line, r = correlation coefficient.
Fig 32. Specific activity of glutathione peroxidase in respiratory muscles, soleus and gastrocnemius. Solid squares represent values for individual muscle samples. Stars represent significant differences obtained using ANOVA. * : p < 0.05; ** : p < 0.01; *** : p < 0.001.

The relationship between previously published data on SDH activity (3,17,23) and the measured GPx activity is also shown. Solid line corresponds to the regression line and r is the correlation coefficient.
Fig. 33. Specific activity of glutathione reductase in respiratory muscles, soleus and gastrocnemius. Bars represent the means ± 1 standard deviation; **: p < 0.01.
Fig. 34. Total SOD activity in respiratory muscles, soleus and gastrocnemius. Solid squares correspond to values for individual muscle samples. Stars represent significant differences among muscles, obtained with ANOVA.

* : p < 0.05; ** : p < 0.01.

The relationship between SDH activity (3,17,23) and total SOD activity is also shown. Solid line is the regression line and r is the regression coefficient.
Fig. 35. Specific activity of catalase in respiratory muscles, soleus and gastrocnemius.

The relationship between previously published data on SDH activity (3,17,23) and catalase activity is also shown. Solid line corresponds to the regression line and r is the correlation coefficient.
4. DISCUSSION.

In this study, the level of glutathione and the specific activity of the antioxidant enzymes are reported in the main respiratory muscles of adult rats. This is the first investigation of the differential antioxidant properties of the muscles primarily responsible for breathing. Large differences in the antioxidant enzyme activities and in the level of glutathione were found between diaphragm and intercostal muscles which appeared to be related to their oxidative capacity. The diaphragm not only exhibits a significantly higher level of total glutathione than the intercostal muscles but also shows a higher activity of a) the enzymes involved in the glutathione redox cycle, b) catalase and c) superoxide dismutase.

Generally, the assessment of the involvement of free radicals in biological systems involves the study of the effects of exogenous free radicals on function and on the antioxidant properties of those systems. Surprisingly, in the case of the respiratory muscles, despite a great deal of interest in the role of free radicals in diaphragmatic dysfunction with excessive mechanical loads (7,25,26), very little information about the diaphragm's inherent antioxidant properties is available and none is available regarding the properties of the intercostal muscles.

Catalase, SOD and the enzymes of the glutathione redox cycle are the primary intracellular antioxidant defense mechanisms responsible for coping with increased oxidant stress. They eliminate $\text{OH}^-$ and $\text{H}_2\text{O}_2$, which may oxidize cellular components. In doing so, they prevent the initiation of free radical chain reactions (13).
4.1. Glutathione and enzymes of the glutathione redox cycle in respiratory muscles: It is well established that glutathione is an important cellular antioxidant acting in collaboration with GPx to break down H₂O₂ and lipid peroxides (21). Unlike catalase, the glutathione redox cycle has the added capacity of metabolizing hydroperoxides other than H₂O₂. The main enzyme is GPx, a 85,000 D tetrameric protein with selenium at the catalytic site that catalyzes the reaction:

\[ \text{ROOH} + 2\text{GSH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \]

The second important enzyme is GR, a 105,000 D dimeric protein that catalyzes the reduction of GSSG back to GSH in the following reaction:

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \]

In relation to glutathione, this study has demonstrated: a) that the level of total glutathione in the diaphragm, obtained with an enzymatic method, is in agreement with that reported by Anzueto et al. using HPLC (1); b) that total glutathione in the parasternal intercostal muscles is only 2/3 of the diaphragm glutathione. No data on glutathione in intercostal muscles was found in the literature. The finding of a 3.9% of the glutathione in the oxidized form (GSSG/GSH\text{total} \times 100) in the diaphragm is in agreement with the level of GSSG reported for other tissues (5,21), but differs from the 22% GSSG recently reported for control diaphragm (1), using different methodology.

With respect to the enzymes of the glutathione redox cycle, a large
difference in the specific activity of GPx between diaphragm and intercostal muscles was found in our study. Similar differences are evident, although not discussed, in the study by Powers et al. on the effects of exercise training (24); approximately 50% less GPx activity was found in the intercostal muscles compared to the diaphragm in that study. We also found that GR was also significantly higher in the diaphragm than in intercostal muscles. In addition, the level of total glutathione (not only in respiratory muscles, but in all four muscles studied) was very tightly correlated with the activity of GPx. This is not surprising, since cellular distribution of glutathione and of the two enzymes of the glutathione redox cycle, has been described to be similar (21). They exist predominantly in the cytosol, although mitochondria also exhibits some glutathione redox activity (21).

4.2. SOD and catalase in respiratory muscles: Cellular SOD activity is represented in the cell by metalloenzymes with different prosthetic groups. These enzymes catalyze the dismutation of $O_2^-$ to $H_2O_2$, $10^4$ times faster than the spontaneous rate of $O_2^-$:dismutation:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The prevalent enzyme is cuprozinc (CuZn)SOD, a stable dimer found mainly in the cytosol, whereas manganese (Mn)SOD is a tetramer that exists mainly in the mitochondria, where it eliminates $O_2^-$ formed during electron transport.
Catalase is a 240,000 D tetrameric hemoprotein, located primarily in the peroxisomes, that catalyzes the reaction:

\[ 2H_2O_2 \rightarrow 2H_2O + O_2 \]

We were unable to find data on specific activity of either catalase or SOD in respiratory muscles. In this study, we found that both muscles have different SOD activity, with the diaphragm having a similar level as the soleus and the intercostals having a similar level as the gastrocnemius. Similar results were obtained for catalase, with significant differences between diaphragm and intercostal muscles. Likewise, the catalase activity in the diaphragm was similar to that of the soleus, whereas intercostal catalase activity was similar to that of the gastrocnemius muscle.

Differences in antioxidant enzyme activity as well as glutathione content in respiratory muscles could not be attributed to different levels of blood contamination of the tissue samples. Although hemoglobin in diaphragm homogenates was three times higher than in intercostal homogenates, contamination of these homogenates with blood antioxidant enzymes was minimal and does not explain the large differences in tissue antioxidant enzyme activity.

4.3. Antioxidant capacity as a function of oxidative capacity: It has been shown that skeletal muscles of different fiber type composition differ in their antioxidant enzyme content \( (2,14,18) \), such that highly oxidative muscles have
higher antioxidant enzymes than largely glycolytic muscles. The diaphragm muscle is continuously active, while the intercostal muscles assist diaphragm function by stabilizing and lifting the rib cage (8). The rat diaphragm has a heterogeneous fiber population, composed of approximately 45% fast twitch glycolytic fibers, 20% fast twitch oxidative and 35% slow oxidative fibers (17). Intercostal muscles have 47-54% fast twitch glycolytic fibers, 15-25% fast twitch oxidative and 28-30% slow oxidative fibers (17). Although fiber type composition is not radically different among the two muscles, studies of oxidative enzymes in respiratory muscles of different species, show that the intercostal muscles have approximately 50% the oxidative capacity of the diaphragm (8). In this regard, Sieck has shown (27) that qualitative histochemical analysis of muscle fibers provides limited insight into the inherent metabolic characteristics of the muscle. In addition, there is supporting evidence that the response of oxidative enzymes to exercise training is different in both types of muscle (10), probably due to different levels of activation of both muscles at a given level of exercise training. We speculated that the differences in enzyme activity between the respiratory muscles could be explained based on their differences in oxidative capacity.

In this study, when the respiratory muscles were evaluated in context with other skeletal muscles with different fiber type composition, glutathione and GPx appeared tightly related to the oxidative capacity of the muscle. Interestingly, GPx is the only antioxidant enzyme that has consistently been shown to be upregulated with increased oxidative activity induced by exercise training (14, 15, 18).
Although we found very significant differences in total SOD activity between highly oxidative and highly glycolytic muscles (i.e. soleus vs gastrocnemius, respectively), the differences were not as obvious when comparing muscles with more similar oxidative capacities (i.e. soleus and diaphragm). Parallel results have been reported by Apple et al. (2). The fact that the relationship between total SOD and oxidative capacity is not as tight as the one for glutathione and GPx is interesting. Exercise training with documented increase in oxidative enzymes, has no effect on SOD activity in most studies (14). The only SOD enzyme that has been shown to be inducible by high intensity exercise is MnSOD, which constitutes a very small part of total SOD (14,15). It is possible that studies of MnSOD in muscles with different oxidative capacity might show a better relationship with oxidative capacity.

