Occasional review

Respiratory muscle function and free radicals: from cell to COPD

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Respiratory muscle dysfunction in chronic obstructive pulmonary disease (COPD)

Dysfunction of the respiratory muscles, especially the diaphragm, is known to occur in patients with severe chronic obstructive pulmonary disease (COPD).[1–3] Weakness of the diaphragm is part of a generalised process involving all (respiratory and peripheral) skeletal muscles. Causative factors for respiratory muscle dysfunction in COPD include disturbances in electrolytes,[4] hypercapnia,[5] forward failure,[6] and prolonged use of oral corticosteroids.[7] In addition, the altered geometry of the thorax in severe emphysema compromises the ventilatory pump function of the diaphragm.[8] Malnutrition, which frequently occurs in moderate to severe COPD,[9] could also play a part in respiratory muscle dysfunction. Recent studies have indicated that wasting of fat free mass in COPD is associated with peripheral skeletal muscle weakness.[10] However, few data are available regarding the effects of malnutrition on respiratory muscle strength. Maximal inspiratory pressure (Pimax) in nutritionally depleted patients with COPD (forced expiratory volume in one second (FEV_1) 45.5 (15.1)% predicted) was lower than in non-depleted patients, but this did not reach statistical significance.[9]

Little is known about the underlying mechanisms of muscle dysfunction and the structural alterations that occur in the diaphragm with COPD. Levine *et al*[11] have shown that the diaphragm in patients with severe COPD (FEV_1 33 (4)% predicted) has a higher proportion of type I (slow) fibres and a lower proportion of type II (fast) fibres than in those without COPD. It has recently been shown that a strong correlation exists between pulmonary functional residual capacity and the proportion of slow myosin heavy chain fibres in the diaphragm.[12] This fast to slow fibre transition in the diaphragm can be regarded as an advantageous adaptation since it will attenuate fatiguability of the diaphragm.[13] However, eventually most patients with COPD die from respiratory muscle failure. Apparently, at some point clinical relevant respiratory muscle dysfunction occurs in COPD. It has been shown that, besides slow to fast fibre transition, other processes also occur in the diaphragm. For instance, Campbell *et al*[14] found that in 17 of 22 patients with none to moderate airway obstruction, morphological changes were present in the intercostal muscles (variation in fibre size, splitting and atrophy) but not in the latissimus dorsi muscle. Fibre atrophy was significantly correlated with airway obstruction. Hards *et al*[15] also found evidence for morphological abnormalities in the internal and external intercostal muscles of patients with mild COPD.

Respiratory muscle dysfunction contributes to dyspnoea and the onset of hypercapnia. The reduction in peripheral and respiratory muscle function contributes to reduced exercise tolerance.[2] Generalised muscle weakness in these patients has been recognised as a main cause of health care utilisation.[16]

Skeletal muscles generate free radicals at rest and production increases during contractile activity.[17 18] Overproduction of free radicals may result in a disturbance between the pro-oxidant and antioxidant balance in favour of the former, and is called oxidative stress. This phenomenon has been found to occur in skeletal muscle under circumstances such as skeletal muscle fatigue and sepsis induced muscle dysfunction.[20 21] A large body of literature indicates that oxidative stress impairs skeletal muscle contractile performance.[18 22–25]

The chronically increased load imposed on the diaphragm in severe COPD may enhance generation of free radicals which, in turn, may further impair contractility of the diaphragm. In this review we will summarise current knowledge on the role of free radicals in respiratory muscle dysfunction and discuss the relevance to patients with COPD. Possible strategies to modulate antioxidant defences in vivo will also be reviewed.

Free radicals and antioxidants in striated muscle contractility

Free radicals are molecules capable of independent existence which contain one or more single electrons in an orbital. Since electrons are usually more stable when paired, radicals are generally more reactive than non-radicals.[26] A frequently used term is reactive oxygen species (ROS) which represents oxygen centred free radicals such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($HO^{\bullet}$) and intermediates in free radical reactions such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

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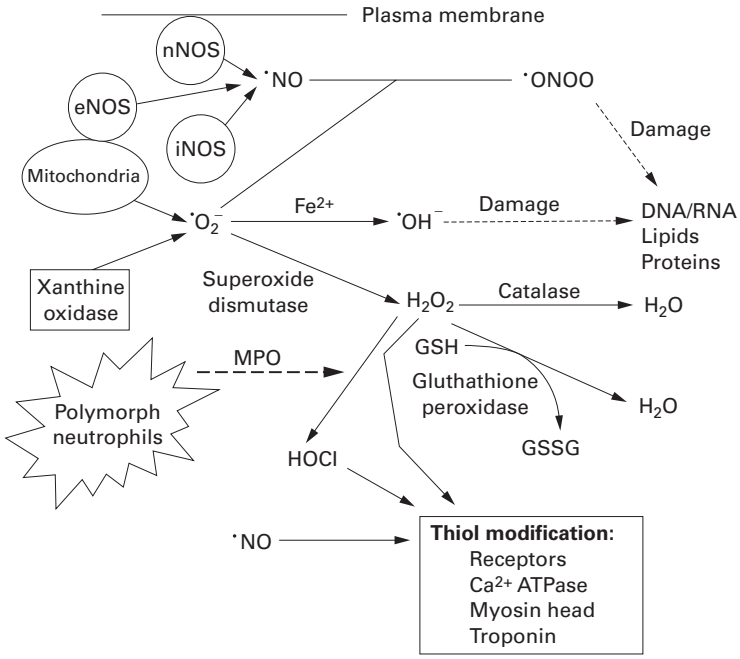

Figure 1 Schematic representation of formation of free radicals in skeletal muscle. Important sources for superoxide ($O_2^{\bullet -}$) include the mitochondrial respiratory chain and cytosolic xanthine oxidase (XO). Superoxide can be converted to the very reactive hydroxyl radical ($^{\bullet}OH^-$), or react with nitric oxide ($^{\bullet}NO$) resulting in the formation of peroxynitrite (ONOO). These free radicals may induce damage to DNA, RNA, proteins, or induce free radical chain reactions in lipid membranes. Free radicals may also induce reversible oxidation of thiols, resulting in modification of receptor/protein function. See text for further explanation. nNOS = neuronal type nitric oxide synthase; eNOS = endothelial type NOS; iNOS = inducible NOS; H_2O_2 = hydrogen peroxide; MPO = myeloperoxidase; HOCl = hypochlorous acid; GSH = reduced glutathione; GSSG = oxidised glutathione.

