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TESI

OXIDATIVE STRESS RESISTANCE IN SKELETAL MUSCLE CELLS: ROLE OF VITAMIN C AND REDOX SENSITIVE TRANSCRIPTION FACTORS NF-KB AND AP-1.

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ABSTRACT

OXIDATIVE STRESS RESISTANCE IN SKELETAL MUSCLE CELLS: ROLE OF VITAMIN C AND REDOX SENSITIVE TRANSCRIPTION FACTORS NF-KB AND AP-1.

During physical exercise skeletal muscle cells are continuosly exposed to oxidative stress. Thus, they compensate environmental challenges by increasing adaptive responses, characterized by AP-1- and NF-kB-mediated transcriptional up-regulation of endogenous enzymatic and non-enzymatic antioxidants. Also exogenous antioxidants, such as vitamin C contribute to cellular defences. The skeletal muscle cells content of vitamin C is determined both by the efficiency of transport systems and by the ability to maintain the vitamin in its reduced form.

Aim of this thesis was to study, in skeletal muscle cells: i) specific antioxidants systems, including glutathione (GSH), thioredoxin reductase (TxR) and antioxidants enzymes; ii) the role of redox sensitive transcription factors, such as AP-1 and NF-kB; iii) the transport of vitamin C, both in the reduced (ascorbic acid, AA) and oxidized form (dehydroascorbic acid, DHA); iiii) the enzymatic systems (NADH-, NADPH-, GSH-, lipoic acid-dependent) involved in vitamin C recycling. To this end, two skeletal muscle cell lines were used (mouse C2C12 and rat L6C5 cells), in both proliferating and differentiated conditions.

We found that muscle cell differentiation was associated with increase in antioxidant ability, and this phenomenon was more evident in the L6C5 cell line. C2C12 myoblasts and myotubes show high levels of NF-kB and thioredoxin reductase, together with AP-1 inhibitory complexes. Furthermore, C2C12 cells have antioxidant enzymes highly active thus allowing survival after exposure to oxidative insults. On the contrary, L6C5 myoblasts show a sensitive phenotype, correlated to low levels of thioredoxin reductase, catalase and NF-kB activity, together with high levels of oxidized glutathione (GSSG) and activating AP-1 complexes. Interestingly, this cell line acquires an apoptosis-resistant phenotype, accompanied by drastic changes in the oxidant/antioxidant balance, when induced to differentiate. Indeed, L6C5 myotubes enhance catalase and NF-kB activities and shift AP-1 complexes from an activating to an inhibitory behaviour.

Concerning the experiments on vitamin C metabolism, our data show that both cell lines import AA by the SVCT2 transporter, while DHA transport is

mediated by glucose carriers GLUT1 and GLUT3. L6C5 myoblasts are more efficient in ascorbic acid transport, while C2C12 cells are more efficient in dehydroascorbic acid transport and ascorbyl free radical/dehydroascorbic acid reduction.

Oxidative stress, induced by glutathione depletion, increased SVCT2 expression and thioredoxin reductase-mediated dehydroascorbic acid reduction, especially in differentiated cells. From these data, SVCT2 and NADPH-thioredoxin dependent DHA reduction appear to belong to an inducible system activated in response to oxidative injury.

RESISTENZA ALLO STRESS OSSIDATIVO NELLE CELLULE MUSCOLARI SCHELETRICHE: RUOLO DELLA VITAMINA C E DEI FATTORI DI TRASCRIZIONE REDOX-SENSIBILI NF-KB E AP-1.

Durante l'esercizio fisico le cellule muscolari sono continuamente esposte allo stress ossidativo e necessitano quindi di efficaci sistemi di difesa antiossidante. Tramite l'attivazione di fattori trascrizionali, quali AP-1 e NF-kB, le cellule muscolari possono compensare le variazioni ambientali incrementando le risposte adattative (antiossidanti endogeni, enzimatici e non). Alle difese cellulari contribuiscono anche gli antiossidanti esogeni, quali la vitamina C. Il contenuto di vitamina C nelle cellule muscolari è determinato sia dall'efficienza dei sistemi di trasporto sia dal mantenimento della vitamina nella sua forma ridotta.

Lo scopo della tesi è stato quello di studiare in cellule muscolari scheletriche: i) specifici sistemi antiossidanti, quali il glutatione, la tioredossina reduttasi ed enzimi antiossidanti; ii) il ruolo di fattori di trascrizione sensibili allo stato redox quali AP-1 e NF-kB; iii) i meccanismi di trasporto sia per la forma ridotta (acido ascorbico, AA) che per la forma ossidata (acido deidroascorbico, DHA) della vitamina C; iiii) i sistemi enzimatici (NADH-, NADPH-, GSH-, acido lipoico-dipendenti) coinvolti nella rigenerazione della vitamina C. A tal fine sono state utilizzate cellule muscolari scheletriche di topo (C2C12) e di ratto (L6C5), sia in fase proliferativa che dopo differenziamento.

I risultati ottenuti dimostrano che il differenziamento è correlato all'aumento della capacità antiossidante, e tale fenomeno è maggiormente evidente nelle cellule L6C5. Le cellule C2C12 (mioblasti e miotubi), possedendo elevati livelli di NF-kB, di complessi AP-1 di tipo inibitorio e di tioredossina reduttasi, mostrano una maggiore espressione di enzimi antiossidanti; perciò, tali cellule sono maggiormente resistenti allo stress ossidativo. Al contrario, i mioblasti L6C5 mostrano un fenotipo sensibile, correlato a bassi livelli di tioredossina reduttasi, catalasi ed attività di NF-kB, ed alti livelli di glutatione ossidato e di complessi AP-1 attivi. Tuttavia, questa linea cellulare, se indotta a differenziare, acquista un fenotipo resistente all'apoptosi, accompagnato da drastici cambiamenti nel bilancio fra specie ossidanti ed antiossidanti. I miotubi L6C5, infatti, aumentano le attività di catalasi e NF-kB, e cambiano i complessi AP-1 da attivanti ad inibitori.

Gli esperimenti condotti per caratterizzare il metabolismo della vitamina

C hanno evidenziato che il trasporto dell'AA è mediato dall'SVCT2 (trasportatore con elevata affinità), mentre il trasporto per il DHA è mediato dai trasportatori del glucosio GLUT1 e GLUT3. I mioblasti L6C5 sono più efficienti nel trasporto dell'acido ascorbico, mentre le cellule C2C12 sono più efficienti nel trasporto dell'acido deidroascorbico e mostrano maggiori attività DHA e AFR (radicale ascorbile) reduttasiche.

Lo stress ossidativo, indotto dalla deplezione di glutatione, induce un aumento dell'espressione di SVCT2 e dell'attività DHA reduttasica tioredossina-dipendente, specialmente nelle cellule differenziate. Da questi dati appare evidente che il trasportatore SVCT2 e l'attività DHA reduttasica NADPH-tioredossina-dipendente appartengono ad un sistema inducibile attivato in risposta allo stress ossidativo.

ABBREVIATIONS:

AA.....ascorbic acid
AA-2P.....ascorbic acid-2 phosphate
AFR.....ascorbyl free radical
AOE.....antioxidative enzyme
AP-1.....activating protein 1
BSO.....buthionine sulfoximine
CM-H_2DCFDA.....5(and-6)chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester
DHA.....dehydroascorbic acid
DOG.....deoxyglucose
DTT.....dithiotreitol
GPx.....glutathione peroxidase
GSH.....reduced glutathione
GSSG.....oxidized glutathione
NF-kB.....nuclear factor-kB
PBS.....phosphate buffered saline
ROS.....reactive oxygen species
RO/NS.....reactive oxygen and nitrogen species
SOD.....superoxide dismutase
Trx.....thioredoxin
TrxR.....thioredoxin reductase

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1. SKELETAL MUSCLE

Owing to the fact that skeletal muscle tissue represent almost 40% of the total body mass (Costantini et al., 1990), the metabolic events in which it is involved, would in turn influence the efficiency and the health of all other tissues.

Skeletal muscle is a complex structure derived by the interaction and association of proliferating myoblasts that, during the differentiation process, fuse together into an unique unit, named myotube (Shen et al., 2003).

Myotubes are long multinucleated fibres with a cylindric (50-100 μm diameter) and extended form. A plasmatic membrane, called sarcolemma, blinds the fibre and the interior of the fibre is filled with sarcoplasm containing nuclei, mitochondria, myoglobin and about 500 threadlike 1-3 μm thick myofibrils continuous from end to end in the muscle fibre.

Two different types of myofilaments are present into the fibre: thick and thin myofilaments, that create a regular structure called sarcomer delimitated by two big Z bands and two sarcoplasmatic T tubules.

The thick filaments contain about 200 myosin molecules (470.000 dalton), each of which is composed of two α-helix twisted high chains (200.000 dalton) and two light chains (15.000-27.000 dalton) associated with the globular zone of the head. The myosin heads, having ATP-ase activity, catalyze the scission of ATP in ADP and Pi, transforming the bound energy in contractile energy. The myosin molecules are bundled together such that their tails form the central portion of the filament and their heads face outwards and in opposite directions to each other. In this way, the myosin globular heads can interact with thin filaments, in order to generate the tension associated with muscle contraction.

The thin filaments are composed by the association of three different proteins: a double helix of actin and two regulatory proteins (troponine and tropomiosine).

Actin is a globular protein (43.000 dalton) bound to ATP. In the presence of Mg^{2+}, the globular (G) monomer polymerizes in fibrous (F) actin (13-14 G-actin), in a double helix structure. ADP still remains bound to actin, stabilizing the whole structure. F-actin interacts with myosin heads (actomyosin) to stimulate the ATP-ase activity of myosin.

Tropomyosin is a fibrous protein (66.000 dalton) formed by two chains

(α and β) blinded in a α-helix structure. This protein strictly associates with F-actin, playing a regulatory role on the interaction between actin and myosin.

Troponin (76.000 dalton) is formed by three specific subunits. Troponin T (Tn-T) is the subunit binding troponin to tropomyosin. Troponin I (Tn-I) is the subunit inhibiting the actin-myosin interaction and preventing the stimulatory action of actin on ATP-ase activity of myosin. Troponin C (Tn-C) is the subunit binding calcium and playing a regulatory role on the interaction between Tn-T and Tn-I, as well as among the three troponin subunits and actin.

Electrical excitation, passing as an action potential, from the motor endplate along the sarcolemma and down the T tubules, leads to calcium release from the sarcoplasmic reticulum (from 10^{-7} M to 10^{-5} M) into the sarcoplasm and the subsequent activation and contraction of the filament array. When calcium and adenosine triphosphate (ATP) are present in sufficient quantities, the filaments interact to form actomyosin and shorten by sliding over each other.

1.1 DIFFERENT TYPE OF MUSCULAR FIBRE

Making an histological examination, different fibres can be seen: **type I** (red fibre, slow) and **type IIa** and **IIb** (white fibre, fast) fibres.

The colour of the fibre mainly depends on the number of mitochondria and myoglobin concentration. Type I fibres contain myoglobin and mitochondria, thus possessing a high capacity of oxidative metabolism. Indeed, glycolysis is utilized only in the first part of exercise, but ATP is mainly produced through fatty acids β-oxidation via Krebs cycle and mitochondrial oxidative phosphorilation. As a result, type I fibres contract slowly and are specialized for the performance of repeated strong actions over prolonged periods.

Type IIa and IIb contain few mitochondria, low concentration of myoglobin and high phosphocreatine stores: ATP principally derives from anaerobic glycolysis. The glucose requirement mainly derives from muscle glycogen stores. As a result, their contraction is fast and they are best suited for delivering rapid, forceful actions for brief periods.

Whole muscles in the body contain a mixture of the three different fibre types, although the proportions in which they are found differ substantially

between different muscles and can also differ between different individuals. Although the fibre type composition is a genetically determined attribute, nonetheless it can change in dependence of the type of work played. Generally, muscle working in strength and speed has more type II fibres, while muscle working in resistance has more type I fibres. Usually, in the middle of muscle, there is a majority of type I fibres, while in the external part there are type II fibres.

Skeletal muscle is a remarkably plastic tissue, as it may adapt in response to different patterns of activity or disuse. This adaptation concerns alteration in muscle size, fibre composition, metabolic capacity and capillary density.

2. PHYSICAL ACTIVITY AND REDOX BALANCE

2.1 EXERCISE-INDUCED OXIDATIVE STRESS

Reactive oxygen/nitrogen species (RO/NS) mainly include free radicals, which are highly reactive, short half-lived (10^{-6}-10^{-12} s) chemical species, produced by the metabolism of aerobic organisms. RO/NS production can also be associated to the presence of inflammatory cytokines (e.g.: IL-1, IL-2, IL-6, TNF-α, EGF, PDGF) (Krieg et al., 2004) or physical and chemical agents (e.g.: high pO_2, metals) (Haddad et al., 2001, Leonard et al., 2004, Shi et al., 2004). Under physiological conditions, these deleterious species are mostly removed by cellular antioxidant systems, which include antioxidant vitamins, protein and non-protein thiols, and antioxidant enzymes. When RO/NS exceed antioxidant defences, oxidative stress will develop. Indeed, if not inactivated, they may damage all biological macromolecules, contributing to several muscle diseases (Murphy et al, 1989, Weindruch 1995). Classical examples are represented by protein damage (altered enzymatic systems), lipid peroxidation (membranes damages) and DNA damage (altered genic expression).

The major source of ROS, at rest and during exercise, is the electron transport chain, which is required for reduction of O_2 to H_2O: indeed, over 90% of the oxygen consumed by a mammal is utilized in mitochondria (Halliwell and Gutteridge 1989, Chance et al, 1979). Electrons from NADH move to complex I (NADH-ubiquinone reductase complex) and electrons from succinate move to complex II (succinate dehydrogenase complex). Ubiquinone, also know as coenzyme Q, accepts electrons from both complexes and is sequentially reduced to ubisemiquinone and ubiquinol. Ultimately, electrons are transferred from ubiquinol to complex III (ubiquinol-cytocrome C reductase), cytocrome C and complex IV (cytocrome C oxidase) to reduce O_2 to H_2O. By electron spin resonance (ESR), the major site of free radical formation appears to be complex I (Herrero et al., 1997, Takeshige et al, 1979) (Fig.1) and, in this context, coenzyme Q in its reduced state, may act as an antioxidant, protecting cellular membranes from free radical damage (Beyer, 1992).

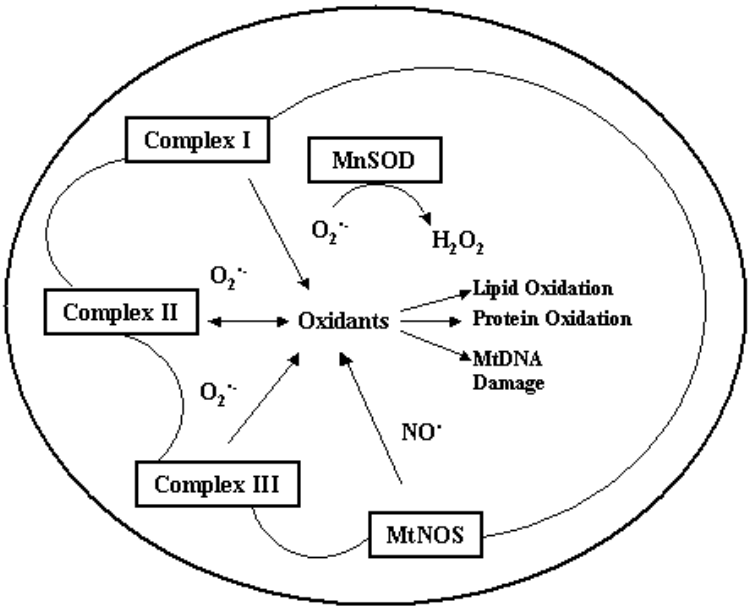

Fig. 1. Mitochondrial sources of ROS (from Leeuwenburgh et al., 2001).

In the last step of respiratory chain, electrons are transferred to O_2, which undergoes a concerted tetravalent reduction to produce water, a reaction catalyzed by cytochrome C oxidase (COX) (Fig.2); this is another source of ROS generation, due to leakage of partially reduced forms of O_2.

The rate of mitochondrial ROS generation is related to the degree of reduction of the oxidizable electron carriers and such a degree of reduction decreases strongly during the state 4 to state 3 transition at the same time when the rate of electron flow is increased (Loschen et al, 1971, Boveris et al., 1972, Boveris et al., 1973, Tzagoloff, 1982). This fact explain why the free radical leak is lower in state 3 (ADP-stimulated) than in state 4 (basal)

respiration. On the other hand, the degree of reduction of the complex I free radical generator is higher than the complex III generator, which is situated further away from the substrate, allowing the complex I to be a more active source of ROS in state 3 (Herrero et al., 1997).

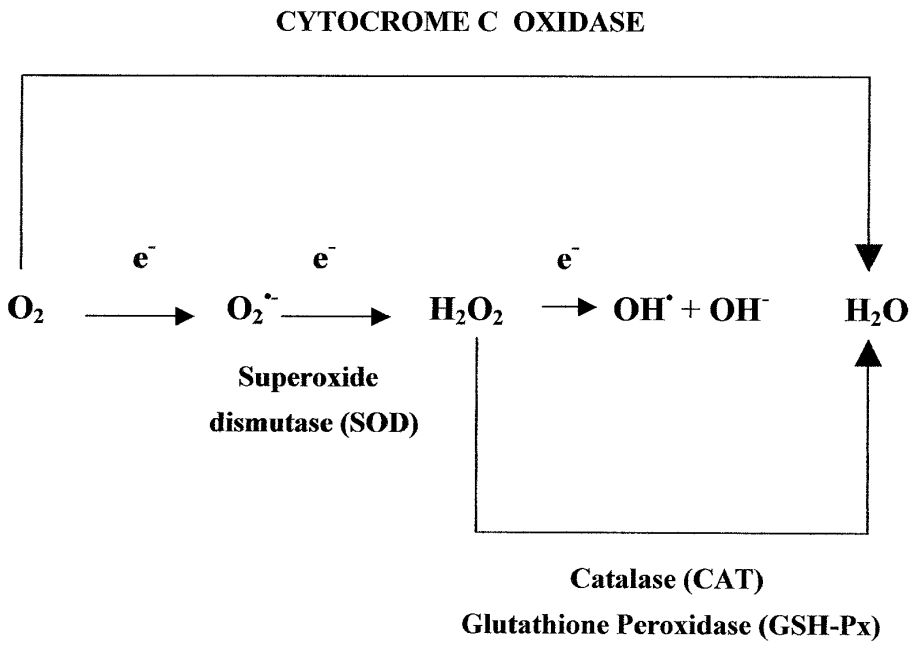

Fig. 2. Enzymes involved in oxygen metabolism.