In conclusion, both the specific activity of the four antioxidant enzymes and the level of glutathione are significantly higher in the diaphragm than in the intercostal muscles. The antioxidant enzyme systems of the diaphragm are closer to those of the soleus, whereas the antioxidant enzyme properties of the intercostal muscles resemble those of the gastrocnemius. A clear relationship between oxidative capacity and antioxidant reserve was found for glutathione and the enzyme of the glutathione redox cycle, glutathione peroxidase. Although less significant, this type of relationship was also found, for total SOD and catalase.
5. LIST OF REFERENCES.


PART B : EFFECT OF INSPIRATORY RESISTIVE LOADING ON THE ACTIVITY OF THE ANTIOXIDANT ENZYMES IN RESPIRATORY MUSCLES.

The effects of endurance training increasing the activity of the oxidative enzymes in limb skeletal muscle are well documented (4). The same kind of effect has been shown for respiratory muscles (9). Recent studies have shown that some of the antioxidant enzyme systems are also affected by endurance training (1,7,8). This increase in activity of the antioxidant enzymes, however, does not seem to be proportional to the increase in oxidative capacity (8). This response of the antioxidant enzyme systems, gives support to the hypothesis that oxidative metabolism is an important source of reactive oxygen species in exercising muscle.

Acute bouts of exercise in untrained animals have shown evidence of tissue lipid peroxidation (2,3,5). In addition, it has been suggested that acute exercise is capable of affecting the activity of some of the limb muscle antioxidant enzymes (5-7).

In this study, the response of the antioxidant enzyme systems of the respiratory muscles, to the incremental inspiratory resistive loading protocol was explored. The specific activity of the enzymes GPx, GR, total SOD and catalase were determined in diaphragm and intercostal muscles following the experimental protocol and compared to data from control animals. No significant differences in specific activity of any of the antioxidant enzymes were found between control and experimental groups. Results are illustrated in figs. 36-39.
Fig. 36: Specific activity of the enzyme glutathione peroxidase (GPx) in respiratory muscles of control and experimental groups.

Open bars represent control animals

Dashed bars represent experimental animals
Fig. 37. Specific activity of the enzyme glutathione reductase (GR) in respiratory muscles of control and experimental groups.

Open bars correspond to control and dashed bars, to experimental animals.
Fig. 38. Specific activity of total SOD in respiratory muscles of control and experimental animals.

Open bars represent control animals, dashed bars correspond to experimental animals.
Fig. 39. Specific activity of catalase in respiratory muscles of control and experimental animals. Open bars: controls, dashed bars: experimental animals.
2. LIST OF REFERENCES.


1. INTRODUCTION.

It is recognized that respiratory muscle dysfunction, due to excessive respiratory muscle loading, may contribute to the development of ventilatory failure in patients with cardiopulmonary disease (27,34). Although the causes for this dysfunction are complex and poorly understood, the possibility of oxidant stress in respiratory muscles exposed to high loads has recently been suggested from evidence, mostly indirect, of free radical generation in skeletal muscle. It has been postulated that oxygen-derived free radicals can be generated in exercising skeletal muscle and may constitute the basis for dysfunction of its contractile elements. Different experimental exercise protocols, as well as models of ischemia-reperfusion, are associated with evidence of free radical generation (6,11,12,14,17,28).

A number of studies have also shown that the electrically stimulated diaphragm is capable of generating oxygen-derived free radicals (13,24,26), and that the use of antioxidants seems to attenuate the fatigue process in *in vitro* and *in situ* experiments (24,26,30). It is not clear however, how relevant these studies
are to conditions in vivo. In a recent study, impaired function associated with lipid peroxidation and changes in the glutathione redox cycle were found in the diaphragm of rats exposed to a short period of inspiratory resistive breathing and attributed to oxidant stress (4).

There are many postulated sites for the production of reactive oxidant species in skeletal muscle including mitochondria, lysosomes, peroxisomes, sarcoplasmic reticulum and the sarcolemma (6). Among these, the mitochondria are likely to be the most important source in exercise, since increased oxygen consumption can indirectly enhance the potential for the formation of oxygen-derived free radicals (6,10). In addition, oxidants may be released by activated polymorphonuclear cells, as has been shown in the delayed response to eccentric skeletal muscle contractions (35) and in the lung with hypovolemic shock (3).

Cellular antioxidant mechanisms normally scavenge oxidants produced by these sources, avoiding the formation of the very reactive hydroxyl radical which is capable of initiating lipid peroxidation (10). Glutathione is one of the most prevalent cellular antioxidants and together with the glutathione-utilizing enzymes, provide cells with a complex defense network against oxidant injury. Loss in total intracellular glutathione is a consistent finding under conditions of oxidant stress and is associated with an increase in glutathione oxidation and protein-mixed disulfide formation (22,33). In addition, the liver is believed to be a potential source of glutathione for the exercising muscle, and exercise-induced depletion of glutathione in the liver has been documented under conditions of very exhaustive
In this study we have:

a) studied the changes in glutathione content and redox state in both diaphragm and intercostal muscles following a prolonged period of incremental inspiratory resistive loading, b) attempted to establish whether a negative correlation exists between the intensity of the respiratory muscle activity and the changes in glutathione, and c) evaluated the glutathione status in the liver in response to the respiratory muscle load. Our model differs from one reported recently (4), in that the animals are exposed to an inspiratory load for a more prolonged period of time, and both normal arterial blood pressure and normal blood oxygenation are maintained throughout the experiment.

Our main finding was a decrease in diaphragm total glutathione, with no increase in oxidized glutathione (GSSG). The level of glutathione a) was significantly correlated with the level of minute ventilation and of PaCO₂ immediately prior to failure, and b) does not seem to be determined by the intensity of respiratory muscle activity.
2. MATERIAL AND METHODS.

2.1. Animals and care: Experiments were done in accordance with guidelines established by the Institutional Animal Care and Use Committee at The Ohio State University. The study was carried out in male Sprague-Dawley rats, fed standard stock chow diets, given water ad libitum and weighing 300-400 g. All animals were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg initially plus 2 small supplements of 10 mg/kg). A 2.2 mm internal diameter tracheal cannula was placed through a tracheostomy and connected to a two-way non-rebreathing valve (model 2300, Hans Rudolph, Kansas City, MO). The animals were randomly assigned to one of the following three groups:

1) the experimental group (n=6) which underwent incremental inspiratory resistive loading while breathing 70% oxygen for 2.5 - 3 hours;
2) a time-matched control group (n=8) which was anesthetized while unloaded and breathing room air (controls on air), and
3) a second time-matched control group (n=9) which was anesthetized and unloaded while breathing 70% oxygen (controls on oxygen).

In our first attempts with the resistive breathing protocol 4 animals breathing air were studied in a similar way as reported by Anzueto et al. (4). However, animals developed severe hypoxemia with resultant hypotension, at about 10 min after loading had started. They remained severely hypotensive developing bradypnea and a progressive decrease in tracheal pressure for another 5 to 8 min,
until apnea. Therefore, seventy percent inspired oxygen was chosen for the experimental group in order to prevent the extreme hypoxemia and resultant systemic hypotension, generally associated with high inspiratory loads (5). This enabled the experimental animals to undergo the resistive breathing protocol for longer than 2.5 hours with higher than normal PaO2. Thus, control animals on 70% oxygen were studied to account for the possible effects of oxygen alone, which could independently result in the production of free radicals and affect the glutathione redox cycle.

2.2. Measurements: Tracheal pressure (Pp) was continuously monitored by way of a side port in the tracheal cannula, which was connected to one side of a differential pressure transducer (Validyne, Northridge, CA). Inspiratory flow was measured with an in-line pneumotachograph (model 0648, A. Fleisch), and inspiratory volume was obtained by integration of the flow signal.

A water-filled catheter, which was connected to a pressure transducer (Validyne, Northridge, CA) was inserted through the mouth and placed in the middle third of the esophagus in order to monitor esophageal pressure (Peo).

The right carotid artery was cannulated for continuous blood pressure measurement and periodic blood sample collection for pH and blood gas analysis (model BMS 3, Radiometer, Copenhagen). Arterial blood pressure, along with Pao2, Pp, and inspiratory volume, were recorded on a four channel recorder. Rectal temperature was continually monitored with a thermistor and maintained at
approximately 38.5°C, using heating pads.

Work of breathing was later calculated from the area under the pressure-volume curves constructed using representative breaths at each level of resistance.