Nitric oxide (NO) is a well known example of a nitrogen centred free radical.

The observation that increased contractile activity enhances generation of free radicals in striated muscle prompted investigators to study the effects of free radicals on skeletal muscle function and excitation-contraction coupling in particular. The latter is defined as the process of coupling chemical and electrical signals at the cell surface to the intracellular release of calcium (Ca^{2+}) and ultimately contraction of muscle fibres.[27] Briefly, upon binding of acetylcholine (ACh) to its receptor on the sarcolemma, an action potential is generated. This action potential activates voltage sensitive receptors in the T tubules. These so-called dihydropyridine receptors (DHPR) are mechanically coupled to ryanodine receptors (RyR) present on the sarcoplasmic reticulum (SR). Activation of the DHPR induces conformational changes in this receptor, thereby opening the RyR and resulting in release of Ca^{2+} from the SR. Increased levels of cytosolic Ca^{2+} ($[Ca^{2+}]_i$) stimulates other RyR not directly coupled to DHPR to release Ca^{2+} from the SR. This positive feedback mechanism is called calcium-induced calcium release. Increased $[Ca^{2+}]_i$ enhances binding of Ca^{2+} to the troponin complex. The latter is the binding site for Ca^{2+} on the contractile proteins and is associated with the actin filament. Binding of Ca^{2+} to troponin is thought to induce a conformational change of troponin resulting in an increase in the availability of myosin binding sites on actin, initiating contraction by facilitating cross-bridge cycling—that is, the cyclic interaction between actin and myosin. Contraction is terminated when $[Ca^{2+}]_i$ is resequestered in the SR by Ca^{2+} ATPases.

Free radicals can interfere with excitation-contraction coupling at several sites. By isolating the SR from skeletal muscle fibres it was found that free radicals affect Ca^{2+} release through the RyR in a dose dependent fashion. Submillimolar concentrations of H_2O_2 activate the RyR, whereas at millimolar concentration H_2O_2 inhibited channel activity.[28] Similar studies revealed that NO inhibits Ca^{2+} release via the RyR channel.[29] As with ROS, the effects of NO are concentration-dependent; at low concentrations NO prevents the RyR channel opening whereas higher concentrations activate the channel.[30] The ability of NO to decrease SR Ca^{2+} release has also been shown in intact fibres.[31] Free radicals exert their effects on SR Ca^{2+} release by modifying thiol groups present on the RyR.[29 30] Other possible targets for free radicals in excitation-contraction coupling include hyperreactive thiol groups present on the myosin head,[32] reactive cysteine residues present on the troponin complex,[33 34] and Ca^{2+} ATPases.[35] Free radicals may also reduce the amplitude of action potentials.[36]

The effects of free radicals generated by skeletal muscle fibres do not only affect excitation-contraction coupling but also other physiological processes. Free radicals affect mitochondrial respiration through competitive interaction with the oxygen binding site of cytochrome oxidase[37] and they affect insulin-independent glucose uptake in muscle fibres.[38] NO generated by skeletal muscle is assumed also to play a role in exercise induced vasodilatation.[39] In addition, free radicals are involved in many cellular process including apoptosis, inflammation, and gene regulation.[40 41] For instance, recent in vitro studies have shown that free radicals are needed for tumour necrosis factor (TNF)-α induced activation of nuclear factor kappa-β (NF-κβ).[42] Although these processes are potentially relevant to respiratory muscle function in COPD, the present review will focus on the direct effects of free radicals on excitation-contraction coupling.

SOURCES AND CHEMICAL PROPERTIES OF FREE RADICALS

Figure 1 shows schematically the well known sources for free radicals in skeletal muscle. The electron transport chain in the mitochondria is an important source for the formation of ROS.[43 44] One to two percent of electron flow "leaks" onto O_2 to form $O_2^{\bullet -}$.[45] Consequently, it has been proposed that increased oxygen consumption results in increased generation of ROS.

Cytosolic xanthine oxoreductase (XOR) has a role in purine nucleotide degradation. This enzyme can be present as the dehydrogenase form (XD) or the oxidase form (XO). Under physiological conditions XOR mainly exists as the former, which uses NAD^+ for electron transfer resulting in the formation of NADH. In contrast, XO uses O_2 for electron transfer resulting in the formation of superoxide:

$$\text{xanthine} + H_2O + 2O_2 \xrightarrow{XO} \text{uric acid} + 2O_2^{\cdot -} + 2H^+ \quad (1)$$

Generation of superoxide by XO plays an important role in ischaemia/reperfusion injury.[46] Expression of this enzyme has been seen in the peripheral and respiratory muscles of rodents[47 48] and in the peripheral skeletal muscle of humans.[49–51] XOR expression in the human respiratory muscles has not yet been confirmed.

Polymorph neutrophils (PMNs) can generate the extremely potent pro-oxidant hypochlorous acid (HOCl). The reaction involves the myeloperoxidase (MPO) catalysed oxidation of Cl^- ions by H_2O_2.[52] PMN infiltration is increased in skeletal muscle after prolonged exercise.[53] Moreover, the potential of PMNs to form ROS is increased after exercise in humans.[54] Thus, under certain conditions free radicals generated by PMNs may induce damage to skeletal muscle fibres. Other sources of free radicals in skeletal muscle include the cytosolic enzyme aldehyde oxidase[44] and the arachidonic acid cyclo-oxygenase pathway.[55]

NO is generated enzymatically by nitric oxide synthase (NOS). Three isoforms of NOS (types I, II, and III) have been identified. Types I and III, neuronal (nNOS) and endothelial NOS (eNOS), respectively, are present in rat[56] and human[57] skeletal muscle. In rodents type I NOS expression is higher in fast than in slow twitch skeletal muscle fibres.[24] Type III NOS co-expresses histochemically with mitochondrial markers,[57] indicating expression in proximity to mitochondria.

Activation of types I and III NOS is dependent on increased $[Ca^{2+}]_i$. While types I and III are constitutive, type II NOS is an inducible form (iNOS). The latter probably plays a part in endotoxin induced muscle dysfunction since it has been shown that injection of *E coli* endotoxin induced expression of iNOS in the diaphragm of mice.[58]

The formation of very reactive free radicals in vivo is of special interest. In the so-called "Fenton reaction" iron (Fe^{2+}) dependent decomposition of H_2O_2 results in the formation of a hydroxyl radical[59]:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\cdot -} + OH^- \quad (2)$$

The Fenton reaction is of importance since it involves the conversion of a moderate reactive free radical into an extremely reactive free radical.[59] The formation of a very reactive free radical from a less reactive one also occurs in the following reaction[60]:

$$O_2^{\cdot -} + {}^{\cdot}NO \longrightarrow ONOO^- \quad (3)$$

Since the rate constant of superoxide to NO is many times higher than the rate constant of superoxide to its endogenous antioxidant superoxide dismutase (SOD),[60] the formation of peroxynitrate ($ONOO^-$) in vivo is likely. These very reactive free radicals are potentially hazardous to normal cell function because of their ability to react with vital cellular components such as lipids, proteins, DNA, and RNA.