Several modifications occuring during exercise could increase the mitochondrial capacity to produce free radicals. First, contractile activity is accompained by increased mitochondrial activity and O_2 consumption (Tyler, 1992, Barja de Quiroga, 1992): it has been demonstrated that, during physical activity, skeletal muscle cells release hydrogen peroxide (Silvera et al., 2003). Second, rise in body temperature associated with intense exercise may modify the activity of crucial enzymes, such as, for example, COX activity (Sjodin et al., 1990). All these changes may lead to increase in

RO/NS production. Third, exercise causes uncoupling of mitochondrial respiration and partial block of the respiratory chain, by changing several factors: expression level of uncopuling proteins (UCP) (Ricquier et al., 1991), cytoplasmatic Ca^{2+} content (McCormack et al., 1990), temperature and ischemia-reperfusion phenomenon (Salo et al., 1991, McCord 1988). Finally, another source of RO/NS in skeletal muscle is due to infiltration of activated neutrophils following strenuous or eccentric exercise (Klebanoff 1980).

ROS (especially superoxide anions) are released into the external medium during contraction (Reid et al., 1992a, Reid et al., 1992b, Kolbeck et al., 1997, Bejma et al., 1999), both after exhaustive aerobic (Davies et al., 1982, Ashton et al., 1998, Ashton et al., 1999) and acute physical exercise (Jackson, 1996, Dillard et al, 1978). Since the antioxidant reserve capacity in skeletal muscle is rather marginal, the remarkable increase in oxygen consumption, with the concomitant production of RO/NS, presents a challenge for antioxidant systems. Prolonged heavy exercise may cause a transient reduction of tissue vitamin E content (Schroder et al, 2001, Sen et al, 1997) and change the glutathione redox state in various body tissues (Urso et al., 2003, Banerjee et al., 2003, Ilhan et al., 2004). It has been suggested that increased amounts of oxidizing free radical species generated by exercising tissues (Brady et al, 1979, Davies et al, 1982, Dillard et al., 1978, Jackson et al.,1985) may be involved in muscle pathogenesis or fatigue (Duthie et al., 1990, Ginsburg et al., 1996, Liu et al., 1999, Rokitzki et al., 1994b).

Physiologically relevant concentration of RO/NS can regulate a variety of molecular mechanisms involved in contraction, metabolism, antioxidant defence pool, cell-cell adhesion, immune response, inflammation, cell proliferation, death and aging (Forman et al., 2002a-b). In skeletal muscle, intensity of exercise and antioxidant concentrations are critical factors in determining alterations in the prooxidant-antioxidant equilibrium (Zembron-Lacny et al., 2000). Acute exercise increases oxidative stress, especially in untrained subjects (Leeuwenburgh et al., 1999, Bejima et al., 1999, Alessio 1999, Lawler et al., 1998, Powers et al., 1999, Venditti et al., 1999), but training has been shown to induce adaptations preventing a massive oxidative damage (Venditti et al., 1977, Venditti et al., 1996). Several reports showed that training and exercise, by influencing the redox state, improve skeletal muscle function (Hayes et al., 1996, Dupont-Versteegden et al., 1994), as well as vascular adaptation, maintenance of muscle mass (Ishihara et al., 1998, Sexton 1995), up-regulation of endogenous

antioxidants and repair systems, including heat shock proteins (HSP 70, HSP 27) (Salo et al., 1992) and heme-oxigenase-1 (HO-1) (Niess et al., 1999, Niess et al., 2000).

2.2 REACTIVE OXYGEN AND NITROGEN SPECIES (RO/NS)

Several cellular activities can lead to radical formation (RO/NS), e.g. mitochondria, peroxisomes, lysosomes, endoplasmatic reticulum, cell membranes and cytoplasm (Maccarrone et al., 1997, Moldovan et al., 2004). Fig. 3 schematically shows the main activities involved in intracellular RO/NS generation.

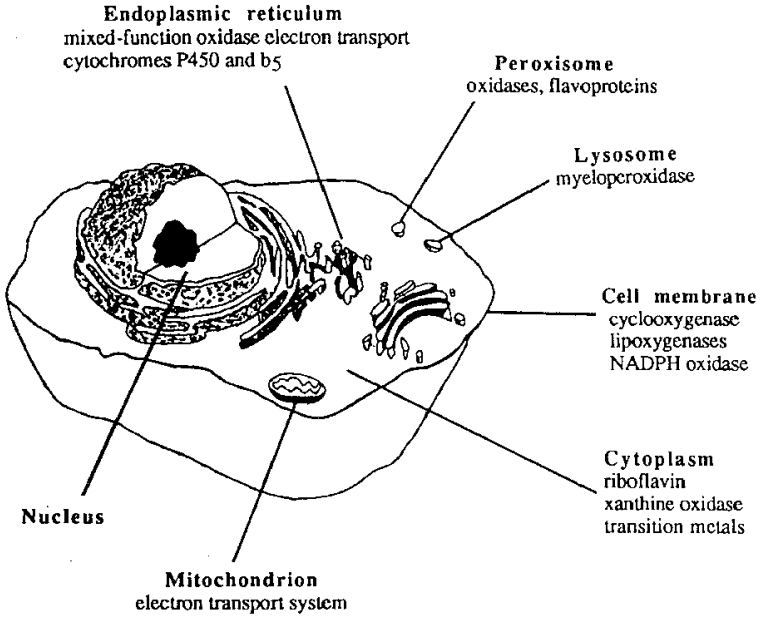

Fig. 3. Intracellular sources of RO/NS (from Maccarrone et al., 1997).

Although a huge RO/NS production is related to pathological conditions (Freeman et al., 1982, Halliwell et al., 1990a), nonetheless small variations in RO/NS levels are related to normal physiological activities.

The most common free radicals presents in our organism are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$) and nitric oxide (NO). Other reactive oxygen species, such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO) are produced, although they are not radicals, as they do not posses unpaired electrons. Nonetheless, they can diffuse across biological membranes and equally produce oxidative damage.

The ***superoxide radical*** ($O_2^{\bullet -}$) is generated by the monovalent reduction of oxygen, in almost all aerobic cells.

$$O_2 + e^- \longrightarrow O_2^{\bullet -}$$

It is mostly produced in activated phagocytic cells (neutrophiles, monocytes, macrophages), as a defence mechanism against pathogens. In this case, the radical is generated by NADPH oxidase, an enzyme associated with the plasma membrane electron transport chain (Forman et al., 2002).

$$2O_2 + NADPH \longrightarrow 2O_2^{\bullet -} + NADP^+ + H^+$$

The $O_2^{\bullet -}$ radical can also be formed in vivo, as modulator of intracellular signaling and growth regulatory pathways.

Several studies have suggested that superoxide is released from skeletal muscle during intense physical activity (Kolbeck et al., 1997). Superoxide is likely to be derived from muscle mitochondria (Jackson, 1996, Reid, 1996). Alternative potential sources of superoxide production include membrane-bound oxidases or endothelial cytosolic xanthine oxidase (Reid et al, 1992, Hellsten, 1994). These mechanisms may be activated in response to different stimuli, including rise in body temperature and reduced blood supply, which somehow mimic the ischemia-reperfusion phenomenon (Di Meo et al., 2001). In vivo generated $O_2^{\bullet -}$ quickly undergoes dismutation to oxygen and hydrogen peroxide, a reaction catalyzed by superoxide dismutase (SOD):

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Hydrogen peroxide (H_2O_2) is highly reactive and toxic. It is not a free radical, but it may accept an electron from superoxide, generating the hydroxyl anion (OH^-) and the hydroxyl radical ($OH^\bullet$):

$$H_2O_2 + O_2^{\bullet -} \longrightarrow OH^\bullet + OH^- + O_2$$

The decomposition of hydrogen peroxide to form water and oxygen is accomplished in the cell by catalase and glutathione peroxidase. This antioxidative enzyme is widely distributed in the cell, with the majority of the activity occurring in the mitochondria and peroxisomes (Powers et al., 1999).

During muscle contraction, production of H_2O_2 from mitochondria increases; this reactive molecule can diffuse across plasma and mitochondrial membranes acting as a substrate for the iron-catalyzed formation of hydroxyl radical.

The ***hydroxyl radical*** ($OH^\bullet$) is the most powerful known oxidant, with a short half-life and ability to attack all biological molecules. $OH^\bullet$ can be formed through the *Fenton reaction* between hydrogen peroxide and transition metals (especially iron). However, in normal conditions the rate of this reaction is very low, thus biologically irrelevant, since active metal ions are bound to proteins and not free to react according to the following equation:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^\bullet + OH^-$$

Nitric oxide (NO) is produced by different NO synthases: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), which convert arginine to cytrulline in the presence of O_2 and NADPH.

$$\text{L-Arg} + O_2 + \text{NADPH} \longrightarrow \text{citrulline} + NO + NADP^+$$

This radical has a key role in maintenance of the vascular tone and neurotransmission. The effects of NO may be either cytoprotective or cytotoxic, depending on concentration, time of exposure and presence of RO/NS. The source of NO released by skeletal muscle during contraction appears to be nNOS (Hirschfield et al., 2000, Balon et al., 1994). This isoform, located at the sarcolemma of skeletal muscle, was found primarily

in type II (fast) muscle fibres (Kobzik et al., 1994), then in all striated fibres (Grozdanovic et al., 1995). At moderate concentrations, NO regulates respiration, but it also reacts with $O_2^{\bullet-}$ producing peroxynitrite ($ONOO^-$), a powerful oxidant (Ischiropulos et al., 1992):

$$O_2^{\bullet-} + NO \longrightarrow ONOO^-$$

Hypochlorous acid (HClO), generated from hydrogen peroxide and chloride ions, is an inflammatory mediator almost exclusively produced in granulocytes. It is also a strong oxidizing and chlorinating compound, which generates other reactive metabolites, such as nitryl chloride (Eiserich et al., 1998). It is a powerful oxidant of amines, aminoacids, thiols and hemoproteins; for this reason it is utilized as a defence system to kill bacteria (Weiss, 1989).

$$H_2O_2 + Cl^- + H^+ \longrightarrow H_2O + HClO$$

Lipids, proteins and DNA are targets for RO/NS injury (Reznick et al., 1998, McArdle et al., 2001 and 2004, O'Neill et al., 1996, Silveira et al., 2003). Polyunsatured fatty acids, possessing weak C-H bounds, are susceptible to radical attack, thus forming C-centered radicals. In this way, RO/NS initiate radical chain reactions leading to lipid peroxidation and membrane damages.

Lipid peroxidation is frequently used as index of oxidative stress during exercise, as by-products of lipid peroxidation are easily measured (Davies et al., 1982, Alessio et al., 1988, Ji et al., 1988, Venditti et al., 1996, Venditti et al., 1977, Rajguru et al., 1993).

RO/NS may attack proteins, modifying aminoacid structures and altering the functional properties of enzymes. O-tyrosine, m-tyrosine, o-o'-dityrosine, 3-chlorotyrosine, 3-nitrotyrosine, mainly produced by $O_2^{\bullet-}$, may be detected as markers of exercise-induced oxidative damage (Pacifici et al., 1990, Leeuwenburgh et al., 1997a-c, Leeuwenburgh et al., 1999).

Genetic RO/NS targets are poly-ribonucleotides and poly-deoxyribonucleotides (Floyd, 1991, Halliwell et al., 1991, Shigenaga et al., 1990). ROS may induce single and double DNA breaks, modify purinic and pirimidinic bases and create deletions or timine dimers. DNA damage is mainly induced by $OH^{\bullet}$ (McBride et al., 1991) and H_2O_2; the latter may diffuse into the nucleus and react with iron or copper ions forming the hydroxyl radical (Halliwell et al., 1990a-b). Genetic alterations lead to

different pathological diseases, including degenerative processes, carcinogenesis and immunodeficiency.

2.3 ANTIOXIDANT DEFENCE MECHANISMS

If the rates of oxidant formation and antioxidant scavenging ability are constant, a redox balance exists inside cells (Fig.4).

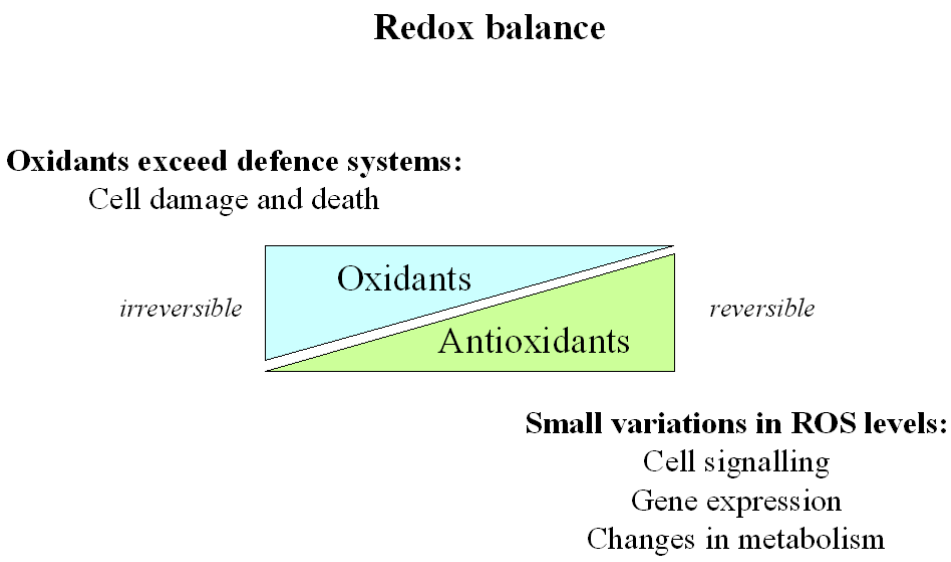

Fig. 4. The intracellular redox balance.

A good antioxidant may be definied as a compound, which acts at low concentrations and significatively delays or inhibits substrate oxidation.

To neutralize the oxidative effects of RO/NS, aerobic organisms are endowed with several systems such as:

1. *Cellular compartimentalization* minimally reduces diffusion of RO/NS from site production to other cellular districts.
2. *Tissue integrity* is an important component of antioxidant properties; in fact, tissue injury may derived from presence of not-bound iron, loss of metal-binding proteins (ferritin, ceruloplasmin), decreased availability of iron and copper ions.
3. *Enzymatic systems* prevent the formation of new free radical species. The most common antioxidant enzymes are: superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase,

catalase and thioredoxin reductase (Nordberg et al., 2001).

4. *Non-enzymatic systems* (ubiquinone, uric acid, melatonin and small thiol-containing molecules, such as glutathione and thioredoxin) directly neutralize RO/NS. Also, some dietary vitamins (vit A, C, E) and polyphenols contribute to cellular antioxidant ability. The tripeptide glutathione (GSH) plays a vital role in protecting tissues from oxidative stress (Meister et al., 1983). Indeed, GSH interacts with different antioxidant molecules (such as α-tocopherol and ascorbic acid), promoting their reduction (hence, their regeneration), by a redox cycling mechanism (Yu, 1994); during this reaction, GSH is oxidized to glutathione disulfide (GSSG).

The antioxidant factors may also be classified according to their mechanism of action:

a) PREVENTIVE ENZYMATIC ANTIOXIDANTS: eliminate the reactive species that are initiators of radicalic chains. This class includes: mitochondrial (Mn-SOD) and cytosolic (Cu-Zn SOD) SOD, glutathione peroxidase (GPx) and catalase (CAT). The SOD enzyme catalizes dismutation of superoxide anion to hydrogen peroxide, which is then transformed to water by other two enzymes: catalase (acting inside peroxisomes) and GPx (which utilizes the reducing equivalents of GSH) Thioredoxin reductase and glutathione reductase basically act to regenerate several antioxidant molecules, including thioredoxin, GSH and ubiquinone. Cytocrome C oxidase (COX) can also be filled in this class, as the amount of RO/NS strictly depends on catalytic efficiency of this enzyme.

b) *PREVENTIVE CHELATING ANTIOXIDANTS:* bind metal ions, thus avoiding free radical chain reactions. Iron- (ferritin, transferrin and the lactoferrin) and copper- (caeruloplasmin) binding proteins belong to this group of chelating compounds.

c) *CHAIN-BREAKING ANTIOXIDANTS:* act breaking off the radical chain propagation, by direct removal of RO/NS. Examples are α-tocopherol (vitamin E) and ascorbic acid (vitamin C). It is noteworthy that α-tocopherol and ascorbic acid cooperate, since ascorbate is able to regenerate vitamin E from its radical (Fig.5).

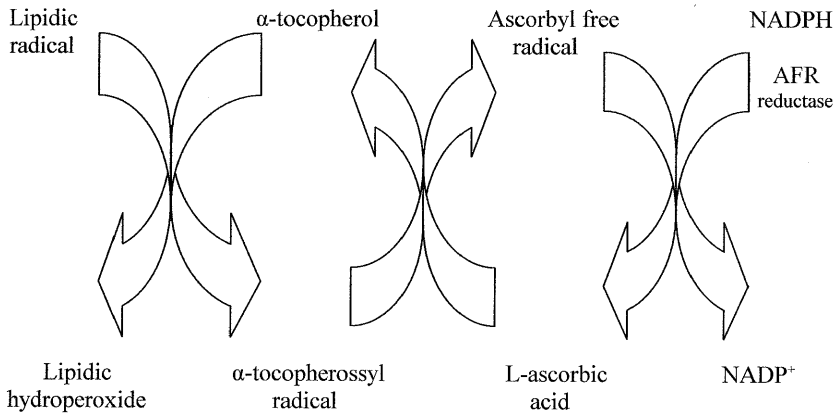

Fig. 5. Vitamin C and vitamin E scavenging systems.