2.3. Incremental inspiratory resistive loading. An estimation of the maximal inspiratory pressure (PImax), generated against an occluded airway, was obtained by occluding the inspiratory side of the two-way valve for 30 sec. or until blood pressure fell to 80 mm Hg. The inspiratory side of the two-way valve was then attached to a variable inspiratory resistance consisting of a fine needle valve. The resistance was set initially to result in a generated P, of 30% of PImax. After 10 minutes, the resistance was increased by an additional 10% of PImax. This procedure was repeated at 10 min. intervals until either tidal volume fell to 15-30% of its baseline value or there was evidence of circulatory collapse. This yielded an average increase in P, of approximately 3.5 cm H2O every 10 minutes. The animals remained on the resistance for 2.5 - 3 hours, at which time they developed evidence of pump failure. Pump failure was defined as marked bradypnea associated with a sudden decrease in peak P, without any change in inspiratory resistance. This was, in general, preceded by a rapid decrease in arterial blood pressure. When blood pressure had declined to 60 mm Hg, the animals underwent thoracotomy in order to obtain tissue samples.
2.4. Sample collection: Costal diaphragm, parasternal intercostal and liver samples were obtained by freeze-clamping with liquid nitrogen. The samples were kept frozen at -70°C and analyzed for total and oxidized glutathione within 24 hours, since storage for longer time was associated with increased levels of GSSG (unpublished observations).

2.5. Glutathione assay: Muscle and liver samples were individually homogenized in 5% sulfosalicylic acid to produce 20% homogenates which were centrifuged at 0°C and 200 g for 8 minutes. The resulting supernatant was centrifuged again for 2 min. at 12,000 g. The DTNB-GSSG reductase recycling assay, modified by Griffith, was then used (2,15). The amount of total glutathione was determined from a standard curve, in which the GSH equivalents (0.25 to 2.0 nmol) were plotted against the rate of change in absorbance at 412 nm.

For the determination of oxidized glutathione (GSSG), derivatization at room temperature was carried out by adding 6 ul of vinylpyridine and 12 ul of triethanolamine to 300 ul of the homogenate supernatant. The derivatized samples were then assayed in the same way as for total glutathione. Results are expressed as nmol/g wet wt. for total glutathione, and as nmol of GSH equivalents/g wet wt. for GSSG. The GSSG/GSH ratio was also calculated.

To account for differences in water content in the muscles with loading, additional tissue samples were taken to determine the dry/wet weight ratio. No differences in the ratio were observed between experimental and control groups.
2.6. Statistical analysis: Data are presented as the mean ± 1 SD. Data were analized with a one-way analysis of variance (34). When a significant F value was found, the Newman-Keuls post-hoc test was performed. Linear or polynomial regression analyses were used to establish correlations between the level of glutathione and relevant experimental parameters, and the critical correlation coefficient was used to determine statistical significance. A p value ≤ 0.05 was considered statistically significant.
3. RESULTS.

3.1. Total glutathione and GSSG in control respiratory muscles: As seen in Fig. 40, the level of total glutathione in the diaphragm was significantly higher than that in the parasternal intercostal muscles, namely 1,149 ± 142 nmol/g wet wt. vs. 690 ± 118 nmol/g wet wt. (p = 0.004).

With rapid processing of the tissue samples, the level of GSSG was very low in control respiratory muscles (Fig. 40). When expressed as nmol of GSH equivalents/g wet wt., the diaphragm showed a significantly higher level of GSSG than the intercostal muscles: 45 ± 14 nmol/g wet wt. vs. 27 ± 12 nmol/g wet wt. (p = 0.05). This is consistent with the increased level of total glutathione in the diaphragm, since when GSSG is expressed as a percent of total glutathione, there is no significant difference between the two muscles (3.9% for the diaphragm and 3.8% for the intercostals).

3.2. Effects of breathing 70% oxygen on total glutathione and GSSH in control respiratory muscles: Figure 40 illustrates that oxygen alone had a very small effect of lowering total glutathione in the diaphragms of control animals (from 1,149 ± 142 nmol/g wet wt. in animals breathing air to 969 ± 131 nmol/g wet wt., p < 0.05). On the other hand, oxygen did not show any significant effect on intercostal muscle total glutathione (624 ± 98 vs. 690 ± 118 nmol/g wet wt.).

No significant differences were found in the level of GSSG or in the GSSG/GSH ratio in either of the muscles, compared to controls on air.
3.3. General physiologic responses to prolonged inspiratory resistive loading:

While on the resistance, animals showed a gradual decrease in their PaO₂ and pH and became increasingly hypercapnic. At the time of pump failure, however, a high PaO₂ was still maintained (183 ± 23 torr), with a very severe acidosis and hypercapnia (pH = 6.97 ± 0.12 and PaCO₂ = 106 ± 36 torr). Although the acidosis was mainly due to CO₂ retention, there was also a metabolic component, since the base excess changed from -3.33 prior to the resistive loading protocol to -12.2 at the end. Arterial blood pressure was maintained well above 100 mm Hg throughout the loading runs, until pump failure. Peak P₁ became progressively more negative with the increase in resistance, reaching a maximum average P₁ of -52.7 ± 8.5 cm H₂O for the group.

3.4. Effect of prolonged inspiratory resistive loading on respiratory muscle total glutathione and GSSG: Total glutathione was significantly decreased in the diaphragms of resistive breathing animals when compared to a) the diaphragm of controls breathing room air and b) the diaphragm of controls breathing 70% oxygen (765 ± 231 vs. 1149 ± 142 nmol/g wet wt., p < 0.01 and 969 ± 131 nmol/g wet wt., p < 0.05, respectively) (Fig. 40).

The level of total glutathione in the intercostals was not significantly affected by the load, when compared to that of the two control groups (Fig. 40).

No significant differences were found in the level of GSSG or in the GSSG/GSH ratio, between experimental and control groups in either muscle studied.
3.5. Liver total glutathione and GSSG: Oxygen administration alone appeared to increase liver total glutathione from 3,881 ± 928 nmol/g wet wt. in the controls on air to 4,375 ± 839 nmol/g wet wt. in the controls on oxygen, but this difference was not statistically significant (p = 0.084) (Fig. 41). No increase in GSSG or in the GSSG/GSH ratio was observed.

3.6. Correlative findings: In order to evaluate which possible physiologic factors could be important in determining the extent of the reduction in total glutathione, correlations were made between total diaphragm glutathione and various physiologic measurements taken during resistive breathing. Although all the animals underwent the same resistive breathing protocol, because of the use of anesthesia, we obtained a broad range of physiologic responses, which allowed us to explore possible correlations. First, we hypothesized that the animals with the largest degree of activity of the respiratory muscles would most likely demonstrate the largest reduction in total glutathione; however, we found no correlation between the physiologic parameters studied (highest peak $P_t$ generated, highest pressure-time product, maximum power output and total P-V work) and the level of glutathione. The four relationships are shown in the following series of figures. In Fig. 42, total glutathione content is plotted against the highest peak $P_t$ generated ($r = 0.12$, NS), and in Fig. 43, against the highest pressure-time product, that is, the change in tracheal pressure integrated over $T_{tot}$ ($r = 0.25$, NS). Figure 44 shows the relationship with maximum power output in Joules/min,
calculated from the pressure-volume (P-V) curves ($r = 0.75$). We did not find the expected negative correlation between the intensity of inspiratory muscle activity and the drop in glutathione, moreover, animals performing more work had higher glutathione levels. Fig. 45 shows the relationship with the total amount of P-V work done over the entire loading period ($r = 0.78$). Interestingly, animals with the highest power output and the largest total P-V work were those with the highest glutathione content at failure. These observations are inconsistent with the hypothesis that the decrease in diaphragm glutathione is related to increased muscle activity.

Next, we tested whether or not the degree of circulatory collapse associated with respiratory failure was related to the drop in total glutathione and found no significant correlation between arterial blood pressure at the end of the experiment and the level of total glutathione ($r = 0.48$, NS, data not shown).

We also tested whether or not the drop in glutathione was related to the degree of hypercapnia and acidosis observed. Although the linear regression coefficient for the relationship between arterial pH and glutathione did not reach statistical significance ($r = 0.74$), there was a strong negative correlation between PaCO$_2$ and glutathione as shown in Fig. 46 ($r = -0.865, p < 0.05$). In addition, there was a significant positive correlation ($r = 0.91, p < 0.05$) between total glutathione content and the tidal volume prior to failure, expressed as percent of its baseline value (Fig. 47). We also found a significant positive correlation between total glutathione content and the level of minute ventilation prior to failure, expressed
as a percent of baseline \( (r = 0.82, p < 0.05) \), (Fig. 48). Although the linear regression coefficient is statistically significant, it appears that the relationship may in fact be curvilinear (Fig. 48, dotted line). There seems to be a particular level of minute ventilation (approximately 30% of baseline) at which glutathione decreases linearly with the decrease in minute ventilation.
Fig. 40. Level of total and oxidized glutathione in GSH equivalents in respiratory muscles. Open bars represent total glutathione and solid bars, GSSG. Data is expressed as the mean ± 1 SD.