Cigarette smoke is a rich source of free radicals, containing over 10^{15} organic radicals and 500 ppm NO per puff .[61] Most of these free radicals are highly reactive and short lived (<1.0 s). Because of the short half life and strong antioxidant capacity of the epithelial lining fluid and blood, it is unlikely that free radicals derived from cigarette smoke directly alter skeletal muscle function. However, it has been shown that circulating neutrophils from smokers have an enhanced oxidative burst.[62] As mentioned above, exercise is known to recruit neutrophils to contracting skeletal muscle.[53] Thus, it is possible that skeletal muscles of smokers have a higher oxidative load than those of non-smokers. Also, a reduction in total antioxidant capacity has been found in the plasma of smokers.[63] It could be speculated that an impaired antioxidant screen as a result of smoking may compromise muscle function. However, no studies have looked at this pathway.

ANTIOXIDANT DEFENCES

In skeletal muscle protection from the deleterious effects of free radicals is provided by many strategies that aim to inhibit the propagation of free radical reactions. An antioxidant is any substance which, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate.[64] Glutathione (L-γ-glutamyl-L-cysteinylglycine) is an abundant and ubiquitous antioxidant. Its antioxidant power is closely associated with its role in providing the cell with its reducing milieu. Intracellular glutathione metabolism is regulated by complex pathways.[65] Reduced glutathione (GSH) serves as an antioxidant by reacting directly with free radicals and by providing substrate for glutathione peroxidase (GPX). Both direct and enzymatic oxidation of GSH results in the formation of oxidised glutathione (GSSG), which is reconverted to GSH by glutathione reductase (GR). Thus, this latter enzyme catalyses equilibrium between GSH and GSSG that greatly favours the former.[65] The intracellular ratio of GSSG/GSH, a common ratio to express the degree of oxidative stress,[66] is therefore usually kept low (±5%) to maintain a reducing state of the cytosol. Thus, tissue glutathione status depends on the direct interaction of GSH with oxidants, the activity of the GPX and GR redox cycle, and on the ability of tissues to synthesise GSH.

In skeletal muscle the glutathione concentration is correlated with oxidative capacity. In the rat soleus muscle (predominantly oxidative fibres) the GSH concentration is higher than in the vastus lateralis muscle (predominantly glycolytic fibres).[67] As with GSH, activity of GPX, GR, and catalase are higher in highly oxidative skeletal muscle fibres than in fibres with a lower oxidative capacity.[67]

Other enzymatic antioxidants include SOD and catalase. These two enzymes essentially act in concert in the following manner:

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + 2O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (5)$$

Non-enzymatic antioxidants include α-tocopherol (vitamin E) and ascorbic acid (vitamin C). The former is a lipid soluble chain breaking antioxidant that reacts rapidly with lipid peroxyl radicals resulting in the formation of the less reactive α-tocopheroxyl radical. In addition, α-tocopherol can scavenge other free radicals such as $^{\cdot}OH$. Ascorbic acid is a water soluble antioxidant which may be important in reducing the α-tocopheroxyl radical, although the relevance of this process in vivo needs to be determined.[68]

Specific NOS inhibitors such as N^G-N^G-dimethylarginine, N^G-N'^G-dimethylarginine, and protein inhibitor of nitric oxide synthase (PIN) exist in vivo.[69 70] PIN expression has recently been demonstrated in the rodent and human diaphragm.[71] However, the functional role of these inhibitors remains to be investigated.

Functional relationship between free radicals and striated muscle contractility

There is good evidence that free radicals are essential for optimal respiratory muscle contractile function. In vitro studies have revealed that the rat diaphragm generates free radicals. Scavenging ROS in this model through the addition of catalase decreased peak isometric force generation.[18] Moreover, exposing non-fatigued skeletal muscle to ROS increases submaximal force generation.[72 73] Intact single fibre studies have shown that short term (three minutes) exposure to H_2O_2 increases submaximal force generation, but does not alter $[Ca^{2+}]_i$ during activation, indicating that H_2O_2 increases Ca^{2+} sensitivity of the fibre.[73] Similarly, blocking endogenous NO synthesis reduces maximal shortening velocity and maximal power output of the unfatigued rat diaphragm in vitro.[74] Together, these studies demonstrate the physiological role of free radicals in maintaining optimal skeletal muscle contractility.

However, overproduction of free radicals is associated with impaired contractile performance. Exposing non-fatigued intact single fibres to H_2O_2 for more than six minutes reduced in vitro force generation and increased $[Ca^{2+}]_i$.[73] This indicates that prolonged oxidative stress reduces Ca^{2+} sensitivity of skeletal muscle fibres—that is, less force production at higher $[Ca^{2+}]_i$. In addition, exposure of peripheral or diaphragm muscle strips to oxidative stress increased fatigue rate.[25 72 75] Conversely, antioxidants such as N-acetylcysteine (NAC), catalase, SOD, and the hydroxyl scavenger dimethylsulphoxide (DMSO) attenuated the rate of fatigue development in vitro.[18 76 77]

Exposure of rat diaphragm strips to the NO donor sodium nitroprusside (S-NP) depressed submaximal force generation.[24] Perkins *et al*[32] showed that, in skinned rat skeletal muscle fibres, S-NP reduced isometric force generation through oxidation of contractile protein thiols.

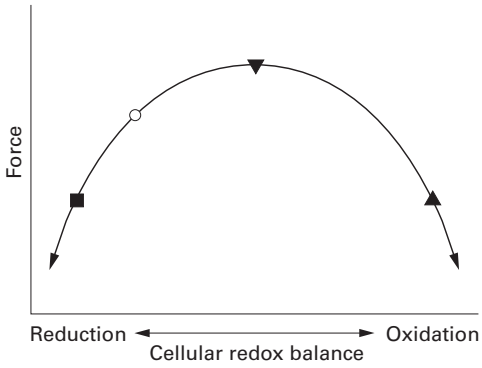

Figure 2 Schematic model of force production as function of cellular redox balance as proposed by Andrade et al.[73] The baseline redox balance (○) is to the left of the peak, into the reduction range. To obtain maximal contractile function free radicals are required (▼). However, overproduction of free radicals, resulting in oxidative stress, impairs contractile function of skeletal muscle (▲). Similarly, scavenging free radicals in unfatigued muscle, resulting in reductant stress, also impairs contractility(■). Reproduced from Andrade et al[73] with permission.