2.4 CELLULAR RESPONSES TO PHYSICAL EXERCISE

Regular performed, moderate exercise has many beneficial effects, whereas acute exercise can produce damage in skeletal muscle and other tissues (King et al., 1970, Kasperek et al., 1982, McCully et al., 1985, Kanter et al., 1988, McCutcheon et al., 1992). Alterations of redox state (by increased RO/NS production or decreased antioxidant activity) induce redox-sensitive signalling cascades, which, in turn, interfere with the expression of endogenous enzymatic (SOD, catalase, GPx) and non-enzymatic (GSH) antioxidants (Svensson et al., 2002, Anuradha et al., 1998, Ji et al., 1993). The protective effects of training are usually associated with increased antioxidant defences, that restore a correct redox balance (Powers et al., 1999, Ji 1999).

One important adaptation, accompanying regular endurance training, is the decrease in H_2O_2 basal levels (20-40%), generated by isolated mitochondria in State 4 (Venditti et al., 1999); this change may have implications for mitochondrial DNA damage. A 10-week exercise program increases SOD and GPx activities, as well as glutathione content (Leeuwenburgh et al., 1994, Leeuwenburgh et al., 1997); *in vivo* studies have shown that exercising animals possess mitochondrial SOD and cytosolic GPx activities higher than those found in sedentary animals (Leeuwenburgh et al., 2001).

Positive relationships have been documented in runners between weekly training distance and resting levels of erythrocyte catalase activity (Ohno et al., 1988; Robertson et al., 1991). At higher training volumes, the increased production of hydrogen peroxide exceeds the capabilities of glutathione peroxidase (GPx). Therefore, catalase production would be expected to increase in response to the demands of training volume, to compensate for the inability of GPx to scavenge hydrogen peroxide. However, several studies demonstrate that catalase activity in response to a single bout of exercise is variable (Marzatico et al., 1997, Tiidus et al., 1996, Aguilo et al., 2000). Other RO/NS-related proteins, such as heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS), change their levels in response to exercise (Niess et al., 2000a-b, Ghosh et al., 2003).

During contractile activity, heat shock proteins (HSPs) are expressed (Locke et al., 1991, Salo et al., 1992) in response to heat or oxidative stress (Freeman et al., 1995, McArdle et al., 2001). Among them, the 70-kDa shock protein (Essig et al., 1997) is crucial, as it is involved in mitochondrial biogenesis (Stuart et al., 1994): substitution of oldest mitochondria with

neoformed ones endows with a greater antioxidant capacity and smaller susceptibility to oxidants (Hamberger et al., 1969, Leikte et al, 1966, Edington et al., 1972, Di Meo et al., 1992). Induced expression of HSP proteins provides short-term cytoprotection and facilitates adaptations depending on “de novo” protein syntesis (Marber et al., 1995, Storz et al., 1996); mechanisms by which HSPs provide cytoprotection may involve refolding of denaturated or aggregated proteins (Plumer et al., 1996).

The mechanisms underlying adaptative changes of SOD and catalase activities, and HSP content, are not fully elucidated. Recently, it has been shown that free radicals activate or modify the activity of redox-sensitive transcripton factors, including nuclear factor-kB (NF-kB) (Janssen et al., 1999), metal response element binding protein-1 (MRE-BP 1) (Dalton et al., 1996), activator protein-1 (AP-1) and heat shock factors (Storz et al., 1996, McArdle et al., 2001). All these transcription factors contain one or more cysteine residues, critical for their activity: the thiol/disulfide redox state is a key determinant of redox homeostasis (Zembron-Lacny et al., 2000, Droge 2002). Indeed, the GSH/GSSG ratio is often used as a marker of intracellular redox state: a decrease indicates that RO/NS production exceeds total reducing capacity.

3. VITAMIN C

Vitamin C is involved in a number of biochemical pathways needed for metabolism and health of exercising individuals, including synthesis and activation of neuropeptides, collagen and carnitine, and protection against exercise-triggered RO/NS generation (Peake 2003).

3.1 BIOCHEMICAL FUNCTIONS

Vitamin C, also referred to as ascorbic acid (AA) or ascorbate, belongs to the water-soluble class of vitamins. It exists in two active forms: the reduced form known as AA and the oxidized form dehydroascorbic acid (DHA). Physiologically, AA provides electrons for enzymes, chemical compounds that are oxidants, or other electron acceptors. Under physiological pH, the predominant form is the ascorbate anion, that undergoes oxidative process, leading to an intermediate compound, the ascorbyl radical (AFR) (Frei 1994, Jacob 1999). The AFR is a radicalic, not dangerous compound, as it is not a strong oxidizing/reducing compound and, moreover, its reactivity with the oxygen (and thus the possibility to generate the superoxide anion) is low (Fig 6).

Vitamin C is involved in many reactions of hydroxylation, involved in the synthesis of collagen (Bremer 1983), carnitine (Nelson et al., 1981), norepinephrine and serotonin; all of them are catalyzed by oxygenases (mono- and di-oxygenases), which contain iron or copper as prostetic group (Dhariwal et al., 1989, Murthy et al., 1987, Padh 1990, Jacob 1994). Since AA is involved in the synthesis of hormones, hormone-releasing factors, and neurotransmitters, it plays important regulatory roles throughout the entire body. Animal models have shown that AA is important in nervous system development, especially in maturation of glial cells and myelin; furthermore, it regulates the activity of some neurons, by modulating the synthesis of neurotransmitter receptors and the whole neurotransmitter dynamics. The vitamin is also important for iron absorption and metabolism. Bile acid formation, and hence cholesterol degradation, are highly dependent on AA. Vitamin C may also has vasodilatory and anticlotting effects within the body, by stimulating nitric oxide release. Physiological effects (such as an antihistamine modified bronchial tone and insulin responses) have been linked to AA.

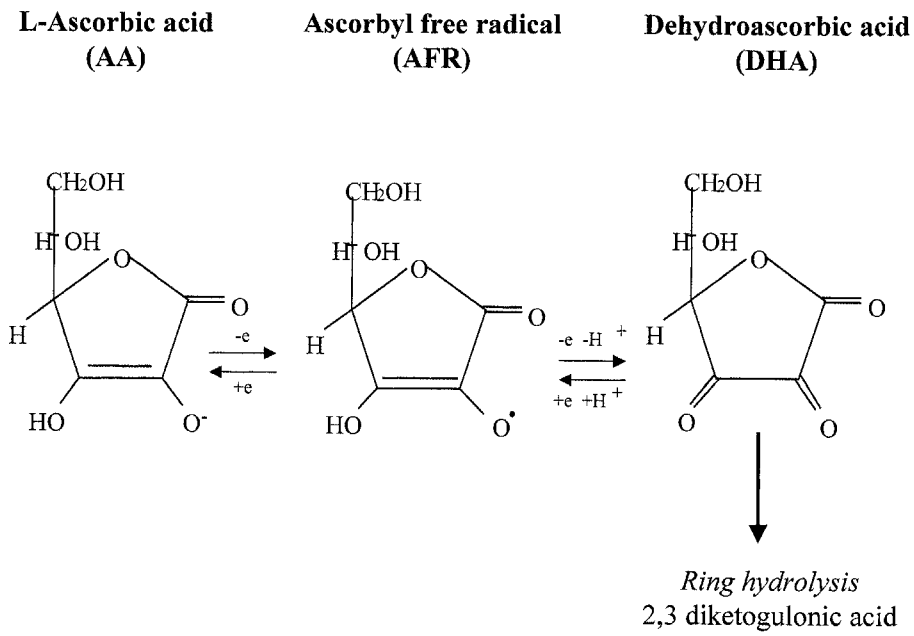

Fig. 6. Vitamin C redox status.

Protection of neural and endothelial tissues, along with effects on cellular tone, can also be attributed to vitamin C. Other functions include regulation of nucleotide concentrations, immune and endocrine functions. Finally, an *in vivo* function of vitamin C is quenching of free radicals: indeed, vitamin C sequesters the singlet oxygen radical, stabilizes the hydroxyl radical and regenerates reduced vitamin E back to the active state. These reactions work together to halt peroxidation of cellular lipid membranes (Sen 2001, Padh 1991, Englard et al., 1986).

3.2 METABOLISM

3.2.1 Transport

Skeletal muscle contains about 40% of whole body vitamin C content. All members of the animal kingdom (except men, guinea pig, primates and some fishes and birds) are able to synthesize ascorbic acid by the glucuronic acid pathway, in liver or kidney (Nishikimi et al., 1994). Since muscle cells cannot *de novo* synthesize the vitamin, they must import it from extracellular fluids and store it inside cells at high concentrations (3-4 mg/100 g) (Peake 2003).

Vitamin C is imported inside cells by two different systems (Fig. 7); a specific Na^+ dependent co-transporter for AA and a facilitative glucose transporter for DHA. There are two Na^+-dependent transporters (SVCT1 and SVCT2) in mammalian cells (Tsukaguchi et al., 1999). SVCT1 and SVCT2 are highly homologous to each other; however, they have different functional characteristics, with SVCT2 exhibiting a higher affinity/lower capacity for AA than SVCT1. Furthermore, the two isoforms show a different tissue distribution: in situ hybridization experiments and northern analysis revealed that SVCT1 is largely confined to bulk transporting epithelia (e.g., small intestine, kidney) (Tsukaguchi et al., 1999 Wang et al., 2000), as well as in other epithelial and endocrine tissues (e.g. skin, liver, lung, prostate, ovary, pancreas, thymus) (Wang et al., 2000, Savini et al., 2002) . The distribution and kinetic parameters suggest that the primary role of SVCT1 transporter is the absorption of dietary AA and renal reabsorption, thus maintaining the whole-body homeostasis. SVCT2 is widely expressed, and its mRNA has been detected in several metabolically active and specialized cells and tissues (neurons, brain, eye, placenta, osteoblasts, chondrocytes) (Tsukaguchi et al., 1999, Castro et al., 2001, Rajan et al., 1999, Kannan et al., 2001, Wu et al., 2004, Clark et al., 2002); the possible role for this transporter may be the widespread uptake of AA required to protect metabolically active cells from oxidative stress. It has been recently demonstrated that mice lacking SVCT2 die within minutes of birth, supporting the important role of this transporter in development (Sotiriu et al., 2002).

Skeletal muscle cells, expressing the hexose GLUT transporters (Korcok et al., 2003) can import DHA (Fig.7). These transporters are unlikely to play a major role in vitamin C uptake under physiological conditions, as the high concentrations of glucose will block the influx; however,

this pathway may be crucial during oxidative stress, when the oxidized vitamin is locally concentrated.

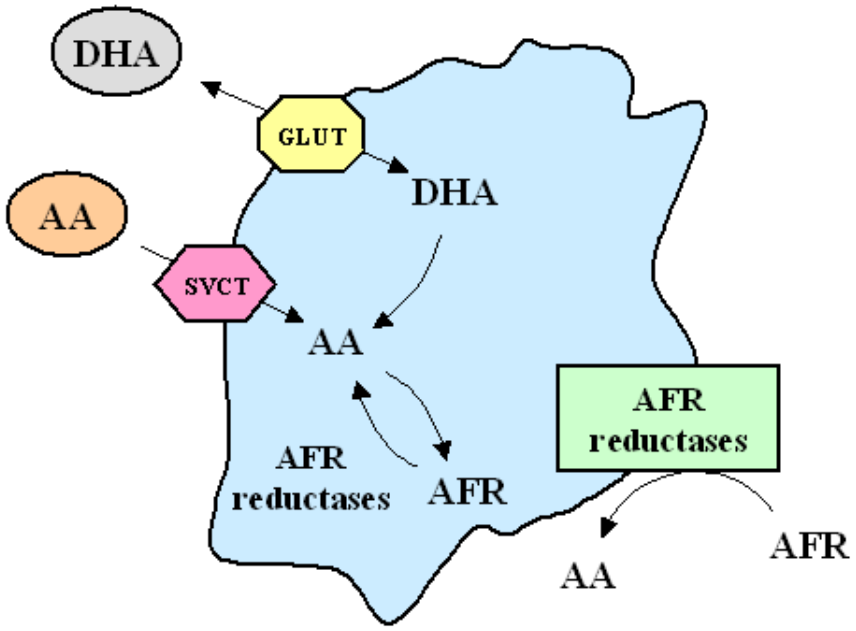

Fig. 7. Vitamin C transport and recycling systems.

3.2.2 Recycling

Playing its physiological role, AA is oxidized to AFR, which then rapidly disproportionates to AA and DHA. Under neutral or alkaline conditions, DHA undergoes an irreversible hydrolysis, with opening of the ring and loss of vitamin activity; the product of this reaction (2,3-dioxo-L-gulonic acid) is splitted in oxalic acid and L-threonic acid.

The active, reduced form of vitamin C can be regenerated both from AFR

and DHA. In those organisms unable to synthesize AA, the efficiency of systems involved in vitamin C recycling is essential to lower dietary requirements (Fig.7).

AFR reduction is mediated by several enzymatic systems, localized in almost all cellular compartements. AFR can be reduced by (i) the plasma membrane NADH-dependent system, (ii) the microsomal cytocrome b_5-cytocrome b_5 reductase system (Hara et al., 1971), (iii) the cytosolic and mitochondrial NADPH-dependent thioredoxin reductase (May et al., 1998).

DHA can be reduced to AA in an enzymatic or non-enzymatic manner through a direct chemical reaction with reduced glutathione (Basu et al., 1979). The exact nature of the enzymatic systems involved in DHA reduction, in human cells, is still under debate, though a tissue-specific difference appears to be implicated. Many studies indicate that different enzymatic systems are involved in DHA reductase activity, though current experimental evidence does not specify the extent to which this occurs *in vivo*. Enzymatic systems of mammalian tissues include: (i) glutathione-dependent DHA reductases, such as glutaredoxin, protein disulfide isomerase (Wells et al., 1990) and glutathione peroxidase (Washburn et al., 1999) (Fig. 8); (ii) NADPH-dependent DHA reductases, such as thioredoxin reductase (May et al., 1997, Mendiratta et al., 1998) and 3α-hydroxysteroid dehydrogenase (Del Bello et al., 1994); (iii) NADH-lipoic-acid-dependent lipoamide dehydrogenase (Xu et al., 1996).

The reduction of DHA to AA by intracellular enzymes keeps the cytosolic concentration of DHA low, thus contributing to a gradient favoring DHA uptake across the plasma membrane (Himmelreich et al., 2002).

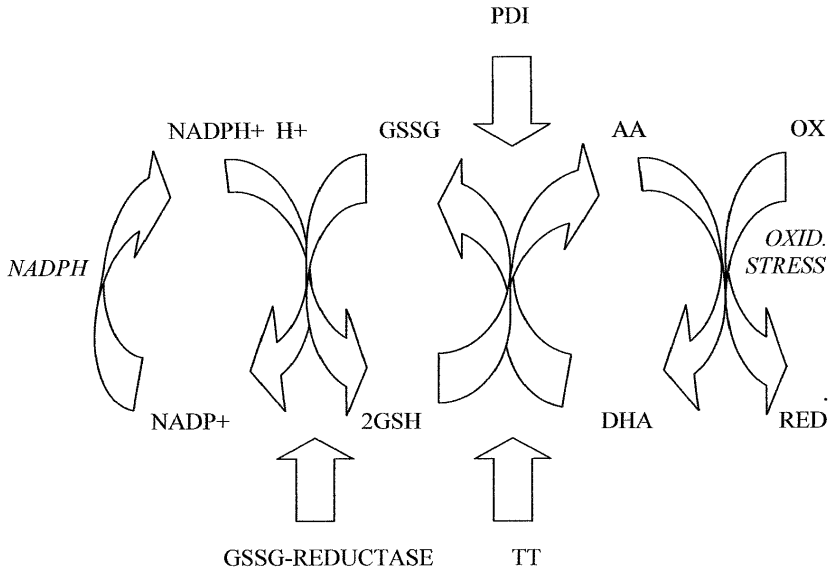

Fig. 8. Model of GSH-dipendent DHA reductase activity, mediated by systems involving thioltransferase (TT) and protein-disulphure isomerase (PDI).

3.3 EFFECTS OF EXERCISE ON VITAMIN C REQUIREMENTS

Since vitamin C is involved in biochemical pathways correlated to exercise metabolism, it is conceivable that physical activity may enhance utilization, metabolism and excretion of AA, thereby increasing dietary requirement for the vitamin. The first evidence of a possible link between vitamin C and exercise came rather unexpectedly from the observation of scurvy symptoms exhibited by explorers on early polar expeditions (Norris 1983). Diet consumed by these explorers was likely to be lacking in foods containing vitamin C, and such a stressful existence could have exacerbated the effects of a vitamin C-deficient diet, so that the vitamin turnover and requirement were increased.

While vitamin C does not appear to improve athletic performance (Gerster 1989), athletes may have increased dietary vitamin C requirements, and supplementation may have important effects on athletes that are more subtle than improvements in aerobic capacity or performance measures (Evans 2000).

Several reports indicate that vitamin C needs are increased in those who exercise (Keith 1997); however conflicting results exist, depending on type of physical exercise and of biochemical analysis performed on markers of oxidative stress. The disparity among these studies may derive from the observation that ascorbic acid is a water-soluble antioxidant, while the best studied markers of oxidative stress concern lipid peroxidation.

Several studies have highlighted that plasma ascorbic acid concentrations fall below baseline, in the days following exercise (Gleeson et al., 1987, Liu et al.,1999, Nieman et al., 2000, Peters et al., 2001b). This decrement may reflect alterations in tissue antioxidant demand following exercise; 4 days post exercise, reduced plasma AA concentrations are parallelled by decreased plasma antioxidant capacity and increased susceptibility of low-density lipoprotein (LDL) to oxidation (Liu et al., 1999). Concentrations of plasma AA have been reported to be significatively higher than baseline values five minutes after a 21 km (13.1 mile) run; levels then fell 20% below pre-exercise values within 24 hours and remained depressed for 2 days (Gleeson et al., 1987). Contradictory results were found by Rokitzki et al. (Rokitzki et al., 1994b) and Glesson et al. (Gleeson et al., 1987), who found that AA levels remained high for 24 hours following a marathon. The fluctuating levels of vitamin C are likely controlled by the adrenal gland (Gleeson et al., 1987).