* \( p < 0.05 \); ** \( p < 0.01 \); *** \( p = 0.004 \).
Fig. 41. Total and oxidized glutathione in GSH equivalents in liver. Open bars correspond to total glutathione and solid bars represent GSSG. Means ± 1 SD are shown.
Fig. 42. Correlation between total diaphragm glutathione and the highest peak $P_i$ generated during the loading protocol.
Fig. 43. Correlation between total diaphragm glutathione and the largest $P$ (integrated $P_t$ during inspiration, over $T_{in}$).
Fig. 44. Correlation between total diaphragm glutathione and the maximum power output calculated from pressure-volume (P-V) curves. An almost significant positive correlation was found. Dotted lines correspond to 95% confidence interval.
Fig. 45. Correlation between total diaphragm glutathione and total P-V work, calculated adding the area under the P-V curve of all inspiratory efforts during the loading protocol. An almost significant positive correlation is shown. Dotted lines correspond to 95% confidence interval.
Fig. 46. Correlation between total diaphragm glutathione and the highest obtained PaCO₂.
Fig. 47. Correlation between total diaphragm glutathione and tidal volume ($V_T$) at the end of the protocol, expressed as percent of pre-load $V_T$. 

$r = 0.5145$
$p < 0.05$
Fig. 48. Correlation between total diaphragm glutathione and minute ventilation at the end of the loading protocol, as percent of baseline minute ventilation (solid line = linear regression; dotted line = possible curvilinear relationship).
<table>
<thead>
<tr>
<th></th>
<th>Total GSH</th>
<th>GSSG</th>
<th>GSSG / GSH\textsubscript{total}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol g\textsuperscript{-1}\ , wet weight</td>
<td>nmol g\textsuperscript{-1}\ , wet weight</td>
<td>( per cent )</td>
</tr>
<tr>
<td><strong>Diaphragm</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control Air (n= 7)</td>
<td>1,149.0 ± 142.0</td>
<td>45.0 ± 14.0</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>Control O\textsubscript{2} (n= 9)</td>
<td>969.0 ± 131.0</td>
<td>21.0 ± 27.0</td>
<td>5.0 ± 2.3</td>
</tr>
<tr>
<td>Experimentals (n= 6)</td>
<td>765.0 ± 231.0</td>
<td>29.0 ± 16.0</td>
<td>3.8 ± 2.4</td>
</tr>
<tr>
<td><strong>Intercostals</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control Air (n= 7)</td>
<td>690.0 ± 118.0</td>
<td>27.0 ± 12.0</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>Control O\textsubscript{2} (n= 9)</td>
<td>624.0 ± 98.0</td>
<td>30.0 ± 18.0</td>
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<tr>
<td>Experimentals (n= 6)</td>
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<td>2.0 ± 1.5</td>
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<td><strong>Liver</strong></td>
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<td></td>
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<tr>
<td>Control Air (n= 7)</td>
<td>3,881.0 ± 928.0</td>
<td>79.0 ± 26.0</td>
<td>2.28 ± 1.31</td>
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<tr>
<td>Control O\textsubscript{2} (n= 9)</td>
<td>4,375.0 ± 839.0</td>
<td>89.0 ± 43.0</td>
<td>1.97 ± 0.91</td>
</tr>
<tr>
<td>Experimentals (n= 6)</td>
<td>3,318.0 ± 462.0</td>
<td>74.0 ± 12.0</td>
<td>2.24 ± 0.17</td>
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## TABLE 15: SUMMARY OF THE PARAMETERS REFLECTING INTENSITY OF RESPIRATORY MUSCLE ACTIVITY

<table>
<thead>
<tr>
<th>Total Glutathione (nmol. g wet weight⁻¹)</th>
<th>Largest $\bar{P}$ (cm H₂O)</th>
<th>Largest Peak $P_T$ (cm H₂O)</th>
<th>Largest Power Output (J.min⁻¹)</th>
<th>Total P-V Work (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,070.0</td>
<td>12.0</td>
<td>48.0</td>
<td>0.331</td>
<td>53.4</td>
</tr>
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<td>953.0</td>
<td>7.8</td>
<td>48.0</td>
<td>0.24</td>
<td>36.0</td>
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<tr>
<td>635.0</td>
<td>10.5</td>
<td>43.0</td>
<td>0.116</td>
<td>19.2</td>
</tr>
<tr>
<td>436.0</td>
<td>8.63</td>
<td>50.0</td>
<td>0.160</td>
<td>19.3</td>
</tr>
<tr>
<td>828.0</td>
<td>8.0</td>
<td>50.0</td>
<td>0.242</td>
<td>32.4</td>
</tr>
<tr>
<td>669.0</td>
<td>11.0</td>
<td>57.0</td>
<td>0.274</td>
<td>49.2</td>
</tr>
</tbody>
</table>

| Mean 765.0 | 9.70 | 49.3 | 0.230 | 35.0 |
| S.D. 231.0 | 1.75 | 4.5  | 0.08  | 14.0 |
4. DISCUSSION.

In this study, we have used the measurements of glutathione content and redox status as indices of free radical generation in the respiratory muscles undergoing a prolonged period of resistive breathing. Our results show a decrease in diaphragm total glutathione of approximately 30% without an increase in GSSG. This decrease in glutathione correlates with the increase in PaCO₂ and the decrease in minute ventilation at failure, and does not seem to be determined by the intensity of respiratory muscle activity.

One of the interesting findings is the difference in total glutathione between the intercostals and the diaphragm in the control animals with the intercostals having only 60% of the diaphragm total glutathione. To our knowledge, there is no data in the literature regarding glutathione in intercostal muscles. This result could not be explained by a different level of blood contamination of the tissue samples, since differences in hemoglobin content between the two muscles were not of sufficient magnitude to account for the different levels of total GSH (unpublished observations). Differences in fiber type composition of the two muscles, on the other hand, could explain the results, since the diaphragm is known to be more oxidative than the intercostal muscles in the rat (18). Since the generation of ATP via oxidative metabolism is a normal source of reactive oxygen species, one could expect that antioxidant enzymes might have higher activities and glutathione might be present at higher concentrations in the more oxidative muscles of a given species. In support of this concept, there is evidence that both the level of
glutathione and the specific activity of the enzyme glutathione peroxidase are related to skeletal muscle oxidative capacity (8,19).

We found that the level of GSSG in control respiratory muscles is below 4% of the total glutathione. This is in agreement with a large body of literature showing that resting tissues have less than 5% GSSG (2,22), but differs from the level of GSSG of 22% reported by Anzueto et al. (4) for control diaphragm. We have found that storage of the tissue samples can affect the level of GSSG (unpublished observations). Thus in our study, all assays were run within 24 hours of sampling. Differences in storage time, as well as a different methodology (enzymatic vs HPLC) for the glutathione determination, may have accounted for the different results.

Oxygen administration alone was found to be associated with a reduction in total diaphragm glutathione of approximately 15%. An enhanced production of oxygen-centered free radicals could be expected under conditions of hyperoxia which would thus affect the glutathione cycle. Loaded animals breathing oxygen showed an additional reduction in total diaphragm glutathione of approximately 20%, thus causing a total decrease of 35% when compared to room air controls. While supplemental oxygen may have also affected total glutathione in the experimental group, its effect is likely to have been smaller than for the unloaded animals due to the decrease in minute ventilation which greatly reduced the PaO₂ in the experimental group. Thus, the loading protocol is likely to explain most of the changes in total diaphragm glutathione found in the experimental group.
We found that the drop in total diaphragm glutathione in loaded animals was not associated with an increase in GSSG. Our results differ from those of the 15 min. resistive breathing protocol of Anzueto et al. (4) in the magnitude of the glutathione depletion. A 65% reduction in total glutathione with normal GSSG and a high GSSG/GSH ratio were reported with a relatively short period of resistive breathing. The main differences between the two studies which could account for the different magnitude of change in total glutathione are: a) blood oxygenation, b) arterial blood pressure and c) time on the resistance. Oxygen was required by our experimental group in order to tolerate the long resistive breathing protocol. In our first attempts with the resistive breathing protocol we found out that without oxygen, high inspiratory resistances could only be tolerated for 10-20 min., due to the severe hypoxemia and hypotension that develop. In our study, anesthetized animals on oxygen were able to sustain very high levels of inspiratory resistance for 2.5 - 3 hours. Because high PaO₂ was maintained throughout the study, blood pressure was maintained above 100 mm Hg, presumably resulting in maintenance of blood flow to the working muscles. A difference in oxygen delivery to the diaphragm could explain the difference in magnitude of the glutathione loss between our study and that of Anzueto et al. (4). A decrease in oxygen delivery could affect ATP concentration in the muscle which could eventually contribute to the glutathione loss. For example, it has been shown in cultured cells that ATP is required to prevent GSH loss (23).