Apparently, force production is a function of redox status of the muscle fibre (fig 2).[73] The baseline redox balance is to the "reduced site" of the balance since exposure of unfatigued skeletal muscle fibres to a low concentration of oxidants increases force generation. However, both oxidant and reductant stress impair force generation. The complexity of the effects of free radicals on force generation is also demonstrated in recent studies by Andrade *et al*.[78] Exposure of intact single skeletal muscle fibres to NO donors reduced Ca^{2+} sensitivity of the fibres which would tend to decrease force generation. However, NO donors also increased $[Ca^{2+}]_i$ during activation which will increase force generation. Since these two effects occurred at the same time, force generation remained unaffected. The authors hypothesised that force production will depend on the fine balance between these opposing effects.[78]

A pivotal question in in vitro studies aiming at modulating the oxidant-antioxidant balance within tissue is the appropriate concentration of the free radical donor or antioxidant. There is currently a lack of knowledge regarding the physiological concentration of free radicals within tissue, and skeletal muscle fibres in particular. Although the release of NO from skeletal muscle preparations has been demonstrated,[79] these data provide limited information as to the NO concentration at the (sub)fibre level. Since NOS has a specific distribution within muscle fibres, it is conceivable that the NO concentration is not uniform within the muscle fibre. Variation in concentration within fibres will be even more pronounced in oxygen centred free radicals because of the extremely short half life and thus the small diffusion distances of these species. It is therefore difficult to establish whether the concentration of free radicals generated by a specific donor equals the physiological concentration of free radicals in tissue.

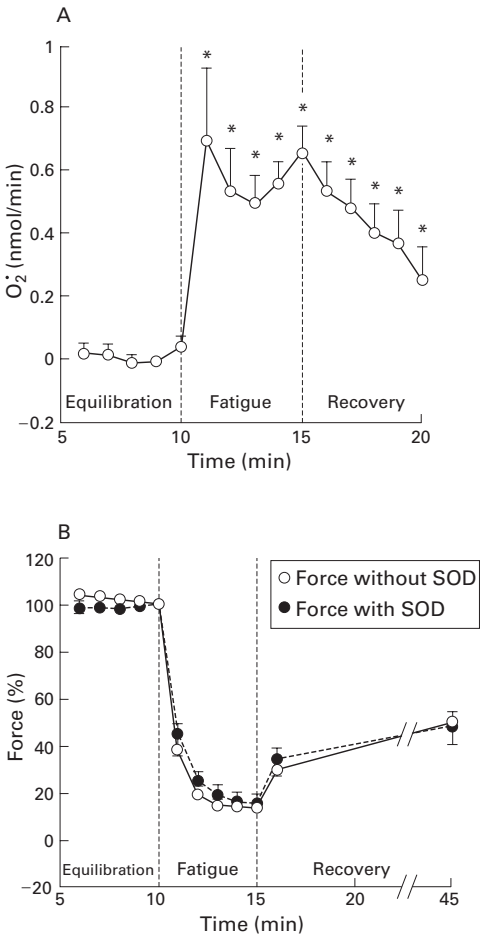

Figure 3 Generation of free radicals starts at onset of contractions. (A) Calculated levels for superoxide produced by isolated perfused rat diaphragm. Superoxide was detected by cytochrome C reduction. Signals were inhibited in the presence of the superoxide scavenger superoxide dismutase (SOD). (B) Isometric force production generated by the diaphragm when subjected to 500 ms of 80 Hz train stimulation at a periodicity of one per minute. At 10 minutes the period of stimulation was increased to one per second. Superoxide generation was significantly increased at the onset of fatiguing contractions ($p<0.05$) and decreased when fatiguing contractions were discontinued. Reproduced from Kolbeck et al[80] with permission.*

Increased generation of free radicals by striated muscle contractions

ANIMAL STUDIES

In vitro studies

Fatiguing contractions of the rat diaphragm increase the rate of free radical generation (fig 3).[18 80 81] Increased generation of free radicals precedes the development of fatigue.[80] Moreover, a significant inverse correlation has been observed between the impairment of force generation in the diaphragm during fatiguing contractions and the amount of superoxide released.[81] Rat skeletal muscle also generates NO at rest which increases after contractile activity.[79]

A limitation of in vitro models in studying the effects of oxidative stress on functional performance is that the tissue under investigation is removed from its physiological environment. This may have important effects on the redox state of the tissue. In vivo, skeletal muscles take up GSH from blood to attenuate intracellular GSH depletion. The absence of GSH and other scavengers in tissue bath experiments may affect functional responses of the tissue under investigation.

In order to circumvent these disadvantages, animal models have been used to study the effects of increased generation of free radicals on skeletal muscle function in vivo.

Exercise

Electron spin resonance (ESR) spectroscopy is a reliable technique to establish generation of free radicals directly. This technique 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 can be detected in samples composed predominantly of other substances. Disadvantages of this technique include its time consuming nature, expense, and in vivo toxicity of some of the spin traps.[82] Using ESR spectroscopy, Davies *et al*[17] were the first to show that strenuous exercise increases free radical generation in peripheral skeletal muscles of rats. Later studies supported these observations. For instance, acute exercise has marked effects on glutathione levels in skeletal muscle. Both glutathione depletion[83] and an increased GSSG/GSH ratio[84] have been observed after an acute bout of exercise. Exercise increased the concentration of GSSG in the soleus and deep vastus lateralis muscles but did not affect its concentration in the superficial vastus lateralis muscle, indicating fibre specific responses. In addition, Ji reported that acute exercise increased GPX, GR, and SOD activity in skeletal muscle,[85] although other studies did not report such upregulation in antioxidant enzyme activity.[83]

Exercise increased lipid peroxidation in contracting muscles[86 87] in a fibre type specific manner.[86] The increase in lipid peroxides after a strenuous bout of exercise in rats was more pronounced in the vastus muscle (fast twitch) than in the soleus muscle (slow twitch, highly oxidative). These differences among fibre types may be the result of muscle fibre recruitment, differences in antioxidant capacity, and amount of free radicals generated.