Many investigations on the potential effects of high-dose vitamin C

supplementation on physical performance have been performed. The variables studied were: maximum oxygen uptake, blood lactic acid levels, heart rate after exercise and, in some cases, performance in competitive events. The obtained results were equivocal and a direct effect could not be demonstrated (Gerster 1989). On the other hand, a sub-optimal vitamin C state seems to be correlated to impaired working capacity, which can be normalized by restoring vitamin C body pools (Bucci 1998).

Ultra long-distance runners experience upper respiratory tract infections; Peters et al. (Peters et al., 1993) reported that vitamin C supplementation may be beneficial in preventing infections in ultramarathoners.

Multiple studies have shown blood vitamin C levels of athletes (including runners) to be adequate (Gleeson et al., 1987, Weight et al., 1988). An intake of 100-500 mg seems to be sufficient to meet needs of exercising individuals (Bucci 1998); this level can easily be obtained through a fruit and vegetable inclusive diet. Conversely, it is known that a vitamin C-lacking diet will inhibit performance (Bucci 1998). However, until an alternative recommended dietary intake (RDI) for athletes will be established, the recommended vitamin C intake shall remain at 75 mg for women and 90 mg for men. Doses of 1500 mg (or greater) will oversaturate the body pool and will be excreted in the urine, therefore, large doses are not warranted.

4. RO/NS AND GENE EXPRESSION

As seen before, the intracellular redox state may influence the transcription of specific genes, conferring to RO/NS a regolatory function on gene expression (Muller et al., 1997, Zhou et al., 2001). A pletora of transcription factors responds to alterations in redox state and, among them, AP-1 and NF-kB are the best studied.

AP-1

The activator protein-1 (AP-1) is a dimeric transcription factor modulated by intracellular redox state (Kroncke 2003; Arrigo 1999). This nuclear transcription factor consists of homo- or heterodimers between Fos (c-Fos, FosB, Fra-1, Fra-2), Jun (c-Jun, JunB, JunD) and activating transcription factor (ATF2, ATF3, B-ATF) family members (Karin et al., 1997).

The Jun and Fos proteins are composed of a leucine-zipper motif and the heterodimer is formed via a twisted helics structure; Fos-Jun or Jun-Jun dimerization is required for DNA binding.

The dimers modulate transcription by binding the so-called 12-0-tetradecanoylphorbol 13-acetate (TPA) response element (TRE; the sequence is TGACTCA) or the cAMP-responsive element (CRE; the sequence is TGACGTCA), present in the promoter region of a wide variety of genes implicated in cell proliferation and tumor promotion (Hai et al., 1991). Thus, distinct heterodimers can be generated, each one with a different DNA binding activity; such an example, cFos-containing heterodimers activate AP-1 target genes, while Fra-containing heterodimers do not. This because Fra proteins lack the C-terminal transactivation domain, thereby acting as negative regulators of AP-1 activity; indeed, they antagonize the transcriptional effects induced by either TPA or expression of c-Jun and c-Fos.

AP-1 is activated by several stimuli, including growth factors, cytokines, hormones, protein kinase C (PKC) activators (such as TPA), UV irradiation and modulators of intracellular redox homeostasis. Most of these inducers (mitogens, TNFα, UV and ionizing radiations, hydrogen peroxide) promote intracellular ROS accumulation and alteration of the thiol state (Marshall et al., 2000).

In resting cells, c-Jun homodimers predominate, as cFos mRNA and proteins have shorter half-lives than cJun. Upon stimulation, c-jun and c-fos

genes are induced and a rapid accumulation of these proteins occurs, although with distinct mechanisms. (Treisman 1992, Rozek et al.,1993)

The abundance of AP-1 components is, thus, controlled by a transcriptional mechanism, which involves a complex signalling cascade. Several serine-threonine (the so-named MAP kinases) are recruited and activated by phosphorylation-dependent mechanisms, which signal extracellular stimuli to the nucleus. (Karin 1995; Minden et al., 1997). At least three sub-groups of MAP kinases have been described (ERK: Extracellular signal regulated kinase; JNK: c-jun N-terminal kinase; p38), and all of them are involved in distinct steps of AP-1 activation. The MAPK pathway also exerts a post-translational control, regulating the half-life and activity of AP-1 proteins.

Targets of redox regulation reside also upstream, as ROS-sensitive kinase cascades (including MAPK, PKC and JNK) can be detected in AP-1 signalling. An intriguing finding concerns the mode of AP-1 regulation by thiols (thioredoxin and glutathione) and antioxidants: AP-1 appears to be an antioxidant-responsive, rather than a ROS-activated, transcription factor (Meyer et al., 1993; Schenk et al., 1994) (Fig.9). Overexpression of thioredoxin or phenolic antioxidant supply increase TPA–induced activation of AP-1; moreover, although hydrogen peroxide leads to a strong induction of the c-jun and c-fos mRNAs, nonetheless AP-1 ability to bind TREs is surprisingly weak. This phenomenon can be explained considering that Fos-Jun complexes possess a conserved cysteine in their DNA binding domain, which must be in the reduced state. Another explanation may be that antioxidants interfere with AP-1 transcriptional activity by triggering the expression of Jun-Fra heterodimers, thus leading to formation of inhibitory complexes.

The conserved cysteine residue contributes to regulation of DNA binding, through reversible oxidation in sulfenic (RSOH) or sulfinic (RSO2H) acids. But, it can also be subjected to S-nitrosylation by NO, thus reversibly inhibiting AP-1 binding to DNA (Pineda-Molina et al., 2001). However, it is not clear whether NO is an activator or inhibitor of AP-1, since NO-donors have been shown to increase AP-1 binding and transcription of c-fos and c-jun (Pilz et al., 1995, Morris 1995). One explanation for the NO effect could be that it is a molecular component in the signal transduction pathway other than AP-1 itself that transduces the NO signal.

NF-kB

The NF-kB/Rel proteins are a family of ubiquitously expressed transcription factors, which modulate genes involved in inflammatory and immune responses, as well as in cellular proliferation (Shishodia & Aggarwal, 2004). The family consists of five members: p50/p105 (also named NF-kB1), p52/p100 (also named NF-kB2), p65 (also named RelA), RelB and c-Rel. NF-kB1 and NF-kB2 are synthesized as large precursors (p105 and p100, respectively), that are cleaved to give rise to the mature form of the protein (p50 and p52, respectively). All the family members share a 300-amino acid, N-terminal domain, named the Rel-homology-domain (RHD), needed for nuclear translocation, DNA binding and dimerization. Indeed, the NF-kB/Rel proteins function as homo- or hetero-dimers, and the most abundant form inside cells is a heterodimer complex containing the p50 and p65 subunits. Dimers bind to consensus kB sequences (GGGPuNNPyPyCC, where Pu is a purine, Py is pyrimidine and N is any base) present in the promoter of NF-kB-responsive genes, in a cell-type- and stimulus-dependent manner, thus leading to specific patterns of transcriptional activation.

Under resting conditions, NF-kB is sequestered in the cytoplasm through interaction with a family of inhibitory proteins, known as IkB (including IkBα, IkBβ, IkBε and IkBγ, BCL-3 and the precursor proteins p100 and p105), which contain five to seven C-terminal ankyrin repeats. This domain binds the dimerization domain of NF-kB, thus preventing nuclear translocation and maintaining NF-kB in an inactive state.

NF-kB signalling can occur through two distinct pathways, which selectively involve different subunits of the IkB kinase (IKK) complex (Quivy et al., 2004; Yamamoto et al., 2004; Hayden et al., 2004). The IKK complex consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ/NEMO). Several stimuli (including cytokines, viruses, endotoxins, phorbol esters, UV light and ionizing radiations) activate the IKK complex through at least two distinct mechanisms: by involvement of "mitogen-activated protein kinase kinase kinase family" (MAP3K) or by direct IKK recruitment to receptor complexes which leads to IKK autophosphorylation and subsequent activation.

In the first pathway, activated IKK phosphorylates IkB proteins, while, in the alternative pathway, IKK phosphorylates the precursor p100 protein. Phosphorylated IkBs are recognized by the ubiquitin ligase machinery; in the first pathway, polyubiquitination leads to IkB degradation by the

proteasome, while, in the alternative pathway, it leads to p100 processing. In any case, free NF-kB dimers are able to migrate in the nucleus, bind DNA and, thereby, induce mRNA synthesis (Fig. 9).

Many events of NF-kB signalling, including phosphorylation and DNA binding, are driven by intracellular redox homeostasis, especially by the thiol-disulfide balance (Arrigo, 1999). Nonetheless, the precise nature of the oxygen sensing and transduction pathways are not fully understood.

Evidences of redox regulation of NF-kB come from the observation that almost all stimuli triggering NF-kB activation produce reactive oxygen species (ROS) or are oxidants by themselves (H_2O_2, superoxide), and that, antioxidants [*N*-acetyl-L-cysteine (NAC), glutathione, catalase, dithiocarbamates, vitamins, glutathione peroxidase, selenium] inhibit activation (Flohè et al., 1997; Schoonbroodt et al., 2000; Janssen-Heininger et al., 2000; Bowie et al., 2000).

Furthermore, p50 contains a critical cysteine residue at position 62, which has to be maintained in the reduced form for DNA-binding activity. Thus, it seems that oxidative conditions in the cytoplasm promote NF-kB activation and nuclear transport, while a reducing environment in the nucleus is required for NF-kB/DNA interaction. Free radicals has been shown to stimulate IkB phosphorylation, but it is not known if they directly stimulate IKK activity or act upstream; also proteasome activity can be modulated by changes in redox state.

Nitric oxide (NO) and reactive nitrogen species also play a regolatory role (Kroncke, 2003; Matthews et al., 1996; Pineda-Molina et al., 2001, Lee et al., 1997). Like for ROS-mediated modulation, the effects appear to be cell type-specific and concentration-dependent (Colasanti et al., 2000; Marshall et al., 2002). NO-donor compounds inhibit the DNA binding activity of both p50-p65 homo- and hetero-dimers, by S-nitrosylation of a critical thiol (Cys62) present in the DNA binding domain of the p50 subunit (DelaTorre et al., 1997; Marshall et al., 2001). In addition, high doses of NO S-nitrosylate IKKβ, thus preventing IkB phosphorylation and degradation, and contributing to NF-kB inactivation (Reynaert et al., 2004). On the other hand, low levels of NO augment NF-kB activity potentially via increased activation of IKKα. NF-kB binding sites are present in the promoter region of the gene encoding inducible nitric oxide synthase (iNOS), one of the enzymes responsible for NO production within the cell (Kleinert et al., 2003; Adams et al., 2002). This negative feedback loop seems to be necessary to block a prolonged activation of NF-kB, thereby limiting chronic inflammation.

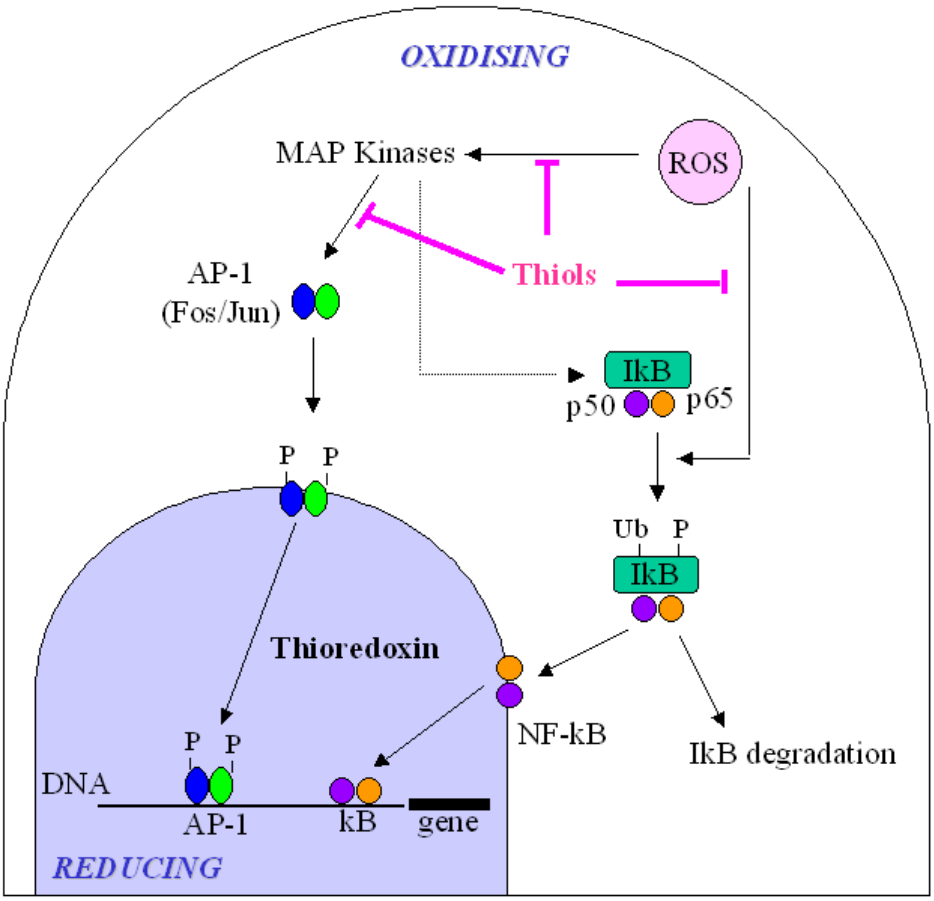

Fig. 9. AP-1 and NF-kB redox signaling patway.

4.1 AP-1 AND NF-kB TRANSCRIPTION FACTORS AND INTRACELLULAR REDOX BALANCE

Cellular redox state regulates AP-1 and NF-kB activities, as cytoplasmic oxidants induce c-jun and c-fos expression (Datta et al., 1992, Nose et al., 1991, Stein et al., 1989, Wenk et al., 1999) and remove the NF-kB/I-kB binding (Baeuerle et al., 1994, Galter et al., 1994). Thus, the two transcription factors are activated in the cytosol by an oxidising environment, while in the nucleus several molecules are present [including thioredoxin (Trx)], which promote a reducing environment needed for the binding of transcription factors to the DNA (Fig. 9). Under oxidative condition, thioredoxin is carried into the nucleus where it reduces cysteine residues, thus increasing transcriptional activity of these redox-sensitive factors (Wei et al., 2000, Hirota et al., 1999, Meyer et al., 1993, Schenk et al., 1994). Indeed, AP-1 and NF-kB contain crucial cysteines in their DNA binding domain, which must be in the reduced state to allow binding to responsive elements located in the promoter of target genes (Abate et al., 1990).

From these findings, it appears clear that oxidative stress induces cellular modifications, which lead to adaptative responses. A mild oxidative stress, generated by non-exhaustive exercise, has been shown to generate changes in the redox balance, by modulating the expression of genes containing AP-1 and/or NF-kB responsive elements in their promoters (Bergelson et al., 1994, Sekhar et al., 1997, Mulchahy et al., 1997). For example, muscle SOD increases during contractile activity and this induction is related to NF-kB and AP-1 activation (Ji 2002). GPx and CAT genes also revealed putative binding motifs for NF-kB and AP-1.

The exposure to low levels of RO/NS exerts dramatic effects not only on the antioxidant defences, but also on several metabolic pathways coupled with contractile activity, including transport of glucose (Kozlovsky et al., 1997), activity of Na^+,K^+-ATPase (Skou et al., 1992) and creatine kinase (Kenyon et al., 1983), and depolarization-induced calcium release (Posterino et al., 1996).

It seems evident that oxidants and antioxidants act in a way more complex than expected (Fig.10). Cellular antioxidants, regulating RO/NS levels, appear as reversible redox modifiers of several signalling pathways (including kinases, phosphatases and transcription factors). From this point of view, a random supplementation of antioxidant molecules might modify the natural balance occurring inside cells, thus not being favourable for

health. More studies are necessary to clarify the exact redox changes occurring during contractile activity and muscle differentiation before any generalization.

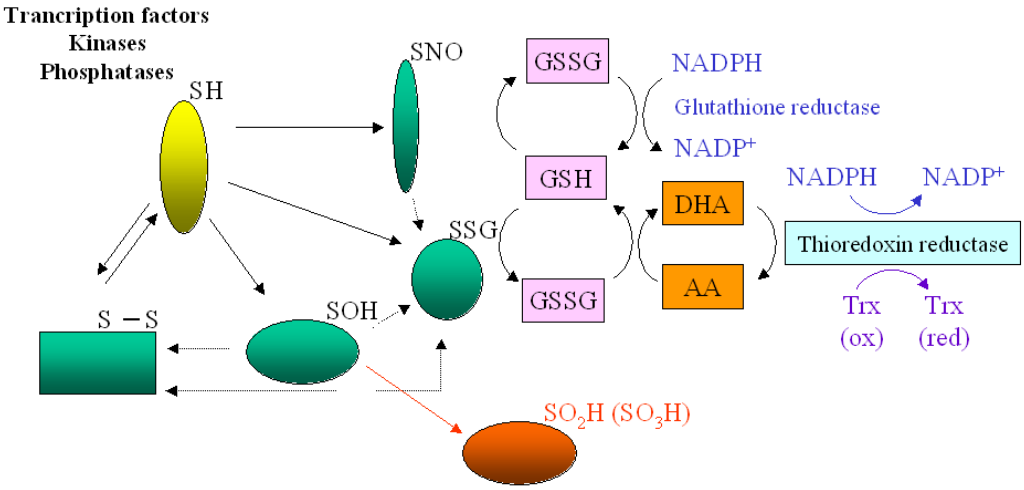

Fig. 10. Cellular antioxidants, regulating RO/NS levels, appear as reversible redox modifiers of several signalling pathways (including kinases, phosphatases and transcription factors).