Loss in cell total GSH associated with an increase in GSSG and in protein-
mixed disulfides are constant findings in models of oxidant stress. Most of the GSSG is normally reduced back to glutathione by the enzyme glutathione reductase. However, under conditions of increased formation of GSSG, a fraction will undergo thiol/disulfide exchange with protein thiols, and part will be exported from the cell to plasma, lymph, or in the case of the liver, to bile (3,22,31), resulting in loss of total cell glutathione. We did not find an increase in GSSG. It is possible that it could have been formed and exported out of the diaphragm, or it may have reacted with intracellular proteins, accounting for the observed loss in total glutathione. However, the lack of an increase in GSSG with our experimental conditions, makes less likely the possibility that production of mixed disulfides could account for all the glutathione loss, since accumulation of GSSG has been reported to be necessary for mixed disulfide formation during oxidant stress (23). Rather, it is possible that some of the systemic effects of resistive breathing might have been responsible for at least part of the changes in diaphragm total glutathione. This is supported by the interesting correlations found between total diaphragm glutathione and the levels of minute ventilation and PaCO₂. At the point of respiratory failure, animals showed severe acidosis, mainly due to CO₂ retention. The significant correlation between PaCO₂ at failure and diaphragm total glutathione suggests the possibility that CO₂ influences the GSH cycle of the working muscle. However, the effects of hypercapnic acidosis on the glutathione cycle have not been explored. A potential effect of acidosis on the glutathione cycle is postulated in a recent study in isolated hepatocytes
undergoing oxidant stress. In that study, acidosis actually improved cell viability under oxidant stress, by decreasing the rate of NAD(P)H oxidation (9). If this mechanism is in any way relevant to \textit{in vivo} conditions in muscle, it is likely that it may contribute to decrease the level of GSSG because more NADPH will be available for GSSG reduction.

Another potential effect of acidosis and/or hypercapnia could be to alter the synthesis of glutathione. Synthesis takes place intracellularly by a two-step reaction requiring ATP and magnesium. Mammalian cells are provided with the two enzymes that are required: \(\gamma\)-glutamylcysteine synthetase and glutathione synthetase. Studies \textit{in vitro} have shown that optimum pH for these enzymes is in the range of 8.0 - 8.4, and that at pH = 7.0, there is more than 50% reduction in activity (21,25). Although we do not have a measurement of intracellular diaphragm pH, studies \textit{in vitro}, with resting mammalian diaphragm have shown that it can maintain intracellular pH between 6.9 and 7.0 under CO\(_2\)-induced acidosis with extracellular pH in the range 7.1 - 7.4 (1). Furthermore, studies of intramuscular pH at exhaustive exercise have shown skeletal muscle pH to be in the range of 6.46 - 6.98 with a blood pH between 7.18 - 7.40 (16). Considering the length of time the animals were exposed to the load and hypercapnic acidosis in our experiment, added to the intracellular acidosis generated in the loaded diaphragm, it is likely that intradiaphragm pH may have been well below 6.9, which would result in an important decrease in the activity of the glutathione synthetases.
Reduction in synthesis could be an important mechanism leading to reduction in cellular glutathione in the time frame of our experiment, provided there is a high turnover in the diaphragm. Glutathione turnover is known to be very fast in liver cells (turnover time = 2 hours) and much slower in other cells. We are not aware of data on glutathione turnover for either resting or exercising skeletal muscle.

Another alternative possibility for the loss in total diaphragm glutathione is that the changes in ATP, ADP and AMP in the working diaphragm could have contributed to the decrease in glutathione synthesis. A decrease in the ATP/ADP+AMP ratio, very likely to occur in our experiment, has been shown to decrease synthesis (29).

We found no effect of inspiratory muscle loading on either the glutathione content or its redox status in the parasternal intercostal muscles. It is possible that the propensity to generate free radicals during muscle contraction or the degree of intracellular acidosis may vary with the level of muscle activation, and that the intercostal muscles in this anesthetized model of respiratory failure might not have been activated as much as the diaphragm.

Glutathione transport from the liver is considered to be a mechanism for interorgan distribution of cysteine (33). Glutathione exported from the liver into the plasma is believed to be degraded in the kidneys providing cysteine moieties to be delivered to other cells or returned to the liver for resynthesis of glutathione. It has been shown that very exhaustive and prolonged exercise can lead to
glutathione depletion in the liver (20), and it has been suggested that the liver might be the major source of glutathione for the muscles during increased activity.

We found no significant changes in the liver GSH content or redox cycle with our model, suggesting that liver glutathione stores are not affected by this level of respiratory muscle activity. The most likely explanation might be that the respiratory muscles, and especially the diaphragm, constitute a relatively small muscle mass and therefore do not measurably affect liver glutathione.

Based on what has been reported in exercise, we expected to find a negative correlation between the level of glutathione and the intensity of respiratory muscle activity. Interestingly, animals performing more work were those with the highest level of glutathione. An explanation for this is not simple, but in fact, animals with the lowest minute ventilation prior to failure (and thus, with the lowest glutathione), were those performing less external work, suggesting that the level of ventilation allowed prior to failure is what more likely determined the level of glutathione in this model.

In conclusion, results of this study show a minor disruption of the diaphragm glutathione status as a consequence of resistive breathing, suggesting that respiratory failure seen with resistive breathing is not attributable to oxidant stress. We are not however, able to say whether or not the resistive breathing protocol is in itself an oxidant stress. Under our experimental conditions, the decrease in total glutathione occurred without a concomitant increase in GSSG, making less likely the possibility of oxidative stress as the cause of the perturbation in the
glutathione status. The fact that we did not find a strong negative correlation between total glutathione content and work of breathing or pressure time product suggests that increased respiratory muscle activity is probably not directly responsible for the glutathione loss. Interestingly, the significant correlations between total glutathione and both PaCO₂ and minute ventilation at failure suggest that some of the systemic effects induced by the resistive breathing protocol may be responsible for the decrease in total glutathione content in the loaded diaphragm.
5. LIST OF REFERENCES


1. INTRODUCTION.

There is current interest in investigating if free radicals play a role in respiratory muscle dysfunction associated with excessive mechanical loads. In support of this: a) there is both direct and indirect evidence of free radical involvement in exercise (1,5,11,13,17,20) b) it has been shown that the diaphragm is capable of producing oxygen-centered free radicals when stimulated electrically in vitro (12,21,22), c) the fatigue process in diaphragm and limb muscles electrically stimulated in vitro is attenuated by the use of free radical scavengers (18,21,22,25), and d) diaphragm glutathione, a major cellular antioxidant, decreases following resistive breathing, in vivo (2,7). No studies have attempted to directly detect free radical species following increased respiratory muscle activity in vivo.

Electron Spin Resonance (ESR) spectroscopy is one of the few techniques capable of the direct detection of free radicals. It measures the energy changes that occur as unpaired electrons align in response to an external magnetic field. A very small population of free radicals and other paramagnetic compounds
(transition metals) can be detected in samples composed predominantly of other substances. It has been widely used in the study of biological problems with isolated proteins and enzymes involving free radicals and paramagnetic metal ions (24). Using whole tissue ESR spectroscopy, a number of studies have provided direct evidence for a burst of free radical production during the early phase of reperfusion of the previously ischemic heart (3,14,27-29).

The purposes of this study are: a) to characterize the ESR spectrum of normal diaphragm under conditions of resting breathing and b) to evaluate the changes in the spectrum when increased diaphragm activity is induced in vivo by prolonged inspiratory resistive loading. Our hypothesis is that excessive contractile activity of the diaphragm in vivo, increases the production of free radical species which can be detected in quickly frozen tissue by ESR spectroscopy.

We found that increased diaphragmatic activity, induced by inspiratory resistive breathing, is associated with an increased formation of free radical species in the diaphragm. The magnitude of the signal is unrelated to the administration of high concentrations of oxygen in the inspired air and reverts to near control values when tested 10 minutes post-mortem.

2. MATERIALS AND METHODS.

2.1 Animals and care: Experiments were done in accordance with guidelines established by the Institutional Animal Care and Use Committee, at The Ohio State University. The study was carried out in male Sprague-Dawley rats, fed
standard stock chow diets, given water ad libitum and weighing 400 ± 100 g. All animals were anesthetized intraperitoneally with sodium pentobarbital (40 mg/kg initially plus 2 small supplements of 10 mg/kg) and randomly assigned to one of the following three groups:

a) the experimental group (n=10) which underwent incremental inspiratory resistive loading while breathing 70% oxygen for 2.5 - 3 hours,
b) a time-matched control group (n=10) consisting of animals examined for 2.5 - 3 hours while unloaded and breathing 70% oxygen (controls on oxygen),
c) a second control group (n=10) consisting of normal animals anesthetized just before sample collection (controls on air).