Increased ventilation during exercise puts an increased load on the respiratory muscles. PMN content, as estimated by MPO activity, was increased in the diaphragm after exercise.[88] Moreover, these PMNs appear to have a greater ability to generate superoxide upon stimulation with cytokines, suggesting that PMNs may contribute to increased free radical generation after exercise. In line with studies in peripheral skeletal muscle, antioxidant enzymes such as GPX and catalase have been shown to be upregulated in the diaphragm after exercise.[88] No data have been published on the effects of exercise on NO production and NOS regulation in animal skeletal muscle.

Loading of respiratory muscles

Inspiratory resistive breathing (IRB) is a well known technique for loading the respiratory muscles. Borzone *et al* demonstrated that IRB until pump failure results in increased generation of free radicals in the diaphragm as

indicated by an increased ESR signal.[89] Subsequent studies indicated that IRB until apnoea has detrimental effects on in vitro contractility of the diaphragm. Impaired force generation is accompanied by increased glutathione oxidation and lipid peroxidation in the rat diaphragm.[19 22 23 90 91]

IRB also has marked effects on NO metabolism in respiratory muscles. It was recently shown that three hours of IRB decreased diaphragmatic and intercostal muscle NOS activity although protein expression of type I and III NOS were not affected.[92] Alternatively, in vivo electrical stimulation of rabbit peripheral skeletal muscle for three weeks significantly increased NOS activity and NOS protein expression.[93] Although the precise mechanism behind these alterations is not yet clear, these studies indicate that NOS activity in respiratory muscles can be modulated by contractile activity.

In situ dog diaphragm stimulation via the phrenic nerve resulted in a decline in force to 20% of the initial value.[94] The drop in force could be attenuated by administration of DMSO or PEG-SOD (a long acting type of SOD). Treatment with these antioxidants also attenuated diaphragm lipid peroxidation.[94]

The emphysematous hamster is a well known animal model for pulmonary hyperinflation. It has been shown that impaired force generation in the emphysematous hamster diaphragm is accompanied by an increase in the GSSG/GSH ratio.[95]

Together, these animal models show that impaired force generation of the respiratory muscles induced by increased loading is accompanied, or even preceded by, increased generation of free radicals. In some studies impairment in force generation was inversely correlated with the markers for free radical generation.[89 95]

Ischaemia/reperfusion injury

Ischaemia/reperfusion injury is a clinical entity which is thought to result from increased generation of free radicals by the cytosolic enzyme XO.[46] Although at first sight ischaemia/reperfusion injury is not a clinical problem of interest to the diaphragm, there might be conditions resulting in areas of deoxygenation—for example, hypoxaemia in concert with a degree of diaphragm hypoperfusion (severe arteriosclerosis, low output failure). Indeed, during hypovolaemic shock the contracting dog diaphragm releases substantial amounts of ATP degradation products such as hypoxanthine[96] which is a primary substrate for XO. Since the diaphragm is relatively rich in XO, generation of superoxide is likely to occur in the diaphragm when ATP degradation products accumulate. Supinski *et al*[97] demonstrated that three hours of ischaemia followed by one hour of reperfusion impaired contractility of the rat diaphragm. The detrimental effects of ischaemia/reperfusion on diaphragm contractility were blunted with DMSO.[97]

HEALTHY SUBJECTS

Muscle cells export GSSG when subjected to oxidative stress.[98] Blood glutathione concentrations may then reflect the glutathione status of less accessible tissues such as skeletal muscle. Supinski *et al*[99] showed that, during IRB, enhanced glutathione oxidation occurs in contracting but not in resting skeletal muscle. Thus, increased GSSG levels in blood after exercise originate, at least partly, from contracting skeletal muscles.

Exercise

In healthy subjects exhaustive physical exercise is associated with overproduction of free radicals as indicated by an increase in glutathione oxidation in the blood[100–102] and skeletal muscle.[103] Furthermore, exhaustive exercise enhances lipid peroxidation as indicated by increased levels of plasma lipid peroxides[104–107] and increased pentane exhalation.[107 108] When exercise is not exhaustive, blood glutathione oxidation and lipid peroxidation do not occur or, at least, to a lesser extent.[105 109 110]

Exercise induced plasma lipid peroxidation is accompanied by decreased plasma nitrite levels.[111] A possible explanation for these findings is that NO scavenges superoxide generated during exercise which will result in reduced plasma nitrite formation after exercise. However, the role of NO formation during exercise needs to be studied in detail. Recent studies have shown nNOS and eNOS expression in the peripheral skeletal muscle[57] and diaphragm[71] of healthy subjects. nNOS co-expresses with mitochondrial markers and a predominance exists for type I fibres. Alternatively, eNOS is equally distributed among different fibre types and is mainly present in endothelium, which suggests a role for vasoregulation. No studies are available on the effects of exercise on NOS activity in human skeletal muscle.

COPD PATIENTS

Exercise

Viña *et al*[112] showed that exercise induced blood glutathione oxidation occurs in patients with COPD as well as in healthy subjects. We have recently found that, in patients with COPD, exercise induced blood glutathione oxidation is accompanied by increased plasma lipid peroxides.[113 114] These observations may have important implications. Firstly, exercise limitation is a prominent feature of severe COPD. In contrast to healthy subjects, patients with severe COPD may get easily exhausted during daily life activities. The above mentioned studies[112–114] suggest that this is accompanied by oxidative stress and free radical induced tissue damage. The respiratory muscles are likely to be a prominent source for increased GSSG and lipid peroxides since, in contrast to healthy subjects, the respiratory muscles in COPD patients use a substantial part (up to 40–50%) of the total oxygen consumption during relatively light exercise.[115] Furthermore, it is remarkable that the degree of exercise induced blood glutathione oxidation is similar in both healthy subjects and patients with severe

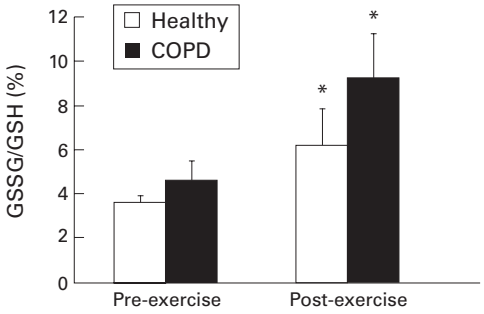

*Figure 4 Exercise causes blood glutathione oxidation in healthy subjects and patients with severe COPD. Data are derived from different studies, but blood was analysed in the same laboratory using the same methods. At rest the GSSG/GSH ratio is similar in both healthy subjects and patients with COPD. In both groups exhaustive exercise is associated with increased blood glutathione oxidation which tended to be more pronounced in patients with COPD. *p<0.05 compared with pre-exercise value. Data for healthy subjects taken from Sastre et al[101] and data for patients with COPD are from Heunks et al.[114] GSH = reduced glutathione; GSSG = oxidised glutathione.*