4.2 AP-1 AND NF-kB TRANSCRIPTION FACTORS AND MUSCLE DIFFERENTIATION

The intracellular interplay between oxidizing and reducing equivalents is also involved in myogenic differentiation. Two groups of myogenic transcription factors allow cell cycle arrest and fusion of myoblasts into multinucleated myotubes: (i) the myogenic regulatory factors (MRFs), including MyoD, myogenin, Myf5 and MRF4, and (ii) members of the myocyte enhancer binding factor 2 (MEF2) family.

The combined action of these two groups of proteins drives the expression of muscle-specific genes, including α-actin, muscle creatine kinase and myosin heavy chain. In vitro, differentiation can be achieved by

removing growth factors from medium, leading to G1-arrest and expression of markers of myocyte fusion.

The nuclear binding activities of AP-1 and NF-kB must be down-regulated before differentition (Lehtinen et al., 1996). Indeed, NF-kB inhibits myogenic differentiation by modulating the activity of MyoD, myogenin and cyclin D1 (Guttridge et al., 1999). Down-regulation of AP-1 activity also precedes activation of the myogenin promoter and initiation of myocyte fusion. Furthermore, during myogenesis, AP-1 changes its subunit composition: in differentiating cells, Fra-2 becomes the major component of the AP-1 complexes and, together with c-Jun and JunD, transactivates the MyoD promoter (Andreucci et al., 2002).

Regarding the involvement of AP-1 and NF-kB in muscle differentiation, it appears clear that redox-dependent mechanisms play a fundamental role during myogenesis: indeed, GSH seems to be necessary to ensure NF-kB inactivation and formation of myotubes (Ardite et al., 2004).

OBJECTIVES OF THE RESEARCH.

Aim of this research was to investigate the oxidative stress resistance mechanisms in skeletal muscle cells, and its impact on adaptative changes, such as the antioxidant profile and the redox intracellular signaling.

We have chosen the mouse C2C12 and the rat L6C5 cell lines, as they are two useful experimental models to study skeletal muscle metabolism and redox changes.

First, we determined the susceptibility of muscle cells to oxidative stress and the activity of specific antioxidant systems, including glutathione, thioredoxin reductase and antioxidant enzymes (e.g. catalase and SOD).

Second, we clarified the functional characteristics of AA and DHA transporters in these cells, as well as the activity of vitamin C recycling systems. Indeed, it is not clear by which mechanism AA is imported inside these cells, since SVCT1 and SVCT2 seemed to be not expressed, as revealed by Northern analysis and in situ hybridization experiments (Tsukaguchi et al., 1999, Wang et al., 2000, Rajan et al., 2000). Furthermore, based on the interrelationship between glutathione (GSH) and AA, we also studied how vitamin C transport and recycling could adapt to oxidative stress induced by glutathione depletion.

Third, evaluation of the possible crosstalk between molecules involved in redox signalling has been done. To this end, the redox sensitive transcription factors (AP-1 and NF-kB), in both myoblasts and myotubes, were chosen to be studied. Finally, we investigated how the redox signaling could be modified by GSH loss.

RESULTS

5. THE RAT L6C5 AND MOUSE C2C12 SKELETAL MUSCLE CELLS SHOW DIFFERENT ANTIOXIDANT CAPACITY AND SUSCEPTIBILITY TO OXIDATIVE STRESS

5.1 L6C5 MYOBLASTS POSSESS LOW ANTIOXIDANT DEFENCES, WITH MYOTUBES SHOWING A GREATER ANTIOXIDANT POTENTIAL.

In unstressed cells, there is a tightly regulated balance between production and removal of ROS. Since muscle cells are continuously exposed to ROS generation, we measured parameters indicative of cellular antioxidant ability in L6C5 and C2C12 cells.

To this end, we measured the expression and/or the activity of antioxidant enzymes (AOEs), including TrxR, SOD, catalase and GPx, and the glutathione content in myoblasts and myotubes.

MYOBLASTS

As shown in Table I, we found significant lower values of catalase activity in L6C5 myoblasts (3.8 vs 7.4 U/mg of protein) with respect to C2C12 cells. Furthermore, although this cell line had higher levels of total glutathione (119 vs 79 nmol/mg of protein), as previously reported (Sen et al., 1993), nonetheless it showed a higher percentage of the oxidised form (8 vs 2% of C2C12 myoblasts). The higher GSSG/GSH ratio in L6C5 myoblasts was in agreement with its higher GPx activity (1.7 vs 0.5 mU/mg of protein measured in C2C12 cells). In contrast, proliferating C2C12 cells showed higher values of catalase activity with a lower GSSG/GSH ratio. Finally, C2C12 myoblasts showed higher levels of TrxR mRNA with respect to L6C5 cells (Fig. 11 A, compare lanes 1 and 3).

Thus, the two cell lines appeared to have a different profile, with C2C12 myoblasts showing a greater antioxidant potential as indicated by low level of GSSG. The reduced glutathione (GSH), a thiol-containing tripeptide, plays a vital role in maintaining cells in the reduced state and in protecting tissues from oxidative stress. By donating a pair of hydrogens, GSH is oxidized to glutathione disulfide (GSSG). The ratio of GSH to GSSG can be used as a hallmark indicator of intracellular redox status, indeed a decrease of this ratio indicates that the production of ROS exceeds the reducing

capacity of GSH and other antioxidants

MYOTUBES

After in vitro cell fusion and differentiation into multinucleated myotubes (Fig.12 A-D) expressing myogenin (Fig. 12 E), the antioxidant profile changed. Indeed, L6C5 myotubes showed a 3- and 1.3-fold increase for catalase and SOD activities, respectively, while GPx activity was reduced by about 50% (Table I). On the other hand, C2C12 differentiated cells showed decreased levels of catalase activity and increased levels of SOD and GPx activities, but these changes appeared to be less pronounced. Finally, both cell lines showed a fall in total glutathione content (Table I) and TrxR expression (Fig. 11 A, lanes 2 and 4). Thus, L6C5 differentiated cells significantly ameliorated the antioxidant state, especially increasing catalase activity (from 3.8 to 11.8 U/mg of protein) and reducing the percentage of oxidised glutathione (from 8.1 to 3.7 %).

Table I: Antioxidant state in L6C5 and C2C12 cells.

	L6C5		C2C12	
	myoblasts	myotubes	myoblasts	myotubes
SOD[a]	1.6 ± 0.08	2.1 ± 0.10	1.7 ± 0.08	2.2 ± 0.10
Catalase[a]	3.8 ± 0.20	11.8 ± 0.70	7.4 ± 0.40	2.2 ± 0.10
GPx[b]	1.7 ± 0.09	0.8 ± 0.04	0.5 ± 0.02	0.9 ± 0.04
Glutathione[c]	119.0 ± 9.50	11.0 ± 0.90	79.0 ± 6.30	8.0 ± 0.60
GSSG/GSH (%)	8.1 ± 0.40	3.7 ± 0.18	2.4 ± 0.12	2.3 ± 0.11
Residual glutathione (after depletion)[c,d]	12.0 ± 0.90	4.1 ± 0.30	8.0 ± 0.60	1.6 ± 0.10

Proliferating, as well as differentiated, muscle cells were assessed for their SOD, catalase, and GPx activities, and glutathione content.
Data are the mean ± S.D. of three independent experiments, each one performed in triplicate.

a: U/mg of protein. One unit of SOD activity is defined as the amount of the enzyme required to inhibit the rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase. One unit of catalase activity is defined as the amount of the enzyme decomposing 1 μmol of H_2O_2 per min.

b: mU/mg of protein. One unit of GPx activity is defined as the amount of the enzyme catalyzing the oxidation by tert-butylhydroperoxide of 1 μmol of GSH per min.

c: nmol/mg of protein.

d: glutathione depletion was achieved by incubating cells with 200 μM (L6C5 cells) or 50 μM (C2C12 cells) BSO for 15 hours.

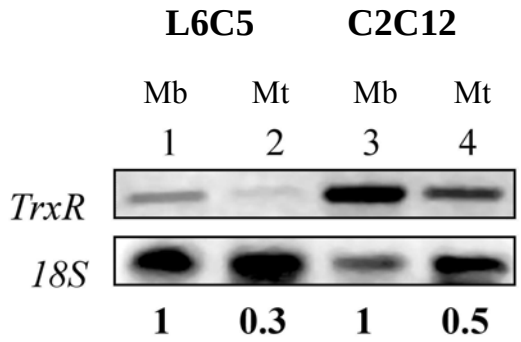

Fig. 11. Analysis by RT-PCR of thioredoxin reductase (TrxR) expression.
Total RNA was purified from L6C5 (*lanes 1-2*) and C2C12 (*lanes 3-4*) cells, in both myoblasts (Mb) (*lanes 1 and 3*) and myotubes (Mt) (*lanes 2 and 4*).
Total RNA was reverse-transcribed and amplified with primers specific for the cytosolic isoform of the enzyme. The blot is representative of two independent experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarily set to 1, after normalization with 18S rRNA.

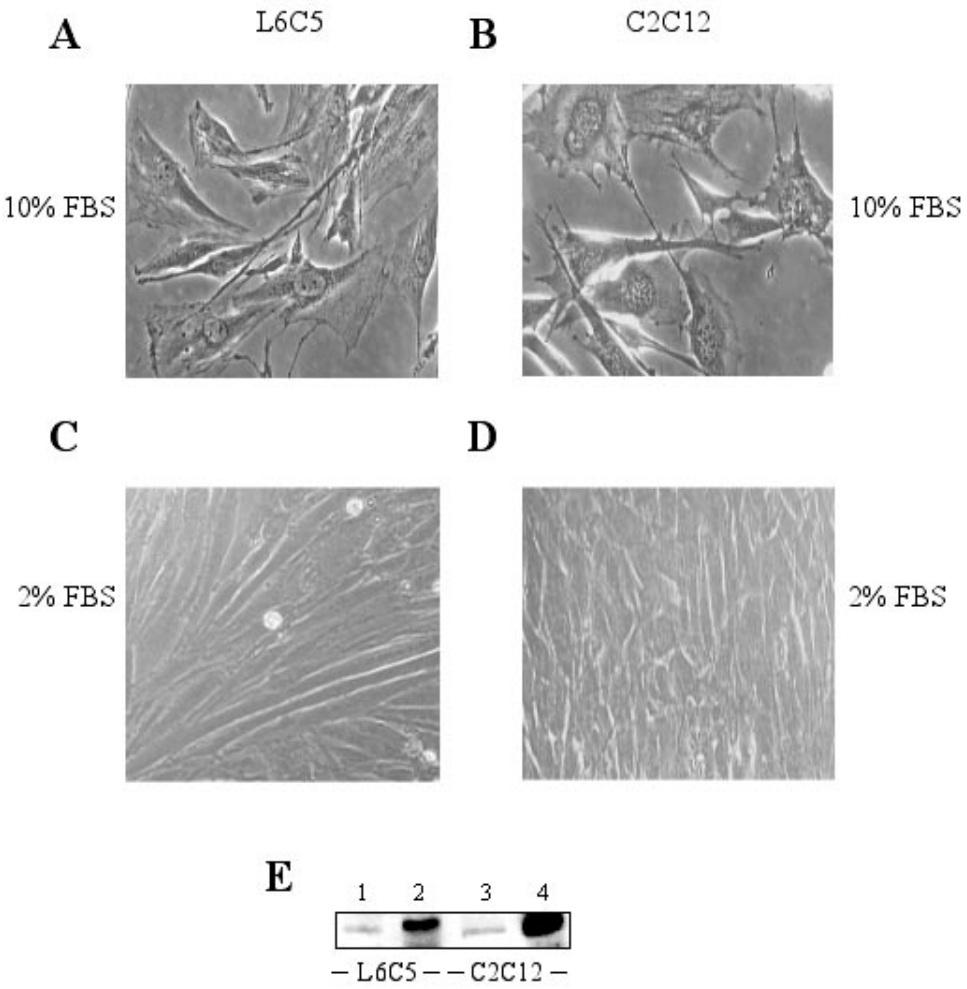

Fig. 12. Analysis of differentiation in L6C5 and C2C12 muscle cells.
(A-D) Phase contrast microscopy images of proliferating **(A-B)** and differentiated **(C-D)** cells. Reduction of serum from 10 to 2% allowed cell to cell fusion and formation of myotubes in both L6C5 **(A** and **C)** and C2C12 **(B** and **D)** cell lines.
(E) Expression of the differentiation marker myogenin by Western blot. Extracts from myoblasts (*lanes 1* and *3*) and myotubes (*lanes 2* and *4*) were immunoblotted with the anti-myogenin antibody. The radiograph is representative of two similar experiments.

5.2 L6C5 MYOBLASTS ARE SENSITIVE TO OXIDATIVE STRESS, BUT BECOME MORE RESISTANT DURING DIFFERENTIATION.

In order to evaluate if the different intracellular redox environment displayed by the two cell lines was reflected in a different response to ROS generation, ROS production in both proliferative and differentiated cells was evaluated.

MYOBLASTS

Corresponding to their lower redox state, L6C5 myoblasts showed endogenous ROS levels higher than C2C12 cells (Fig. 13 A). Furthermore, the two cell lines clearly differed in sensitivity to oxidative stress: exposure to increasing concentrations (50-300 μM) of H_2O_2 exerted differential apoptotic effects. As shown in Fig. 13 B, L6C5 myoblasts were 10-20 times more sensitive than C2C12 cells: in the presence of 100 μM H_2O_2 almost all L6C5 cells were dead, while essentially all C2C12 cells were still alive at 300 μM H_2O_2.

The different susceptibility to H_2O_2–mediated cell death was further confirmed by caspase-3-like activity. As shown in Fig.14 A, protease activity was induced by H_2O_2 dose-dependently only in L6C5 myoblasts and it was significantly inhibited by the pancaspase-inhibitor Z-VAD-FMK. The pancaspase–inhibitor did not completely abrogate caspase activity, suggesting that more than one type of cell death could be involved.

Exogenous supplementation with the hydrophylic antioxidant vitamin C improved the redox balance by lowering basal ROS levels: this effect was more pronounced in L6C5 myoblasts leading to 50% inhibition of free radical generation (Fig. 13 A). As a result, vitamin C exerted protective effects, especially in the sensitive cell line, reducing L6C5 cell death by about 90% (Fig. 14 B).

MYOTUBES

Accordingly to their improved redox balance, L6C5 myotubes acquired an apoptosis-resistant phenotype, characterized by lower basal ROS levels (Fig. 13 A) and improved survival after H_2O_2–triggered cell death (Fig. 13 B), with respect to proliferating cells; indeed, longer incubation times (24 hours instead of 1 hour) were necessary to see a significant number of

apoptotic nuclei, nonetheless the percentage of death was lower than that seen in myoblasts.

Accordingly, vitamin C protection was no longer evident in resistant myotubes. The sensitivity to ROS generation and oxidative stress remained unchanged in C2C12 myotubes (Fig. 13 A and B), again in line with the less pronounced changes in their antioxidant profile.

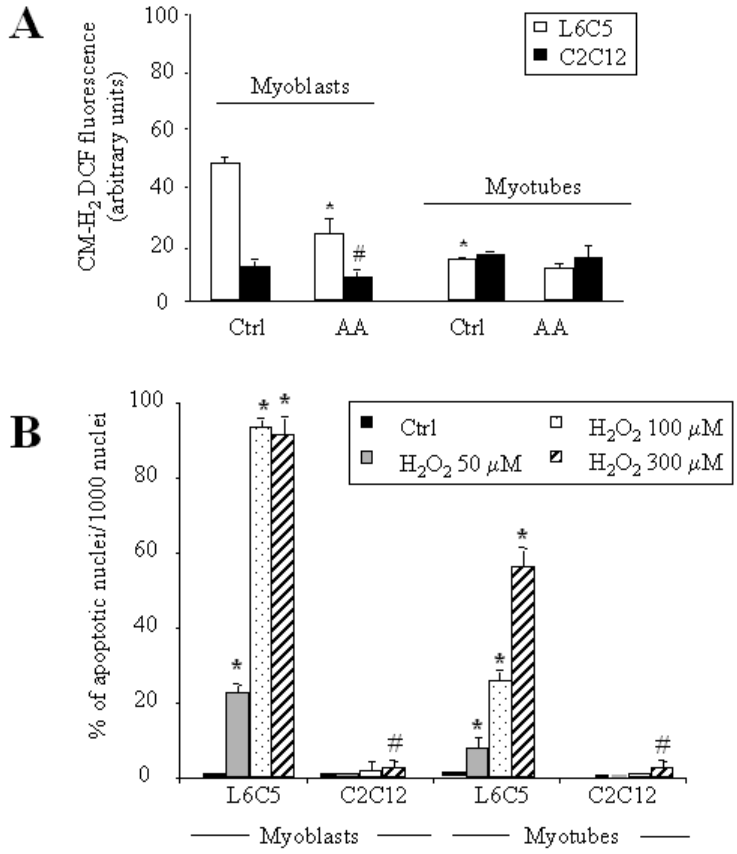

Fig. 13. C2C12 cells have a greater antioxidant potential and are more resistant to oxidative damage than L6C5 cells.

(A) Generation of intracellular peroxides in myoblasts and myotubes. L6C5 (white bars) and C2C12 (black bars) cells were left untreated or supplemented with 1 mM AA-2P for 24 hours before collecting them, and peroxides generation was assessed by flow cytometry, using CM-H_2DCFDA as probe. Results are means $\pm$ S.D. of three independent experiments, each one performed in duplicate. *: $p < 0.01$ vs control cells; $^{\#}$: $p < 0.05$ vs control cells (T-test).

(B) Induction of apoptosis in myoblasts and myotubes. Cells were left untreated or treated with 50-300 μM H_2O_2 for 1 (myoblasts) and 24 hours (myotubes). After incubation, cells were stained with Hoescht 33258 and condensed/fragmented nuclei were counted. Results are means $\pm$ S.D. of three independent experiments, each one performed in triplicate. *: $p < 0.01$ vs control cells; $^{\#}$: $p < 0.05$ vs control cells (T-test).