Seventy percent inspired oxygen was chosen for the experimental group in order to prevent the extreme hypoxemia and resultant systemic hypotension, which we observed in previous studies whenever the loading protocol was applied to animals breathing air. High levels of resistance could only be tolerated for 10 to 15 minutes without the use of oxygen. With the administration of 70% oxygen, the experimental group was able to undergo the resistive breathing protocol for periods longer than 2.5 hours with higher than normal PaO₂. This response to the resistive breathing protocol had previously been documented for the awake sheep model of inspiratory resistive breathing (4). Thus, control animals on 70% oxygen were studied to account for the possible effects of oxygen alone, which could independently result in the production of free radicals.
Every experimental animal had its two controls studied on the same day, under the same spectrometer conditions. Therefore, data was analyzed comparing each experimental result to its two control results for a particular day. This was necessary because of the observed day-to-day variations in instrument performance.

2.2 Measurements: A 2.2 mm internal diameter tracheal cannula was placed through a tracheostomy and connected to a two-way non-rebreathing valve (Model #2300, Hans Rudolph, Kansas City, MO). Tracheal pressure was continuously monitored by way of a side port in the tracheal cannula, which was connected to one side of a differential pressure transducer (Validyne, Northridge, CA). Inspiratory flow was measured with an in-line pneumotachograph, and inspiratory volume was obtained by integration of the flow signal.

A water-filled catheter, which was connected to a pressure transducer (Validyne, Northridge, CA) was inserted through the mouth and placed in the middle third of the esophagus in order to monitor esophageal pressure (Pes). The right carotid artery was cannulated for continuous blood pressure measurement. Rectal temperature was continually monitored with a thermistor and maintained at 38.5° C, using heating pads.

2.3. Protocol for incremental inspiratory resistive loading: At the beginning of each study, an estimation of the maximal inspiratory pressure (PImax) was obtained by occluding the inspiratory side of the 2-way valve for 30 seconds,
or until blood pressure fell to 80 mm Hg. The highest pressure attained was considered PImax. The inspiratory side of the 2-way valve was then attached to a variable inspiratory resistance consisting of a fine needle valve. The resistance was set initially to result in a generated tracheal pressure (Pt) of 30% of PImax for 10 minutes. Then, the resistance was increased stepwise by 10% of PImax at 10 min: intervals until tidal volume fell to 15-20% of its baseline value. This protocol yielded an average increase in Pt of approximately 3.5 cm H2O every 10 minutes. The animals then remained on the resistance for at least 2.5 hours before developing evidence of pump failure. Pump failure was defined as marked bradypnea associated with a sudden decrease in peak Pt without any change in applied inspiratory resistance. This was, in general, associated with a rapid decrease in arterial blood pressure. When blood pressure had declined to 60 mm Hg, the animals underwent immediate thoracotomy under mechanical ventilation, in order to obtain tissue samples.

2.4. Sample collection: Diaphragm samples were taken using a freeze clamping technique at liquid nitrogen temperature. Samples were obtained within 0.5 to 1.5 min. of opening the thorax and removal of the resistive load. During sample collection, the animals were mechanically ventilated while the heart maintained contraction. Four PVC-covered metallic clamps were used to collect flat samples approximately 1.5 x 8 mm in size (2 to 10 mg dry weight). Samples were collected in the same order in all animals and were kept in liquid nitrogen.
until they were transferred intact into an ESR dewar, with no mechanical processing such as chopping or grinding.

2.5. ESR measurements: All ESR spectra were obtained at liquid nitrogen temperature on a Varian E-9 X band spectrometer, equipped with an EPR-Ware™ data acquisition system. Three transients were averaged for each sample and corrected for any baseline signal present in the liquid nitrogen dewar. Conditions were: microwave power, 10 mW; frequency, 9.15 GHz; frequency modulation, 100 kHz; modulation amplitude, 5 G; central magnetic field set, 3,265 G; scan width, 400 G; scan rate, 100 G/min; time constant, 0.128 sec. At the end of each experiment, the diaphragm samples were placed in an oven at 98 °C, until a constant dry weight was obtained.

2.6. Statistical analysis: Data are presented as the mean ± 1 SD. ANOVA for repeated measures was used to analyze the data. When a significant F value was found, the Tukey post-hoc test was applied (26). The Wilcoxon test was used to identify differences between pre- and post-mortem data. A p value < 0.05 was considered statistically significant.
3. RESULTS.

3.1. General physiologic responses to inspiratory resistive breathing:

Peak Pt became progressively more negative with the increase in resistance, reaching a maximum of \(-52.7 \pm 8.5\) cm H2O in the first hour on the incremental load and remaining approximately at that level for the next 2 hours, until pump failure. Arterial blood pressure was maintained well above 100 mm Hg throughout the loading runs, until failure. Minute ventilation decreased progressively, from \(189 \pm 82\) ml/min to \(49 \pm 29\) ml/min at failure (29 \(\pm 22\)% of baseline minute ventilation). Averaged tracheal pressure, over the entire breath cycle (P) increased to 10 cm H2O.

3.2. ESR spectrum of normal diaphragm at 77K: All control diaphragm samples exhibited a well defined ESR spectrum (fig. 49), consisting of two components: a) a symmetric line at \(g = 2.004\) (similar to that found in heart and skeletal muscle) suggestive of a semiquinone radical (11,15,29) and, b) a signal at \(g = 1.94\), which has also been described in the heart and is believed to represent an iron-sulfur center (29).

These spectra were reproducible, with a coefficient of variation of 19%, which is somewhat lower than previously reported for other tissues (8). A direct linear relationship was found as expected, between the dry mass of diaphragm and the intensity of the ESR signal at \(g = 2.004\) (Fig. 50). Although not shown, a similar relationship was found for the \(g = 1.94\) line (\(r = 0.69, p < 0.01\)).
3.3. ESR spectrum of diaphragm after resistive breathing ("experimental diaphragm"): A typical spectrum is shown in fig.49-C. The ESR spectra are quite similar to those of the control diaphragm, with no additional lines. However, the signal intensity of the $g = 2.004$ line was consistently larger in "experimental diaphragms", while signal intensity of the $g = 1.94$ line did not change.

Three different quantitative approaches were chosen to evaluate the magnitude of the change in the two signals of the spectrum with resistive breathing, since ESR spectra varied somewhat from day to day depending on instrument performance:

3.3.1. the absolute signal intensity approach: four samples obtained with 4 different forceps that give slightly different sample sizes were studied for each animal. For each sample, an averaged spectrum of three scans was obtained. Then, the grand mean of the signal intensity from the 4 averaged sample spectrums was used to compare control and experimental diaphragms. Signal intensity from peak to peak in cm was used for comparison since the average weight of the 4 samples taken from the same animal was not significantly different between controls and experimentals.

3.3.2. the intensity ratio approach: the averaged signal intensity in an experimental animal was normalized by the averaged signal intensity for the control studied on the same day.

3.3.3. the intra-sample normalization approach: the signal intensity ratio for the $g = 2.004$ line to the $g = 1.96$ line was also used, since the latter signal did
not change with the resistive breathing protocol but changed directly with sample size. Therefore, the signal at $g = 1.94$ was considered an indirect method of normalizing for sample size.

Overall, these results showed that: a) the signal intensity in experimental diaphragms was significantly higher than in both controls on air and controls on oxygen ($p < 0.01$), fig. 51; b) the normalized intensity for the $g=2.004$ line (experimental/control) was always greater than 1, as shown in Fig. 52 (a signal ratio=1 means no difference between control and experimental), c) the intensity of $g = 2.004$ signal / intensity of $g = 1.94$ signal was also significantly higher in experimental than in control diaphragms ($p < 0.01$ when compared to controls on air and $p < 0.05$ when compared to controls on oxygen, fig. 53). With any of these three approaches the standard deviation in the experimental group was much larger than in the two control groups (19% coefficient of variation in controls), reflecting the different intensity of respiratory muscle activity.