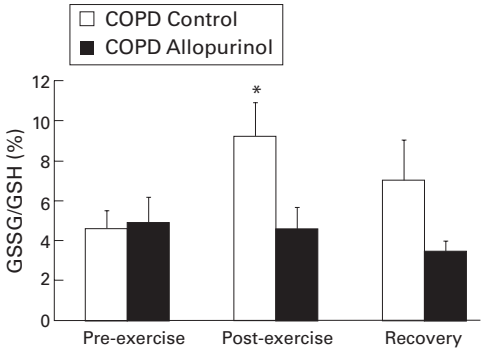

Figure 5 Xanthine oxidase is involved in exercise induced oxidative stress in COPD. Changes in GSSG/GSH ratio in arterial blood of control subjects and allopurinol treated patients with COPD. Blood was collected at rest, three minutes after incremental cycle ergometry (post-exercise), and 60 minutes after incremental cycle ergometry (recovery). The GSSG/GSH ratio was significantly increased after exercise in control subjects but not in the allopurinol treated patients. GSH = reduced glutathione; GSSG = oxidised glutathione.

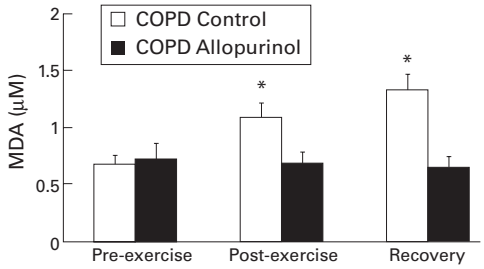

*Figure 6 Changes in malondialdehyde (MDA) concentration as an indicator of free radical induced lipid peroxidation in control and allopurinol treated patients with COPD. Plasma was collected at the same time points as in fig 5. In control patients exercise significantly increased MDA levels after exercise and a further increase was observed at recovery. In patients treated with allopurinol exercise had no effect on MDA levels (*p<0.05 compared with pre-exercise levels).*

COPD (fig 4). At first sight this is surprising since it is generally assumed that oxidative metabolism in the mitochondria is the most prominent source for generation of free radicals during exercise. The reduced rate of oxygen consumption at maximal exercise that frequently occurs in COPD would be expected to attenuate formation of free radicals. Possible explanations for this obvious oxidative stress in patients with COPD during exercise include disturbances in the mitochondrial respiratory chain, contribution of other sources besides the mitochondria to generation of free radicals during exercise, and impaired antioxidant defences in COPD.

Impaired mitochondrial metabolism may occur in COPD. It has been shown that cytochrome C oxidase activity in the quadriceps muscle of patients with COPD is increased compared with healthy subjects.[116] This may enhance generation of free radicals during exercise in COPD. On the other hand, release of partially reduced oxygen from cytochrome C oxidase is unlikely because of its high binding affinity.[117] Further studies are needed to determine the contribution of mitochondria to exercise induced oxidative stress in COPD.

An interesting possibility is that other sources besides the mitochondria contribute to free radical generation during exercise. As mentioned, generation of free radicals by XO plays a key role in ischaemia/reperfusion injury.[46] At the tissue level similarities exist between strenuous exercise and ischaemia/reperfusion. During strenuous exercise accumulation of ATP degradation products such as xanthine and hypoxanthine occurs in skeletal muscle,[118] thereby providing a substrate for XO. This implies that, in conditions of metabolic stress resulting in ATP degradation, XO may generate superoxide. The release of hypoxanthine and urate from contracting skeletal muscles has been confirmed in humans.[119–121] In addition, XO expression in human skeletal muscle is increased after strenuous exercise,[51] which also favours the generation of free radicals by XO. We have recently investigated the contribution of XO to exercise induced free radical generation in patients with COPD (FEV_1 1.1 (0.1) l).[114] Treatment with the XO inhibitor allopurinol (300 mg/day for two days) prevented exercise induced blood glutathione oxidation and lipid peroxidation (figs 5 and 6), indicating that XO plays a prominent role in exercise induced free radical generation in COPD. Preliminary data indicate that XO also plays a prominent part in exercise induced free radical generation in healthy subjects (J Viña, personal communication).

No studies have been published on the antioxidant status of the diaphragm in patients with COPD. Levine *et al*[11] showed that type I fibre composition of the diaphragm in patients with COPD is increased. Whether this is associated with increased generation of free radicals or upregulation of the antioxidant screen remains to be investigated. In addition, little is known about NOS expression in peripheral or respiratory muscles in COPD. A preliminary study has shown that NOS expression in the quadriceps muscle of seven patients with COPD was no different from that in healthy controls.[122] However, the role of NOS in skeletal muscle dysfunction in COPD needs further investigation.

Modulation of antioxidant balance

Because of the increased awareness of the deleterious effects of free radicals on tissue function, many studies have been performed with the aim of either reducing the generation of free radicals or improving antioxidant balance.

OXYGEN

Oxidants are generated during hypoxia and antioxidants attenuate the deleterious effects of hypoxia on force generation in rat diaphragm.[123] It has therefore been speculated that oxygen treatment reduces generation of free radicals. Indeed, under certain circumstances oxygen supplementation appears to be effective in reducing oxidative stress. IRB induced GSH depletion of the diaphragm was less severe in rats breathing 100% oxygen than in rats breathing room air.[124] Task endurance was also significantly longer in oxygen supplemented rats. In humans oxygen has been shown to reduce exercise induced oxidative stress. In patients with severe COPD performing exhaustive cycle ergometry, oxygen supplementation attenuated exercise induced blood glutathione oxidation.[112] The underlying mechanisms are poorly understood. Supplementation with oxygen attenuates exercise induced hypoxaemia in patients with severe COPD. This might have beneficial effects on glutathione status since hypoxaemia inhibits glutathione synthesis.[125] Furthermore, it is likely that oxygen supplementation delays ATP degradation during exercise and thereby limits accumulation of xanthine and hypoxanthine. This reduces the ability of XO to generate superoxide. Thus, oxygen supplementation might be a useful strategy for reducing exercise induced oxidative stress, at least in severe COPD. The functional consequences of this mechanism in humans have not yet been investigated.