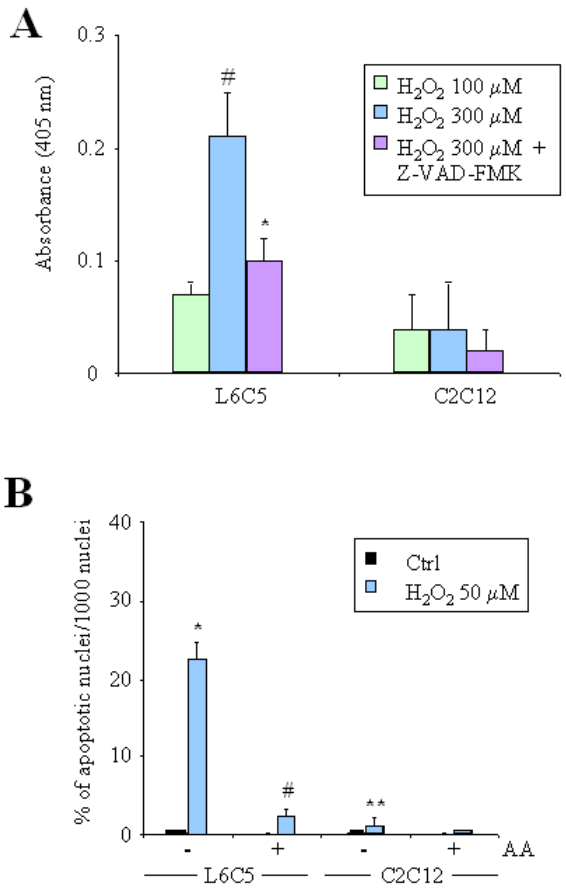

Fig. 14. C2C12 cells are more resistant to oxidative damage than L6C5 cells.
(A) Caspase-3-like protease activity in L6C5 and C2C12 myoblasts. Cells were treated with 100 and 300 μM H_2O_2 for 1 hour, lysed and assayed for caspase-3-like activity by spectrophotometric measurements (405 nm). For the higher dose, the assay was performed both in the absence or in the presence of pancaspase inhibitor (Z-VAD-FMK). Values are reported as net increase of absorbance with respect to untreated cultures and represent the mean ± S.D. of three experiments each one performed in duplicate. #: $p < 0.01$ vs control cells; *: $p < 0.01$ vs H_2O_2-treated cells (T-test).
(B) Protection against cell death exerted by ascorbic acid. Myoblasts were subjected to oxidative treatment as described above. When indicated, cells were pre-incubated for 24 hrs with 1 mM AA-2P and maintained in the presence of the vitamin until the end of treatments. Apoptotic nuclei were visualized by Hoescht 33258 staining. Results are means ± S.D. of three independent experiments, performed in duplicate. *: $p < 0.01$ vs control cells; #: $p < 0.01$ vs H_2O_2-treated cells; **: $p < 0.05$ vs control cells (T-test).

6. THE RAT L6C5 AND MOUSE C2C12 SKELETAL MUSCLE CELLS SHOW DIFFERENT VITAMIN C METABOLISM

6.1 TRANSPORT

AA is the first hydrophilic non-enzymatic antioxidant involved in RO/NS scavenging (Frei et al., 1989). As exogenous vitamin C supplementation has been shown to exert protective effects against oxidative stress (Fig. 13 and 14), we characterized vitamin C metabolism and homeostasis in the two cell lines.

First, we evaluated the characteristics of vitamin C transport. As the vitamin can be imported inside the cells both in the reduced and oxidized form, we looked at AA and DHA uptake.

6.1.1 ASCORBIC ACID

To assess the expression of AA transporters in C2C12 and L6C5 muscle cells, we performed RT-PCR analysis with primers specific for SVCT1 and SVCT2. The SVCT2 transcript was expressed in both cell lines and it was down-modulated by differentiation (densitometric analysis revealed 70 and 30% down-regulation in L6C5 and C2C12 cells, respectively).

The SVCT1 transcript was detected only in L6C5 myoblasts; however the expression levels were low, as a faint band could be seen by doubling the amount of target cDNA and increasing the number of cycles from 35 to 40, with respect to the conditions used for SVCT2 amplification (Fig. 15).

We then analyzed the functional characteristcs of AA transport by performing time-dependent and concentration-dependent experiments. As shown in Fig. 16, the time course of AA transport, performed at 100 μM substrate concentration, was linear for at least 15 minutes in both cell lines. Proliferating L6C5 cells were more efficient than C2C12 myoblasts.

When differentiated, both cell lines displayed a decreased AA transport, and this effect was more evident in L6C5 cells.

AA uptake was sodium dependent, since 97% of inhibition was achieved when choline chloride replaced NaCl. Michaelis-Menten analysis of dose-response studies revealed that C2C12 myotubes displayed a decreased V_{max} (0.27 nmol/min/mg protein) with respect to myoblasts (0.42 nmol/min/mg protein), although the affinity for AA remained unchanged (apparent K_m

values: 25 μM for myoblasts versus 24 μM for myotubes) (Fig. 17 A). Also L6C5 myotubes changed V_{max} values (0.20 nmol/min/mg protein for myotubes versus 0.66 nmol/min/mg protein for myoblasts), whereas K_m values were unchanged: 17 μM for myotubes versus 22 μM for myoblasts (Fig. 17 B). Eadie-Hofstee plots, accordingly to RT-PCR analysis, showed that only one component with SVCT2-like kinetic parameters (Wu et al., 2003, Korcok et al., 2000) was present in all examined cells (Fig. 18 A-B, C-D).

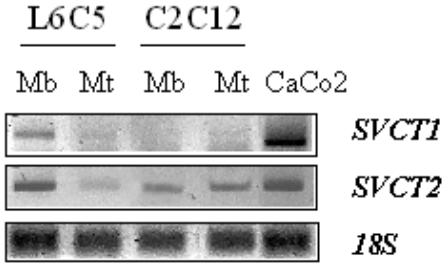

Fig. 15. RT-PCR analysis of SVCT1 and SVCT2 expression in C2C12 and L6C5 myoblasts (Mb) and myotubes (Mt).

Total RNA was reverse transcribed and amplified either with SVCT1 (*upper panel*) or SVCT2 (*middle panel*) specific primers; amplification of the 18S rRNA (*lower panel*) was also shown as loading control. CaCo2 cells were used as positive control. Blots are representative of three different experiments.

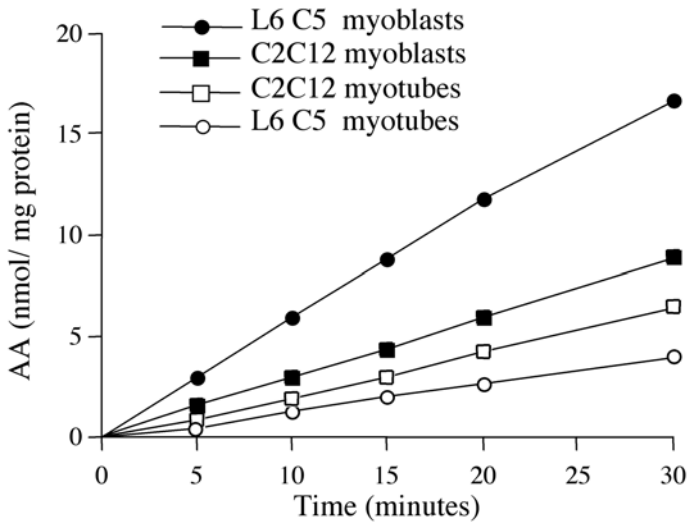

Fig. 16. Time-course of ascorbic acid transport in proliferating and differentiated C2C12 and L6C5 cells.

Cells were incubated with 100 μM AA for the indicated times. The AA uptake was determined by HPLC. Values are the mean of four independent experiments (S.D. ≤ 7%).

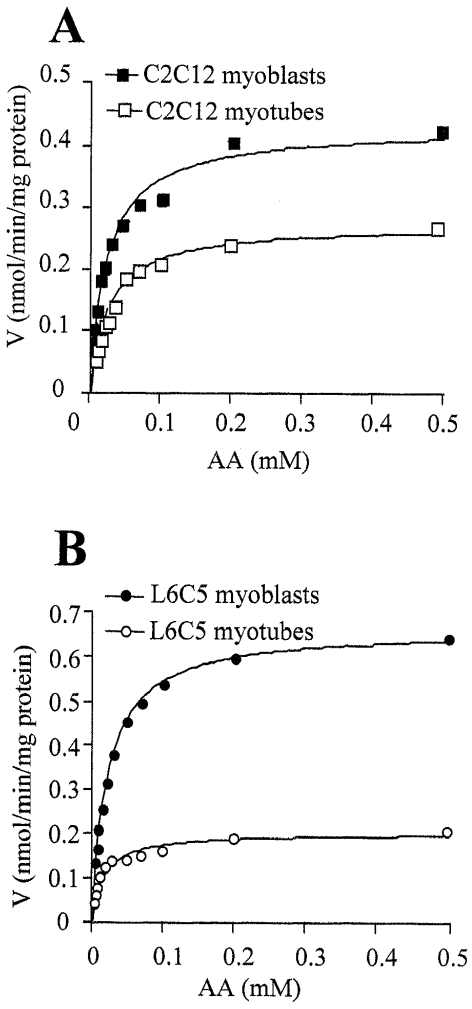

Fig. 17. Concentration dependence of AA transport in C2C12 (A) and L5C5 (B) cells.
Cells were incubated with different concentrations of AA (5-500 μM) for 10 minutes. Intracellular AA concentration was determined by HPLC.
Values are the mean of five independent experiments (S.D. ≤ 8%).

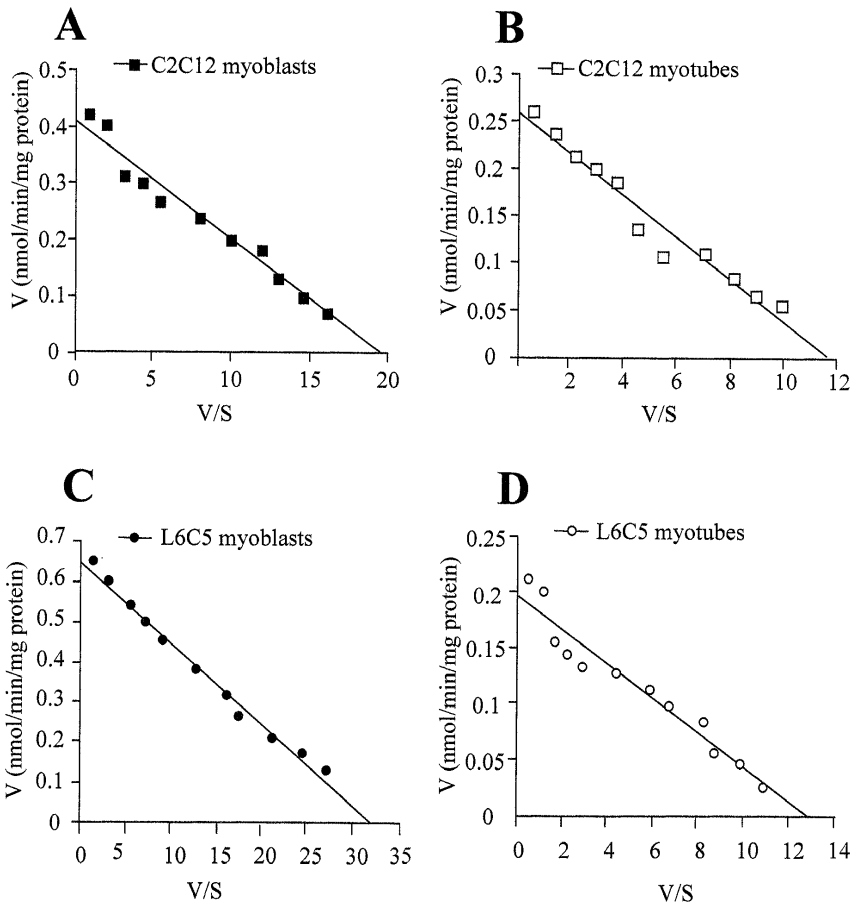

Fig. 18. Eadie-Hofstee plots of AA transport in C2C12 (A and B) and L5C5 (C and D) cells.

Cells were incubated with different concentrations of AA (5-500 μM) for 10 minutes. Intracellular AA concentration was determined by HPLC.
Values are the mean of five independent experiments (S.D. ≤ 8%).

6.1.2 DEHYDROASCORBIC ACID

Next, we assessed the ability of muscle cells to take up DHA. Dehydroascorbate uptake depends both on transport and reduction efficiency (Vera et al., 1995). To evaluate the intracellular DHA content, cell extracts have been incubated with or without the reducing agent dithiotreitol (DTT) for 10 minutes before HPLC analysis.

Time-course experiments (performed at 100 μM DHA) showed that, at the maximum accumulation time (30 minutes), proliferating C2C12 cells had an intracellular concentration of AA 3.7-fold higher than that found in L6C5 myoblasts (Fig. 19 A and C filled symbols); furthermore, all the imported DHA was reduced to AA intracellularly, as shown by superimposable curves of cell extracts with and without DTT treatment (Fig. 19 A and C).

Dose response experiments showed that the apparent K_m for DHA was comparable in the two cell lines (0.36 mM and 0.39 mM for C2C12 and L6C5 myoblasts, respectively), whereas the V_{max} was different (23.8 nmol/min/mg protein and 5.2 nmol/min/mg protein for C2C12 and L6C5 myoblasts, respectively) (Fig. 19 B and D). Above 100 μM DHA, the vitamin was all present in the reduced form in C2C12 cells (Fig. 19 B), while about 40% was in the oxidized form in L6C5 cells (Fig. 19 D). These data indicate that in L6C5 cells the reduction was a rate limiting-step, especially at high extracellular DHA concentrations. On the contrary, the reduction was not a limiting step in C2C12 cells, as demonstrated by the linearity of Eadie-Hofstee plots (Fig. 19 B, inset).

Both cell lines showed a decreased DHA transport during differentiation: indeed, C2C12 and L6C5 myotubes displayed 3 and 5 times less DHA uptake, respectively (Fig. 19 open symbols).

These results were compatible with the activity of GLUT transporters checked by measuring the deoxyglucose transport; as shown in Fig. 20, the decrease in deoxyglucose transport paralleled that observed for DHA, consistently with the reported down-modulation of GLUTs expression in skeletal muscle cells by differentiation (Aebi 1984, Del Maestro et al., 1985). DHA uptake was almost completely inhibited (95%) by the presence of 10 μM cytochalasin B (a specific glucose-carrier inhibitor) (data not shown), further supporting that the transport was exclusively mediated by GLUT transporters.

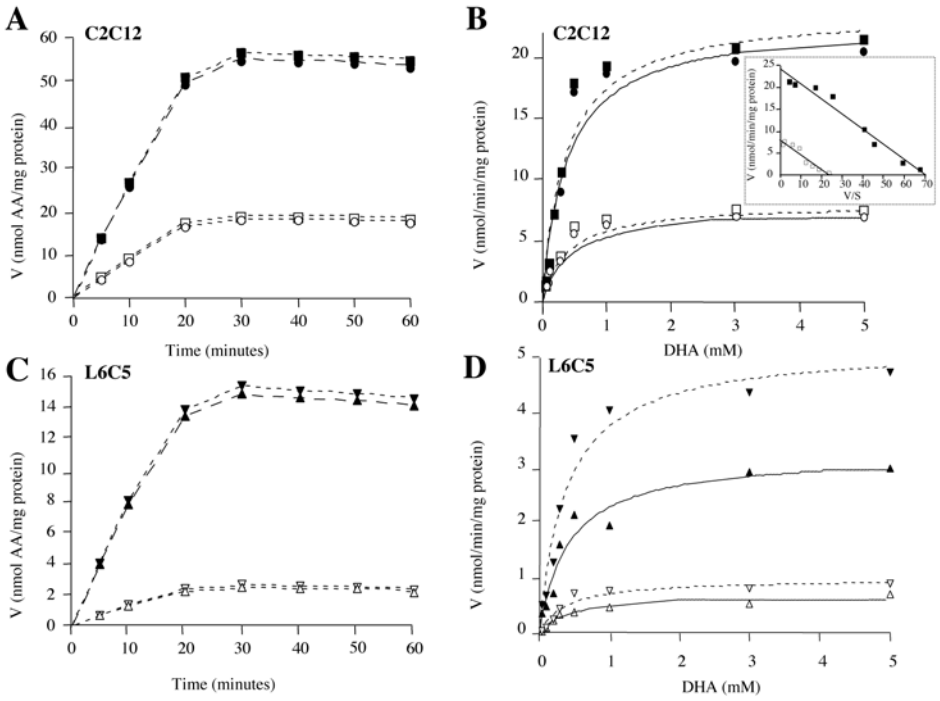

Fig. 19. Kinetic analysis of dehydroascorbic acid transport in proliferating and differentiated C2C12 and L6C5 cells.

(A) Time-dependent uptake of 100 μM DHA by C2C12 cells.

(B) Concentration dependence of DHA uptake in C2C12 cells. ■ myoblasts with DTT; ● myoblasts without DTT; □ myotubes with DTT; ○ myotubes without DTT. Inset: Eadie-Hofstee plot.

(C) Time-dependent uptake of 100 μM DHA by L6C5 cells.

(D) Concentration dependence of DHA uptake in L6C5 cells. ▲ myoblasts without DTT; ▼ myoblasts with DTT; △ myotubes without DTT; ▽ myotubes with DTT.

Cells were incubated with different concentrations of DHA (0.05-5 mM) for 20 minutes. Intracellular AA content was determined by HPLC.

Values are the mean of five independent experiments (S.D. ≤ 6 %).