3.4. Relationship between index of respiratory muscle activity and intensity of signal at $g$ value = 2.004: A significant positive correlation was found between the largest averaged tracheal pressure per breath ($\bar{P}$) and both signal intensity and intensity ratio ($p < 0.05$, Fig. 54). Averaged tracheal pressure refers to the integrated tracheal pressure distributed over the entire respiratory cycle. This parameter is generally considered a good reflection of respiratory muscle oxygen consumption (23).
3.5. **Effect of oxygen administration on the ESR spectrum of control diaphragm:** a) signal intensity in the diaphragm of control animals breathing 70% oxygen and anesthetized for the same period as the experimental group was no different than the signal intensity in controls breathing air and studied immediately after induction of anesthesia (Fig. 51); b) control oxygen / control air intensity ratio was close to one. As a group average, controls on oxygen had 99% of the intensity of the signal seen in controls breathing air (Fig. 52-B). These results demonstrate that oxygen administration alone and/or prolonged anesthesia have no effect on the diaphragm ESR spectrum.

3.6. **Effect of death on the ESR signal at g value = 2.004 (Fig. 55):** We studied the diaphragm of two experimental animals (not included in the analysis) whose hearts inadvertently stopped beating 2 to 8 minutes prior to sampling. In these animals we found no increase in intensity of the signal at g = 2.004. To determine whether these animals should be legitimately excluded from the analysis, we followed the g = 2.004, signal post-mortem for 10 minutes after surgical removal of the heart, in the last 5 animals in each group. We found that the g = 2.004 signal measured 10 minutes post-mortem decreased 9 to 45 per cent (mean 25% ) from the pre-mortem signal (p < 0.05) in the experimental group, approaching values seen in control diaphragms. The g = 2.004 signal remained the same or slightly increased in both control groups.
Fig. 49. The ESR spectrum of frozen diaphragm obtained at 77°K. A: is a typical spectrum of diaphragm from control animals breathing air, B: corresponds to that of a control breathing 70% oxygen, and C is the spectrum of the diaphragm from an animal having undergone resistive loading. Spectrometer conditions: microwave power: 10 mW, frequency: 9.15 GHz, 100 kHz frequency modulation, 5 G modulation amplitude, central magnetic field: 3.265 G, scan width: 400 G, scan rate: 100 G/min, time constant: 0.128 sec.
Fig. 50. Linear regression for the relationship between sample size (dry weight) and intensity of the signal at $g = 2.004$. Dark squares correspond to controls breathing air, whereas dark triangles correspond to controls breathing oxygen. Continuous line corresponds to the linear regression for both control groups ($r = 0.72, p < 0.01$). Open circles represent samples from experimental animals and the dotted line is the regression line for those points ($r = 0.40, p < 0.05$).
Fig. 51: Intensity of the signal at $g = 2.004$. Bars represent means ± 1 SD ($n = 10$). **: $p < 0.01$. 
Fig. 52: Signal intensity at $g = 2.004$.

A. Intensity in experimental/intensity in control on air

B. Intensity in control on oxygen/intensity in control on air.

Bars represent the ratio for individual experiments. Ratio $=1$ means no difference between control and experimental animals.
Fig. 53: Signal intensity at $g = 2.004$, normalized by signal at $g = 1.94$. Bars represent means ± 1 S.D. ($n = 10$)
Fig. 54: Relationship between the largest averaged tracheal pressure (P) and intensity ratio (experimental/control on air) of signal at $g = 2.004$. 

$r = 0.67$

$p < 0.05$
Fig. 55: The effect of death on the signal intensity at $g = 2.004$. Signal intensity pre and post-mortem is shown for controls on air ($n = 5$), controls on oxygen ($n = 5$) and experimentals ($n = 5$). Open bars: pre-mortem. Solid bars: post-mortem. *: $p < 0.05$. 
4. DISCUSSION.

In this study we have applied ESR spectroscopy to directly detect the presence of free radicals in the diaphragm following *in vivo* loading. We found an increase of approximately 30% in the signal at $g = 2.004$ with the resistive breathing protocol. The reduction in signal intensity seen 10 minutes post-mortem in animals previously exposed to the load supports the proposition that the increase in signal seen immediately after loading represents free radical species, which by nature are very labile.

ESR spectroscopy allows the direct detection of free radical species in biological phenomena. The use of frozen samples at liquid nitrogen temperature has helped in overcoming some of the problems of studying tissue with ESR, such as the water content of the samples, and the stability of organic free radicals (24). A large body of literature has accumulated regarding ESR spectral changes in the heart with reperfusion after ischemia, providing direct evidence for the involvement of free radicals in this condition. However, much less is known concerning the effects of various physiological states on the ESR characteristics of skeletal muscle (11,15) and nothing is available on respiratory muscles. Jackson et al. (15) examined the ESR spectra obtained from normal resting mouse gastrocnemius and human abdominal muscles, reporting a signal at $g = 2.0036\text{--}2.004$ at liquid nitrogen temperature, that disappeared at room temperature. Davies et al. (11) described a similar ESR signal in rat skeletal muscle that increased with exercise. Jackson also observed that 30 min of excessive contractile
activity of rat hind limb muscles was associated with an increase in the intensity of the g = 2.004 signal, providing support to a large body of research suggesting the involvement of free radicals species in exercise (1,5,11,13,17).

A possible role for free radical species in the respiratory muscle dysfunction that accompanies excessive muscle overload has recently been suggested by indirect evidence from studies both in vitro and in situ (18,21,22,25) and from the reports of a decrease in total diaphragm glutathione with resistive breathing (2,7).

The current study shows that the ESR spectrum of the normal diaphragm is very similar to that reported for other skeletal muscles (11,15) and heart (3,29) and establishes that a linear relationship exists between diaphragm mass and intensity of the two main ESR signals. This relationship has been shown previously for the g = 2.004 signal in the heart (3) and illustrates the need for normalized data when interpreting changes in tissue ESR signals. Another interesting finding in this study is the lack of effect of breathing high oxygen concentrations on the diaphragm ESR spectrum. Our results are in agreement with previous work showing that the administration to an animal of high concentrations of oxygen in the inspired air for prolonged periods of time, has no effect on tissue ESR spectrum (24). It has been shown, however, that oxygen does have an important effect increasing the intensity of the signal at g value = 2.004 when it is bubbled while tissue is ground or chopped under liquid nitrogen, due to the reaction of molecular oxygen with radicals generated by mechanical bond breakage (14,29).

We have shown that diaphragm overload induced by resistive breathing is
associated with an increase in the \( g = 2.004 \) signal, in the same way that electrical stimulation induced an increase in signal in Jackson's study (15). In addition, a significant positive correlation exists between one indicator of the magnitude of respiratory muscle activity (P) and signal intensity.

Despite the ubiquity of the \( g = 2.004 \) signal, its nature in normal muscle is not completely understood. It has been suggested that this line comes mainly from a semiquinone type of molecule present in the mitochondria and is believed to represent a combination of free radical species and other stable paramagnetic species, since it persists in tissue even under conditions in which cell metabolism stops. (8,16,24).

Although skeletal muscle has many potential sites of free radical generation (ie. mitochondria, peroxisomes, sarcoplasmic reticulum sarcolemma), increased electron transport chain activity in the mitochondria has been postulated as the primary site for free radical generation during increased muscle activity. At least two percent of the total mitochondrial oxygen consumption is estimated to go to the formation of superoxide anion from the interaction of various mitochondrial components with molecular oxygen, linking the production of oxygen-centered free radicals to cellular metabolism (9). The use of electron transport inhibitors has allowed the identification of possible sites of oxygen-centered free radical generation in the mitochondria, as flavoproteins, coenzyme Q and cytochrome b. A large body of research has supported the semiquinone form of coenzyme Q as the primary compound responsible for interaction with molecular oxygen, forming
superoxide anion during both normal and increased electron transport chain activity (6). However, an antioxidant role for coenzyme Q has also been postulated (6,10) and a recent study in vitro has suggested that coenzyme Q in normal mitochondria may not be capable of interacting directly with molecular oxygen (19).

In reperfused hearts after ischemia, investigators have shown, using ESR spectroscopy of frozen tissue associated with temperature annealing studies, that a signal at \( g = 2.003 \), corresponds to oxygen-centered free radicals. This is a temperature labile signal that decreases when superoxide dismutase is used prior to reperfusion (14). It has been shown that although the intensity of this signal increases with reperfusion, it is also dependent on the extent of mechanical fracturing of the tissue and the concentration of oxygen bubbled directly during tissue fracture (29). Another study, was not able to identify this signal in ischemia-reperfusion studies of the heart when tissue fracturing was reduced to a minimum (3). We have not been able to identify that signal in this study, using unfractured, flat diaphragm samples, once the spectrum of liquid nitrogen alone was subtracted from the sample spectrums. This could be due to the small muscle mass used (2-10 mg dry weight) and the fact that there was no muscle grinding.