NUTRITION

The diet provides an important source of antioxidants. Well known examples include vitamin E and C, β-carotene, and flavonoids.

Animal studies

Vitamin E plays a prominent role in respiratory and peripheral muscle function. Deficiency of vitamin E increases lipid peroxidation and glutathione oxidation in the rat diaphragm.[90] In addition, vitamin E deficiency is associated with impaired in vitro force generation of the diaphragm. IRB induced impairment in in vitro force generation and increased GSSG levels in the diaphragm were more severe in vitamin E deficient rats than in control rats.[90] In limb muscle an acute bout of strenuous exercise decreased vitamin E content.[126] Vitamin E deficiency increases oxidative stress in peripheral skeletal muscle, as indicated by increased ESR signal[17] and increased lipid peroxidation.[17 127]

There are no data on the effects of vitamin C on respiratory muscle function. Vitamin C supplementation cannot counteract reduced exercise performance in vitamin E deficient rats.[128] This is not surprising since these two antioxidants are proposed to act in concert; vitamin C can serve as a donor antioxidant for oxidised vitamin E but, because of its hydrophilic properties, it is not able to scavenge lipid peroxides directly.[129] Vitamin C supplementation before treadmill exercise partially prevented blood glutathione oxidation.[101] Packer *et al*[130] have shown that vitamin C deficiency, and also high dose vitamin C supplementation, impairs exercise endurance in guinea pigs. In the presence of ferric ions vitamin C can act as a pro-oxidant. It is uncertain whether this explains the reduced exercise capacity in vitamin C supplemented animals.

Since selenium is an essential component for the synthesis of GPX, selenium deficiency attenuates skeletal muscle GPX activity.[131] Selenium supplementation increases skeletal muscle GPX content but did not, however, prevent exercise induced lipid peroxidation in rat skeletal muscle.[131]

Healthy subjects

Encouraged by the beneficial effects of antioxidant supplementation in animals, similar studies have been conducted in humans, although the effects of nutritional supplementation on respiratory muscle function in humans has not yet been studied.

In healthy subjects supplementation with vitamin E increased the skeletal muscle vitamin E content[132] and decreased the baseline level of plasma lipid peroxides.[133] Vitamin E supplementation reduced exercise induced lipid peroxidation estimated by plasma lipid peroxides, pentane exhalation, or excretion of lipid peroxides in urine.[108 132 133] Moreover, supplementation accelerated recovery from downhill running induced muscle damage[134] and attenuated exercise induced lipid peroxidation in muscle.[132]

Healthy subjects supplemented with a vitamin mixture (vitamin E, vitamin C, and β-carotene) had lower baseline levels of exhaled pentane and plasma lipid peroxides than those given placebo.[107] The increase in exhaled pentane and plasma lipid peroxides after exercise was significantly lower in subjects supplemented with vitamins than in placebo treated subjects. Ingestion of GSH and vitamin C for seven days before exhaustive cycle ergometry attenuated exercise induced blood glutathione oxidation in athletes.[101]

Together, these studies indicate that skeletal muscle antioxidant deficiency predisposes to free radical induced tissue damage. Both animal and human studies have shown that dietary antioxidant supplementation attenuates exercise induced oxidative stress. However, no study has reported any beneficial effect of antioxidant supplementation on exercise physiological parameters in humans.

COPD patients

Nutritionally depleted COPD patients have lower respiratory and skeletal muscle strength than non-depleted patients.[9] No literature is available on the antioxidant status of respiratory or limb skeletal muscles in COPD. Few

data are available on the blood antioxidant capacity in patients with COPD. From studies by Viña *et al*[101 112] it can be deduced that the blood glutathione concentration is similar in healthy subjects and in patients with COPD. Also, Trolox equivalent antioxidant capacity (TEAC), an assay used to determine antioxidant capacity of plasma, was no different in healthy subjects and patients with stable COPD.[63] However, markers of plasma lipid peroxidation were increased in stable COPD patients.[63]

Besides malnutrition, commonly used drugs in COPD may also contribute to deficient antioxidant screen. For instance, theophylline has been shown to decrease vitamin B6 activity. The latter facilitates the availability of selenium for GPX synthesis.[135]

TRAINING

Exercise training has marked effects on skeletal muscles—for example, increased oxidative capacity, estimated by citrate synthase activity, is increased after training in both rats[87] and humans.[136] Since increased oxidative capacity may enhance the generation of free radicals, the effects of training on antioxidant capacity have been the subject of many studies.

Animal studies

Intermittent inspiratory muscle training increases the type II fibre cross sectional area of the diaphragm.[137] Alternatively, chronic loading of the respiratory muscles induced by pulmonary hyperinflation[138] or tracheal binding,[139] and also exercise training[88 140–143] increased the oxidative capacity of the diaphragm. This increase was accompanied by increased activity of antioxidant enzymes such as GPX[88 144–146] and catalase.[88]

Enzymatic antioxidant activity such as GPX,[48 83 85 127 140 147 148] GR,[83 149] and SOD[140 147 150] has been shown to increase after treadmill training in rat limb skeletal muscle. In contrast, training did not alter catalase activity.[48 140] The response of antioxidant enzymes differs between muscles. Upregulation is more pronounced in highly oxidative muscles,[83 140 147 148] although the opposite has also been shown.[151] Nevertheless, in general it appears that training induced increases in skeletal muscle oxidative capacity are accompanied by increased antioxidant enzyme capacity.

Non-enzymatic antioxidants also respond to training. Training increased the glutathione content of the gastrocnemius muscle in dogs and attenuated exercise induced glutathione depletion in skeletal muscle.[83] The vitamin E content of skeletal muscle has been shown to be unresponsive[152] or to be reduced[153] after training.

To our knowledge, only one study has been published on the effects of training on the generation of nitric oxide in skeletal muscle. Balon and Nadler[154] showed that eight weeks of treadmill running increased both nNOS and eNOS expression in rat soleus muscle.

Healthy subjects

Respiratory muscle training may improve inspiratory and expiratory muscle strength in healthy subjects.[155] However, no data have been published on the effects of training on the oxidative or antioxidant capacity of the respiratory muscles in healthy subjects.