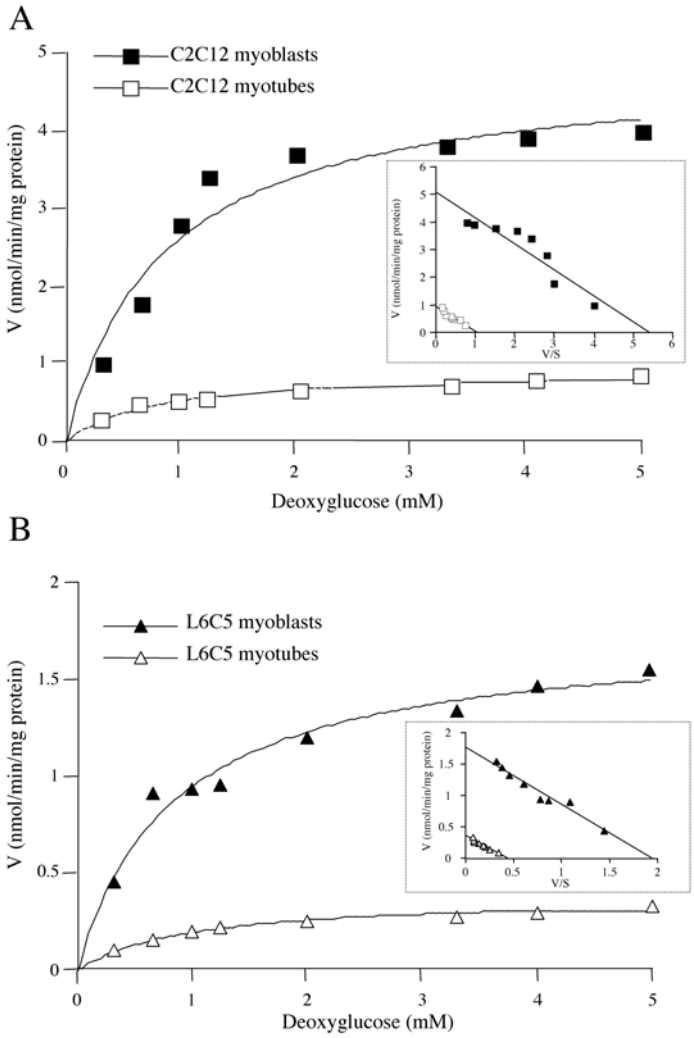

Fig. 20. Deoxyglucose uptake in proliferating and differentiated C2C12 and L6C5 cells.
Cells were incubated with different concentrations of DOG (0.3-5 mM for 10 minutes). DOG uptake was carried out by scintillation spectrometry measurements.
(A) Concentration dependence of DOG uptake in C2C12 myoblasts and myotubes.
(B) Concentration dependence of DOG uptake in L6C5 myoblasts and myotubes.
Values are the means of three independent experiments (SD. ≤ 6%).
Insets: Eadie-Hofstee plots.

6.2 RECYCLING

To study the systems involved in vitamin C recycling, we assayed DHA and AFR reductase activities, both in proliferating and differentiated cells.

As shown in Fig. 21 A, in proliferating C2C12 cells intracellular AFR reductase activity was higher (528 %) than in L6C5 myoblasts, and was up-modulated after differentiation. On the other hand, transmembrane AFR reductase activity was similar in the two cell lines and it was unaffected by differentiation (Fig. 21 B).

Basal DHA reductase activity was higher (164%) in proliferating C2C12 cells with respect to L6C5 myoblasts (Fig. 21 C) and it was lowered in both differentiated cells. To determine the contribution of specific enzymatic systems on DHA reduction, we measured DHA reductase activity in the presence of co-substrates required for the putative enzymatic systems involved. In both C2C12 and L6C5 myoblasts, the relative contribution was: NADPH-GSH dependent < NADPH-thioredoxin dependent < NADH-lipoic acid dependent system (Fig. 22).

Both differentiated cells showed decreased DHA reductase activities, with the NADH-lipoic acid system being less affected. Indeed, in C2C12 and L6C5 myotubes, we found respectively 24% and 22% decrease for the NADH-lipoic acid system, 68% and 69% decrease for the NADPH-GSH dependent system, 68% and 59% decrease for the NADPH-thioredoxin system (Fig. 22).

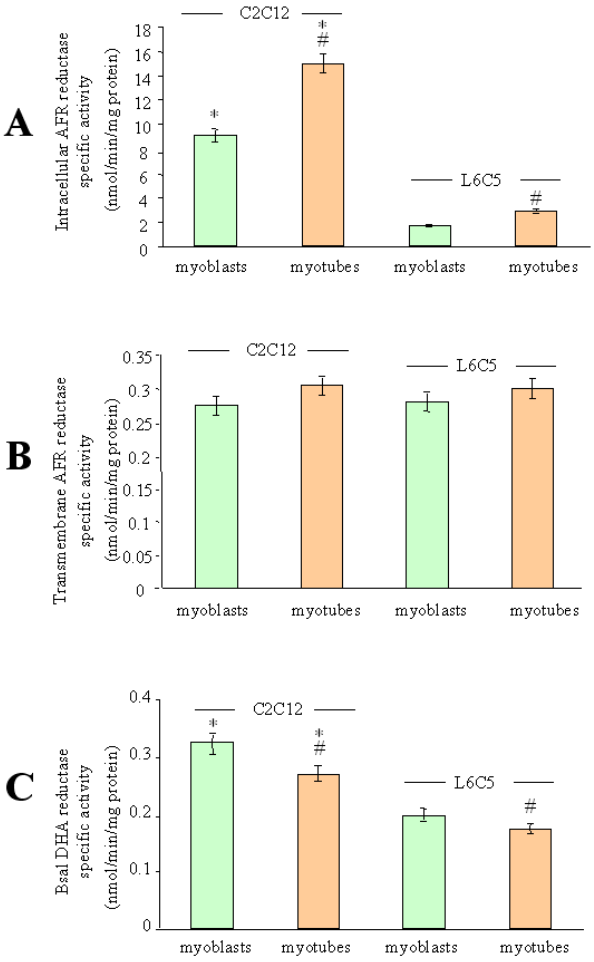

Fig. 21. Vitamin recycling ability of C2C12 and L6C5 cells.
(A) Intracellular AFR reductase activity. This activity was measured spectrophotometrically, through the rate of ascorbate free radical-dependent oxidation of NADH. Corrections were made for direct oxidation of NADH by homogenates.
(B) Transmembrane AFR reductase activity. This activity was monitored by analysing the prevention of AA autoxidation in the presence or in the absence of cells.
(C) Basal DHA reductase activity. This activity was evaluated by HPLC. Corrections were made for non-enzymatic reduction of DHA by a concentration of GSH corresponding to its cellular content. This reaction corresponded to: 15-18% for L6C5 myoblasts; 10-12% for C2C12 myoblast and less than 2% for both L6C5 and C2C12 myotubes.
Values are the mean of four independent experiments, each one performed in triplicate.
* $p<0.0001$ versus L6C5 cells. $^{\#}$: $p<0.0001$ versus myoblasts.

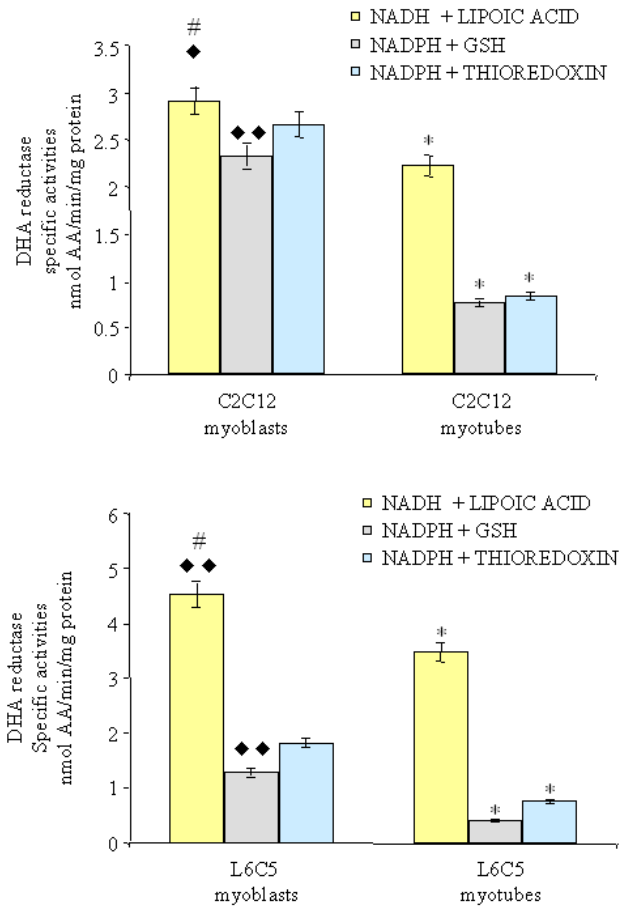

Fig. 22. Specific activities for NADH-lipoic acid, NADPH-GSH and NADPH-thioredoxin dependent systems in C2C12 (A) and L6C5 (B) cells.

NADPH-GSH-dependent DHA-reductase activity was measured in the presence of 0.4 mM NADPH + 2 mM GSH.

NADH-lipoic acid-dependent DHA-reductase activity was measured in the presence of 0.5 mM lipoic acid + 0.4 mM NADH.

NADPH-thioredoxin-dependent DHA-reductase activity was measured in the presence of 0.4 mM NADPH + 6 µM thioredoxin.

Correction were made for direct reaction of DHA with NADH-lipoic acid (1-2% of total activity), NADPH-GSH (30-40% of total activity) and NADPH-thioredoxin (4-8% of total activity). Data are the mean of three independent experiments, each one performed in triplicate. ◆: $p=0.0008$ versus NADPH-thioredoxin dependent activity. #: $p<0.0001$ versus NADPH-GSH dependent activity. ◆◆: $p<0.0001$ versus NADPH-thioredoxin dependent activity. *: $p<0.0001$ versus myoblasts.

6.3 GLUTATHIONE DEPLETION STUDIES

We performed GSH-depletion studies, as they allow to evaluate the direct role of GSH on vitamin C metabolism, as well as the adaptive responses to oxidative stress of systems involved in vitamin C homeostasis.

To this end, we investigated the effects of buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamyl-cysteine syntase.

Intracellular glutathione content was 119 ± 9 and 79 ± 6 nmol/mg protein for L6C5 and C2C12 myoblasts respectively, whereas the values for myotubes were 11 ± 0.9 and 8 ± 0.6 nmol/mg protein for L6C5 and C2C12 cells, respectively. After 15 hr incubation with BSO, both cell lines showed a GSH content corresponding to 10% of starting levels. The same percentage of depletion was reached with different concentrations: L6C5 cells required amounts of BSO (200 μM) higher than that used for C2C12 cells (50 μM), probably reflecting a different glutathione metabolism.

Experiments on AA transporters revealed that the SVCT2 gene expression was up-regulated only in differentiated cells (about 2.7 and 1.3 fold over untreated cells for L6C5 and C2C12 cells, respectively) (Fig. 23). In agreement with SVCT2 expression, functional analysis of AA transport showed 35% and 18% V_{max} increase in BSO-treated L6C5 and C2C12 myotubes, respectively; no changes in K_m values were observed.

DHA uptake increased in GSH-depleted L6C5 cells (130% V_{max} increase), whereas was unaffected in C2C12 cells. Moreover**,** at variance of untreated cell, imported vitamin C was all present in the reduced form in L6C5 cells. No changes in deoxyglucose transport were found in GSH depleted cells, indicating that the increase of DHA uptake observed in L6C5 cells was correlated to increased DHA reduction.

Indeed, recycling experiments demonstrated increased basal DHA reduction, especially via the NADPH-thioredoxin dependent system, in both cell lines (Table II). This increase overwhelmed the decreased activity of the other enzymatic systems, thus improving the storage of vitamin C as showed by the import experiments.

Finally, GSH-depletion had no effect on AFR reduction: both intracellular and transmembrane AFR reductase activities were unchanged (Table II).

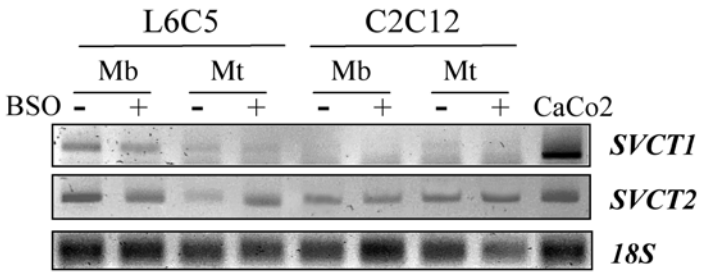

Fig. 23. RT-PCR analysis of SVCT1 and SVCT2 expression in C2C12 and L6C5 muscle cells.

Myoblasts (Mb) and myotubes (Mt) were grown for 15 hours with or without BSO. Total RNA was reverse transcribed and amplified either with SVCT1 (*upper panel*) or SVCT2 (*middle panel*) specific primers; amplification of the 18S rRNA (*lower panel*) was also shown as loading control. CaCo2 cells were used as positive control. Blots are representative of three different experiments.

Table II: Effect of GSH depletion on vitamin C recycling

	L6C5		C2C12	
	myoblasts	myotubes	myoblasts	myotubes
DHA reductase activities *(% over untreated cells)*				
Basal	132 ± 5*	110 ± 8#	128 ± 7*	110 ± 6♦♦
NADH, lipoic acid-dependent	66 ± 4*	86 ± 5*	96 ± 6°°	110 ± 5♦
NADPH, GSH-dependent	90 ± 6**	24 ± 2*	87 ± 5*	83 ± 4*
NADPH, thioredoxin–dependent	133 ± 8*	196 ± 12*	122 ± 10	278 ± 16*
AFR reductase activities *(% over untreated cells)*				
Intracellular	98 ± 5	94 ± 6	98 ± 5	93 ± 5
Transmembrane	100 ± 4	97 ± 3	99 ± 5	102 ± 5

Data are the mean of three independent experiments, each one performed in triplicate.
A paired *t*-test was used to compare the different systems between untreated and BSO-treated cells.
*: $p<0.0001$; #: $p=0.0035$; **: $p=0.0037$; °°: $p=0.1202$; ♦: $p=0.0184$; ♦♦: $p=0.0114$

7. THE REDOX SIGNALLING PATHWAY IS DIFFERENT IN RAT L6C5 AND MOUSE C2C12 SKELETAL MUSCLE CELLS

7.1 AP-1 INHIBITORY COMPLEXES ARE PRESENT IN RESISTANT C2C12 MYOBLASTS AND L6C5 MYOTUBES.

Since the balance between pro- and anti-oxidant conditions regulates several steps of gene expression (signal transduction, nuclear migration, transcriptional and post-transcriptional events), we investigated whether differences in the cellular redox state and susceptibility to oxidative stress correlated with differences in NF-kB and AP-1 signalling pathways.

From mobility shift experiments, we found that proliferating L6C5 cells had AP-1 DNA-binding activity lower than C2C12 myoblasts (compare lanes 1 in Fig. 24 A and B), but they were more susceptible to vitamin C-mediated upregulation of basal activity (compare lanes 2 in Fig. 24 A and B). An oxidative insult (myoblasts treated with H_2O_2; Fig. 24 A and B, lanes 3) increased the DNA binding activity in both cell lines, but a slight synergistic effect between oxidant and antioxidant molecules could be seen only in L6C5 myoblasts (Fig. 24 A, lane 4).

A remarkable difference between the two cell lines could be seen also during differentiation: the AP-1 DNA binding activity was doubled in L6C5 myotubes (Fig. 24 A, lane 5) and halved in C2C12 myotubes (Fig. 24 B, lane 5). Moreover, in the apoptosis-resistant L6C5 myotubes, the H_2O_2-mediated induction of DNA binding activity was completely abolished (Fig. 24 A, lane 6), while this effect was less pronounced in C2C12 differentiated cells (Fig. 24 B, lane 6); in L6C5 myotubes, the enhanced catalase activity probably led to stronger H_2O_2-scavenging.

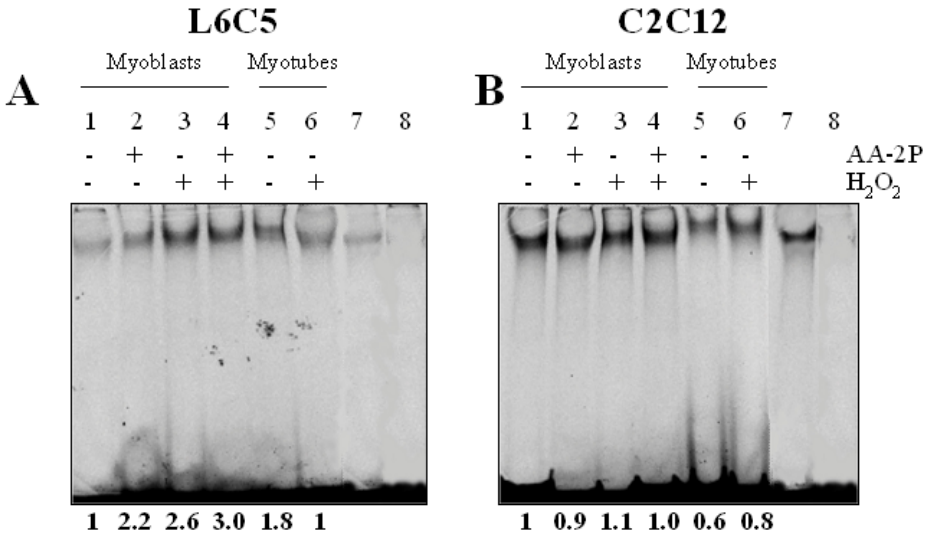

Fig. 24. AP-1 bandshift analysis of nuclear extracts purified from L6C5 (A) and C2C12 (B) cells.

Myoblasts (*lanes 1-4*) and myotubes (*lanes 5-6*) were left untreated (*lanes 1 and 5*) or pre-incubated with AA-2P for 24 hours (*lanes 2 and 4*); where indicated, L6C5 and C2C12 cells were also incubated for 1 hour with H_2O_2 (*lanes 3, 4 and 6*). Specificity controls consist of binding competition experiments with a 50-fold molar excess of unlabeled mutated (*lane 7*) and consensus (*lane 8*) AP-1 oligonucleotide.

The radiograph is representative of three independent experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarly set to 1.