The ESR signal at \( g = 2.004 \) in normal tissue is known to be relatively stable (8,24). However, the stability of the signal induced by increased muscle activity is not known. We have shown that the increase in ESR signal intensity in the loaded diaphragms reverses and is no longer seen in samples taken 10 min
after death. In fact, two experimental animals that died 5 to 8 minutes before diaphragm samples were taken, did not show an increase in signal after resistive breathing. At 10 min post mortem, signal intensity in loaded diaphragms had returned to the level seen in control diaphragms, while the signal decreased in none of the control samples. This suggests that a critical feature in this type of study is the time that elapses between intense contractile activity and sampling.

In conclusion: a) the normal frozen unloaded diaphragm exhibits the same ESR characteristics as described for other skeletal muscles and for the heart, b) the intensity of the free radical signal is directly related to muscle mass and independent of the use of high concentrations of oxygen or prolonged anesthesia, c) the ESR free radical signal increases with increased diaphragm activity in a linear fashion, and this increase is completely reverted 10 min post mortem. Since ESR of frozen tissue does not allow the identification of the specific free radical species involved, it is not possible with this data to distinguish between an increase in common reactive metabolic intermediates and an increase in the formation of oxygen-centered free radicals in the loaded diaphragm. Further studies, using ESR technology associated with spin trapping, as used in the heart, may help to identify the types of free radical species involved and their role under conditions of respiratory muscle overloading in vivo.
5. LIST OF REFERENCES.


To keep blood gases within acceptable limits, air must be moved rhythmically in and out of the lungs. This activity is accomplished through the expansion and deflation of the thorax, which results from the coordinated action of a number of skeletal muscles. The diaphragm and the parasternal intercostal muscles, generally co-activated during inspiration, represent the most important inspiratory muscles. They play an important role in the clinical manifestations of different disease states. For instance, respiratory muscle dysfunction is known to play a primary role in the development of ventilatory failure in patients with neuromuscular disorders. Chronic obstructive pulmonary disease, a highly prevalent condition, is associated with pathophysiologic conditions which place the respiratory muscles at mechanical disadvantage. These conditions result in excessive effort relative to the strength and endurance of the respiratory muscles, which very likely contributes to the development of ventilatory failure. It is also likely that respiratory muscle dysfunction contributes to the difficulties that some patients encounter when being weaned from mechanical ventilation (4).

Although substantial progress has been made in understanding the function
of the respiratory muscles in both physiologic and pathophysiologic conditions, much remains unknown regarding the cellular mechanisms involved in respiratory muscle dysfunction.

The model of respiratory failure induced by incremental inspiratory resistive loading which was developed in these studies was intended to contribute to the understanding of respiratory failure. It creates conditions of mechanical disadvantage to the respiratory muscle and a pathophysiologic environment which are similar to what is seen in patients with respiratory failure. The model has provided an opportunity to study the contribution of the changes in the metabolism of oxidant and antioxidant species to the respiratory muscle dysfunction associated with excessive workloads. Although this model of respiratory failure is developed in an anesthetized animal, the results of the physiologic studies differ very little from those obtained with larger awake animals (1,3). The use of incremental loading associated with the administration of supplemental oxygen, provides the opportunity of stressing the muscles for a relatively prolonged period of time, allowing the adjustments to the loads to develop gradually. A complete characterization of the physiologic response obtained with this model has been done in the same way it has been done with other models. The major emphasis however, was put on the quantitation of the external work performed by the respiratory pump, in order to be able to correlate the metabolic responses with the work performed by the muscles. Most of the mechanical changes and acid base disturbances induced by the protocol are a reflection of the changes often seen in
disease states; therefore, this model represents a relatively good approximation to conditions of respiratory failure seen with lung or thoracic diseases.

The status of the high energy phosphate compounds in the respiratory muscles was examined to determine if changes in their levels could be responsible for the respiratory dysfunction at high workloads. Very small changes in phosphocreatine and almost no change in the adenine nucleotide pool were found in both diaphragm and intercostal muscles, indicating that the development of pump failure is not associated with significant changes in the concentration of high energy phosphates. In addition, any cell redox change is probably not attributable to lack of high energy phosphates.

The main objective of this study was to characterize the response of some of the main antioxidant systems available to the respiratory muscles, in response to the overloading conditions. Since no data is available in the literature regarding the antioxidant systems of the respiratory muscles, a complete characterization of the antioxidant enzyme systems in the animal model was required prior to the study of the changes induced by overloading conditions. Large differences in the glutathione level and in the specific activity of all the antioxidant enzymes were found between diaphragm and intercostal muscles. These differences are not explained by the different level of blood contamination present in the muscle samples. The results obtained in the respiratory muscles were analyzed in the context of the results obtained in muscles of known different oxidative capacity, like the soleus, a predominantly oxidative skeletal muscle, and the gastrocnemius,
a muscle with a mixed population of fibers. It became clear that there is a strong relationship between muscle oxidative capacity and antioxidant systems, which is likely to explain most of the differences in antioxidant capacity between the diaphragm and the intercostal muscles. This relationship, highly significant for the glutathione cycle and less significant for catalase and SOD, was found to exist when analyzing antioxidants as a function of fiber type composition or the specific activity of the oxidative enzyme, succinate dehydrogenase.

Although changes in activity of the antioxidant enzymes of the magnitude seen with exercise training were not expected, because of previous reports of changes in enzyme activity in limb muscles in acute exercise, the responses of the antioxidant enzymes to the resistive loading protocol was studied. The resistive loading protocol did not affect the specific activity of the antioxidant enzymes.

Despite the fact that antioxidant enzyme activities did not change, glutathione, one of the main cell antioxidants showed a decrease of approximately 30%, following the resistive loading protocol. This decrease in total glutathione was not accompanied by an increase in oxidized glutathione. This finding, added to the fact that the changes in glutathione were unrelated to the level of contractile activity developed by the respiratory pump, and that the level of glutathione is linearly related to the level of acidosis, CO₂ retention and hypoventilation experienced with the loading protocol, suggests that at least part of the changes in glutathione in the working diaphragm could be due to the systemic effects of the load. That is, the acidosis or CO₂ retention associated with the loading protocol
may have played a contributory role to the changes in glutathione.

One of the working hypotheses was that the loss of glutathione could be secondary to drops in available high energy phosphates. However, the level of high energy phosphates was not significantly altered, and therefore, it is unlikely that the loss in glutathione is due to lack of energy substrates.

For whatever mechanism glutathione was lost from the muscle cells, the mild disruption of the glutathione cycle observed in this study cannot be used as evidence of severe oxidative stress associated with respiratory failure. Furthermore, it is unlikely that the loss in glutathione, in any way contributed to failure of the muscle pump.

It is possible to speculate, however, that because of the importance of glutathione in cellular defense mechanisms, depletion of glutathione associated with ventilatory failure induced by resistive breathing may increase the susceptibility of the diaphragm to oxidant stress induced by other mechanisms.

The response of the antioxidant systems to increased respiratory muscle overload represents an indirect way to determine a possible role for oxidant stress in the production of respiratory muscle dysfunction. A more direct method of detecting involvement of free radical species was also utilized. ESR spectroscopy was used to directly detect the presence of free radical species in the diaphragm. An increased formation of free radical species was observed to be linearly related to the intensity of respiratory muscle contractile activity.

In summary, the respiratory muscles appear to be particularly well suited
to protect themselves from oxidative stress by having an abundant source of antioxidants. This protection appears to be related to the muscle oxidative capacity. In the animal model developed to study clinically relevant conditions of respiratory failure, it appears that the diaphragm produces oxidant species which can be detected by ESR studies of frozen tissue. At the same time, most of the protective antioxidant systems are preserved, but there is a significant drop in total glutathione. Where diaphragm glutathione goes remains unknown, but it is unlikely that the relatively small decrease in glutathione represents evidence of a serious impingement on the antioxidant reserve of the muscles. These studies do not provide evidence that oxidant stress plays a direct role in respiratory muscle dysfunction associated with failure. However, oxidative stress may be occurring concurrently and perhaps under other experimental conditions may play a more important role.

The physiologic data from the experimental model suggests that ventilatory failure and associated cardiovascular collapse may occur prior to conditions in which the muscles themselves have reached contractile failure. The relative preservation of high energy phosphates in the muscles at failure is in support of this notion.

The relevance of the information provided by these studies is that although oxidant stress and even injury can be observed in different types of in vitro preparations, following intense muscle stimulation, it appears that under in vivo conditions, these mechanisms may be of less importance than is currently believed.
Secondly, by comparison with results from another *in vivo* model of respiratory failure in the rat (2), it is now clear that changes in antioxidant systems associated to respiratory failure *in vivo*, are not solely due to the intensity of muscle contraction, but may more likely involve conditions of circulatory collapse, hypoxemia and suffocation associated with the model used to study respiratory failure.

**LIST OF REFERENCES.**