The effect of short term exercise training on skeletal muscle antioxidant enzyme activity in healthy subjects is controversial. Seven weeks of sprint cycle training increased skeletal muscle GPX and GR activity but did not alter SOD activity.[156] Alternatively, eight weeks of aerobic cycle training increased citrate synthase activity of the vastus lateralis muscle but did not alter GPX and SOD activity, nor GSH and vitamin E content.[157]

COPD patients

Exercise training in patients with COPD increases the effectiveness of ventilation without improving lung function,[158] indicating that extrapulmonary factors contribute to exercise limitation in COPD. It has been shown that, under certain conditions, respiratory muscle training increases respiratory muscle force in COPD[159] which may contribute to effectiveness in ventilation. However, only one study has been published on the oxidative capacity of the diaphragm in COPD. Levine *et al*[11] showed that the percentage of type I fibres in the diaphragm in patients with COPD is higher than in healthy controls. This is in line with observations from animal studies which revealed that increased load on the respiratory muscles is associated with increased oxidative capacity.[138 139] No data are available on the antioxidant status of the respiratory muscles in COPD.

Maltais *et al*[160] reported that 12 weeks of exercise training increased citrate synthase activity of peripheral muscles in patients with severe COPD (FEV_1 0.99 l). Again, no data are available on antioxidant enzyme activity in limb skeletal muscle in patients with COPD.

PHARMACOLOGICAL INTERVENTIONS

No drug aiming to improve respiratory or peripheral skeletal muscle antioxidant capacity has been registered. NAC is an antioxidant drug commonly used in clinical practice. Two possible antioxidant mechanisms have been proposed for this thiol containing antioxidant. Firstly, NAC may have direct free radical scavenging properties. ROS may react with NAC resulting in the formation of NAC disulphide.[161 162] The significance of this mechanism of action in vivo is questionable since the bioavailability of total NAC is only ~9%[163 164] and of the reduced form is even lower (~4%),[164] which is probably due to the extensive first pass metabolism.[164] A direct scavenging action of NAC in vivo is therefore only likely to be significant when administered intravenously or by inhalation. Secondly, and of more importance, NAC may also exert its antioxidant effects indirectly by facilitating GSH biosynthesis.[165]

Animal studies

NAC administration improves efficiency of the glutathione redox cycle and contractile performance of the rat diaphragm. For instance, intravenous administration of NAC before IRB attenuated glutathione depletion of the diaphragm.[22 91] Furthermore, NAC treated animals tolerated IRB better than untreated rats, as indicated by increased loading endurance and higher pressure generation of the inspiratory muscles during IRB.[91] Alternatively, the deleterious effects of IRB on in vitro force generation of the diaphragm were not reduced by NAC.[22 91] This is not surprising since, in both groups, IRB was continued until apnoea and thus a same critical level of fatigue of the inspiratory muscles was reached. In situ fatiguability of the diaphragm imposed by repetitive stimulation of the phrenic nerve was significantly reduced in NAC treated rabbits.[166]

Administration of the XO inhibitor allopurinol before an acute bout of exercise shortened the duration of GSH depletion in the soleus muscle in mice.[167] More important, allopurinol attenuated exercise induced morphological damage such as irregularities in myofibrillar organisation, intrafibre oedema, and mitochondrial swelling in the soleus muscle.[167] This is an important finding since it indicates that inhibition of free radical generation attenuates morphological damage to skeletal muscle. However, allopurinol did not alter the course of IRB, nor did it prevent the IRB induced increase in lipid peroxidation in the diaphragm.[168]

Treatment of rats with the NOS inhibitor L-NAME before IRB decreased NOS activity of the diaphragm.[169] However, it did not affect the course of IRB—that is, endurance and pressure developed—nor in vitro force development of the diaphragm after IRB.

Healthy subjects

No studies are available on the effects of treatment with NAC on glutathione levels in respiratory muscles. However, NAC may have beneficial effects on respiratory muscle function in healthy subjects. Breathing against an inspiratory resistance induces fatigue of the diaphragm. The fall in force can be assessed by measuring the twitch tension of the diaphragm via electrical stimulation of the phrenic nerves. Intravenous NAC (150 mg/kg) given one hour before IRB increased loading endurance and attenuated the fall in twitch tension of the diaphragm after loaded breathing.[170] NAC did not, however, affect the rate of recovery from diaphragm fatigue, but this is to be expected since the antioxidant properties of NAC will attenuate the deleterious effects of free radicals on muscle function, but free radicals are not assumed to play a key role in recovery from fatigue.

Similarly, NAC has been shown to reduce the fatigue rate of limb skeletal muscle in vivo. Reid *et al*[171] found that the rate of development of fatigue could be attenuated by pretreatment with NAC. Fatigue was induced by repetitive electrical stimulation of the tibialis anterior muscle. Force development was measured throughout the fatigue induction. In subjects treated with intravenous NAC (150 mg/kg) the rate of development of fatigue was significantly lower than in saline treated subjects.

Oral administration of NAC (800 mg/day for two days) did not affect blood GSH concentrations but attenuated exercise induced blood glutathione oxidation in healthy subjects.[102]

COPD patients

No studies are available on the effects of NAC treatment on skeletal muscle function and exercise induced oxidative stress in patients with COPD. It was recently reported that allopurinol inhibits exercise induced blood glutathione oxidation and plasma lipid peroxidation in severe COPD (fig 5).[114] The effects of NOS inhibition on muscle function in COPD have not yet been investigated.

It is not easy to determine whether the administration of agents aimed at attenuating perturbation in the oxidant-antioxidant balance should be acute or chronic, although this is of major clinical and physiological interest. If the imbalance between oxidants and antioxidants during exercise in COPD is the result of depletion in endogenous antioxidants—that is, is a result of malnutrition—administration should be continued until the antioxidant screen is restored. However, if overproduction of free radicals is the result of upregulation of enzymes involved in free radical generation, enzyme inhibitors such as allopurinol should probably be administered chronically.

Conclusions and future research

Free radicals are important modulators of skeletal muscle contractility. Although required for optimal contractile function, overproduction of free radicals may have opposite effects. The generation of free radicals by skeletal muscle is increased during contractile activity. Since the load imposed on the diaphragm in patients with severe COPD is increased, it might be speculated that this is accompanied by an increase in the generation of free radicals. This may in turn contribute to respiratory muscle dysfunction. However, no direct data are available to support these speculations.

Future research should focus on the antioxidant status of respiratory muscles in humans, particularly in patients with COPD. The effects of long term antioxidant supplementation on peripheral and limb skeletal muscle must be assessed. Also, the role of specific pathways for the generation of free radicals should be assessed in COPD and may result in the application of drugs targeting these pathways.

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