We also found that AP-1 complexes of the two cell lines contained different activating (c-Fos and c-Jun) and inhibitory (Fra-1 and Fra-2) subunits. L6C5 myoblasts showed high levels of c-Fos protein, and low levels of Fra-1 and Fra-2 proteins (Fig. 25 A, lane 1); in particular, Fra-1 expression was very low in L6C5 myoblasts, if compared with protein levels found in the apoptosis-resistant C2C12 cell line (compare lanes 1 in Fig. 25 A and B). As expected, H_2O_2 exposure led to a significant increase in c-Jun levels (Fig. 25 A and B, lane 3).

Interestingly, vitamin C supplementation significantly increased Fra-1 expression, paralleled by inhibition of c-Fos levels only in L6C5 myoblasts (Fig. 25 A, lane 2). The positive effect of vitamin C was still evident in the presence of H_2O_2 (Fig. 25 A, lane 4).

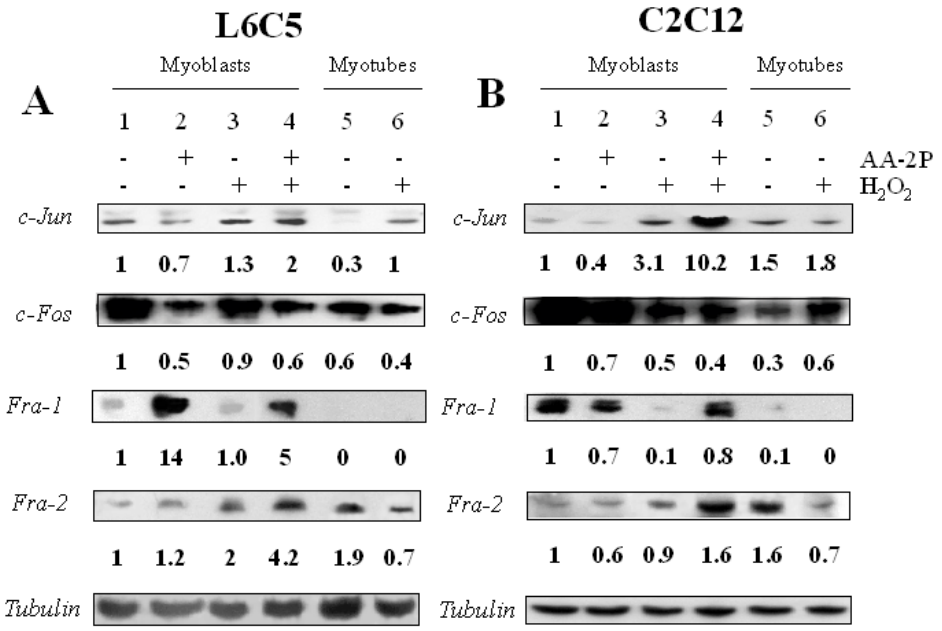

Fig. 25. Western blot analysis of AP-1 components in L6C5 (A) and C2C12 (B) cells. Myoblasts (*lanes 1-4*) and myotubes (*lanes 5-6*) were treated as above and cellular extracts were purified and immunoblotted with anti-c-Jun, anti-c-Fos, anti-Fra-1 and anti-Fra-2 antibodies. The radiograph is representative of two similar experiments. Densitometric analysis of the blots is represented in bold: the band intensity of untreated cells was arbitrarly set to 1. Statistical analysis of densitometric values was <0.05%.

These results were confirmed by supershift experiments: AP-1 heterodimers of C2C12 cells contain both Fra-1 and Fra-2 components (Fig. 26 B, lanes 1 and 7), while L6C5 myoblasts did not show shift with either antibody (Fig. 26 A, lanes 1 and 7).

Interestingly, in L6C5 cells, the presence of Fra-1 and Fra-2 components in AP-1 complexes well correlated with vitamin C supplementation (Fig. 26 A, lanes 4 and 8-10).

All these findings suggested that C2C12 myoblasts, containing huge amounts of Fra-1 protein, counteracted the H_2O_2–mediated activation of AP-1 by forming inhibitory complexes. On the contrary, L6C5 myoblasts contained almost exclusively c-Jun/c-Fos heterodimers, which could be responsible for H_2O_2-mediated cell death.

The protective effect of vitamin C could be achieved by shifting AP-1 from activating to inhibitory complexes. Both myotubes decreased c-Fos and Fra-1 protein levels and increased the Fra-2 component, thus maintaining the AP-1 complexes in an inhibitory state (Fig. 25 A and B, lane 5 and Fig. 26 A and B, lane 5). This effect could explain the acquired apoptosis-resistant phenotype of L6C5 differentiated cells.

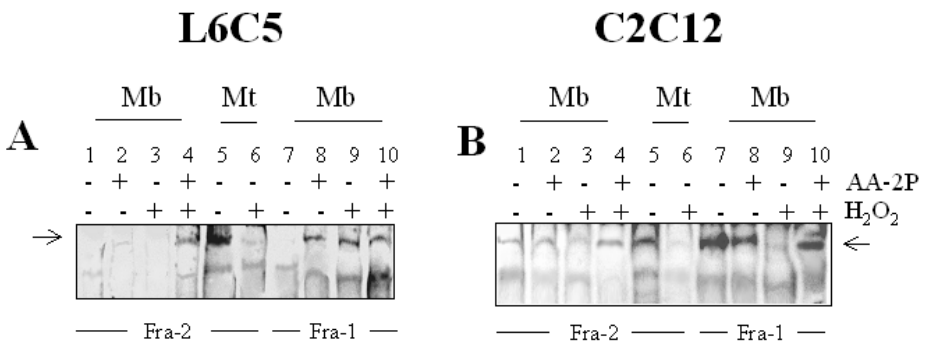

Fig. 26. Supershift experiment of AP-1 components.
L6C5 **(A)** and C2C12 **(B)** nuclear extracts were incubated with AP-1-specific labelled oligonucleotide together with antisera raised against Fra-2 (*lanes 1-6*) or Fra-1 (*lanes 7-10*) proteins in myoblasts (Mb) (*lanes 1-4 and 7-10*) and myotubes (Mt) (*lanes 5-6*). Lanes 1 and 7, control cells; lanes 2 and 8, AA-2P-loaded cells; lanes 3 and 9, H_2O_2-treated cells; lanes 4 and 10, cells treated with H_2O_2 in the presence of AA-2P; lane 5, differentiated cells; lane 6, differentiated cells treated with H_2O_2. The arrows indicate the band supershifted by the antibodies.

7.2 NF-kB ACTIVITY IS HIGH IN RESISTANT C2C12 MYOBLASTS AND L6C5 MYOTUBES .

Similar results were obtained when we looked at the NF-kB DNA binding activity. Proliferating L6C5 cells had NF-kB activity lower than C2C12 myoblasts (compare lanes 1 in Fig. 27 A and B). Again, the activity was increased by H_2O_2 treatment in both cell lines (Fig. 27 A and B, lane 3), while it was almost unaffected by vitamin C supplementation only in C2C12 myoblasts (Fig. 27 B, lanes 2 and 4). On the contrary, in L6C5 myoblasts, the low basal activity was significantly up-modulated by vitamin C alone (Fig. 27 A, lane 2) and this effect was synergistically enhanced by the combination of oxidant and antioxidant compounds (Fig. 27 A, lane 4). Finally, L6C5 myotubes retained almost the same levels of NF-kB activity observed in myoblasts, while the DNA binding activity was greatly reduced in C2C12 myotubes (Fig. 27 A and B, lanes 5); in both differentiated cell lines the increase in NF-kB binding activity after H_2O_2 exposure was marginal (Fig. 27 A and B, lanes 6).

In conclusion, L6C5 cells, containing less amounts of NF-kB, were more susceptible to H_2O_2-induced cell death, but, during differentiation, they became more resistant avoiding the down-modulation of DNA binding activity of this survival factor.

In order to investigate if NF-kB DNA binding activity and AP-1 composition correlated with resistance to oxidative stress, myoblasts were grown in the presence of specific inhibitors, which block the two signalling pathways, and tested for H_2O_2-mediated cell death.

A specific inhibitor of the c-Jun N-terminal kinase (L-JNKI1) partially decreased L6C5 susceptibility to oxidative stress, whereas an inhibitor of NF-kB nuclear translocation (anti-NF-kB inhibitory ligand) exerted opposite effects on C2C12 myoblasts, increasing the number of dead cells after H_2O_2 treatment (Fig. 28, grey columns).

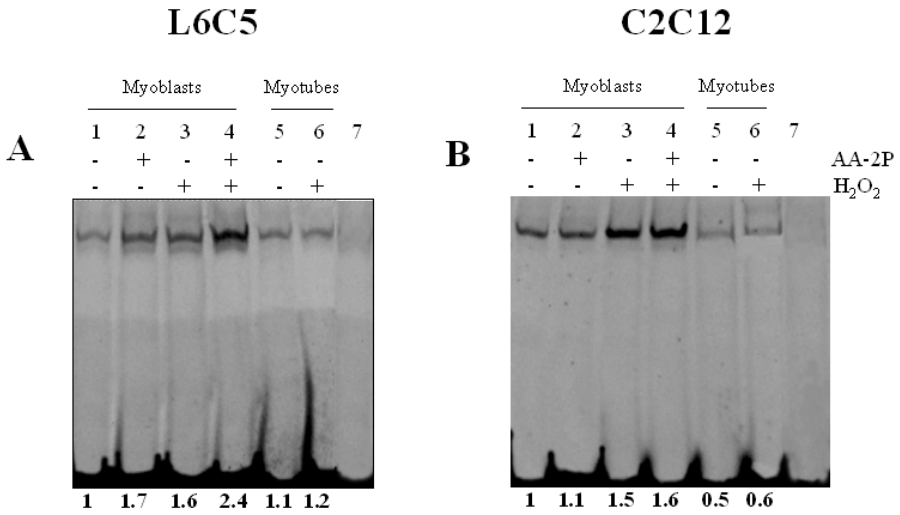

Fig. 27. NF-kB bandshift analysis of nuclear extracts purified from L6C5 (A) and C2C12 (B) cells.

Myoblasts (*lanes 1-4*) and myotubes (*lanes 5-6*) were left untreated (*lanes 1 and 5*) or pre-incubated with AA-2P for 24 hours (*lanes 2 and 4*); where indicated, L6C5 and C2C12 cells were also incubated for 1 hour with H_2O_2 (*lanes 3, 4 and 6*). The specificity control consists of a binding competition experiment with a 50-fold molar excess of unlabeled oligonucleotide (*lane 7*). The radiograph is representative of three independent experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarly set to 1.

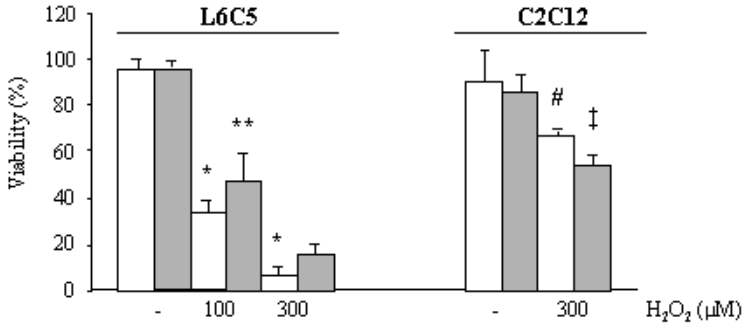

Fig. 28. Susceptibility to oxidative stress after blocking AP-1 and NF-kB activities.
L6C5 and C2C12 cells, grown in the absence (white bars) or presence (grey bars) of inhibitors of AP-1 (L-JNKI1, which inhibits the c-Jun N-terminal kinase) and NF-kB signalling pathways (anti-NF-kB inhibitory ligand, which inhibits NF-kB nuclear migration), were treated with 100 or 300 μM H_2O_2 for 1 hour and collected 24 hours after. After collection, viability was assessed by trypan blue uptake.
Data are means ± S.D. of two experiments each one performed in triplicate. *: $p < 0.01$ vs control cells; **: $p < 0.01$ vs H_2O_2-treated cells; ‡: $p < 0.05$ vs H_2O_2-treated cells; #: $p < 0.05$ vs control cell (T-test).

7.3 GLUTATHIONE DEPLETION DOES NOT AFFECT AP-1 AND NF-kB ACTIVITIES.

Since glutathione, and more exactly its oxidised form (GSSG), negatively modulates NF-kB DNA binding activity (Galter D. et al., 1994, Flohe L. et al., 1997), we investigated the effects of BSO, on the two redox-sensitive transcriptional factors.

Both L6C5 and C2C12 myoblasts were sensitive to GSH loss, as demonstrated by the increase in ROS production (2-3 fold over control cells) (Fig. 29). Vitamin C pre-loading significantly reduced cellular ROS levels in C2C12 cells (48.8% of reduction with respect to BSO-treated cells), while its effect was less pronounced on L6C5 cells (30% of reduction) (Fig. 29).

We also investigated AP-1 and NF-kB binding activities in glutathione-depleted myoblasts.

GSH depletion had no effect on L6C5 NF-kB and AP-1 DNA binding activities (compare lanes 1 and 2 in Fig. 30). Only slight effects were seen in glutathione-depleted C2C12 myoblasts: we found slight up-modulation of NF-kB activity (compare lanes 4 and 5 in Fig. 30 A) and down-modulation of AP-1 activity (compare lanes 4 and 5 in Fig. 30 B). This was in agreement with the observed increase of ROS, especially in C2C12 cells after GSH depletion (Fig. 29). The drastic fall in glutathione content also abrogated the vitamin C-mediated activation of DNA binding activities (Fig. 30 A and B lanes 3 and 6), suggesting a potential crosstalk between these two hydrophylic antioxidants.

Thus, GSH depletion did not have dramatic effects on redox-responsive transcription factors, in agreement with results obtained in differentiated cells, where the drastic fall in total glutahione content did not impair the resistance to oxidative stress.

Interestingly, BSO treatment increased TrxR expression (Fig. 31), suggesting that alternative endogenous defence mechanisms could be activated, depending on the oxidative insult employed.

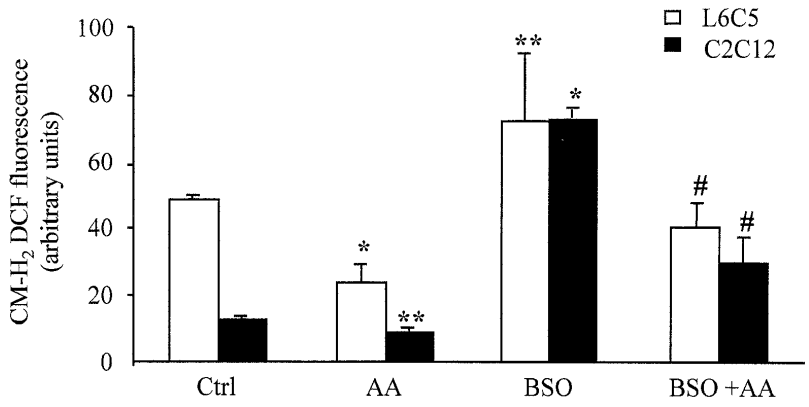

Fig. 29. Generation of intracellular peroxides in glutathione-depleted cells.
L6C5 (white bars) and C2C12 (black bars) myoblasts were left untreated or supplemented with 1 mM AA-2P; where indicated, cells were depleted of glutathione by incubation with BSO, an irreversible inhibitor of γ-glutamyl-cysteine synthase. As the two cell lines show a different glutathione metabolism, the same level of GSH depletion (corresponding to 10% of GSH starting levels) was obtained by incubating L6C5 and C2C12 cells with 200 μM and 50 μM BSO, respectively.
Peroxides generation was assessed by flow cytometry, using CM-H_2DCFDA as probe (see *Materials and Methods* for details).
Results are means $\pm$ S.D. of three independent experiments, each one performed in duplicate. *: $p < 0.01$ vs control cells; **: $p < 0.05$ vs control cells; $^{\#}$: $p < 0.01$ vs BSO-treated cell (T-test).

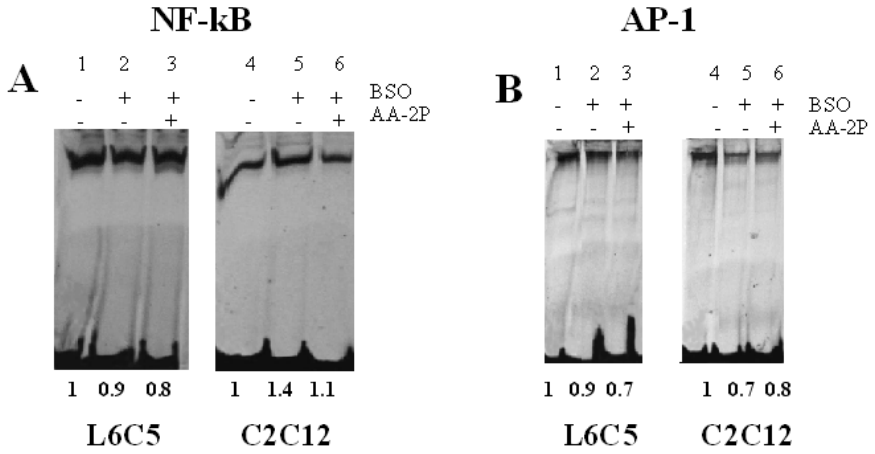

Fig. 30. AP-1 and NF-kB binding activities in glutathione-depleted myoblasts.

(A) NF-kB bandshift analysis of nuclear extracts purified from L6C5 and C2C12 cells. Myoblasts were left untreated (*lanes 1 and 4*) or depleted of glutathione (*lanes 2 and 5*); where indicated, L6C5 and C2C12 cells were also pre-incubated with 1 mM AA-2P (*lanes 3 and 6*). The radiograph is representative of two independent experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarly set to 1.

(B) AP-1 bandshift analysis of nuclear extracts purified from L6C5 and C2C12 cells. Myoblasts were left untreated (*lanes 1 and 4*) or depleted of glutathione (*lanes 2 and 5*); where indicated, L6C5 and C2C12 cells were also pre-incubated with 1 mM AA-2P (*lanes 3 and 6*). The radiograph is representative of two similar experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarly set to 1.

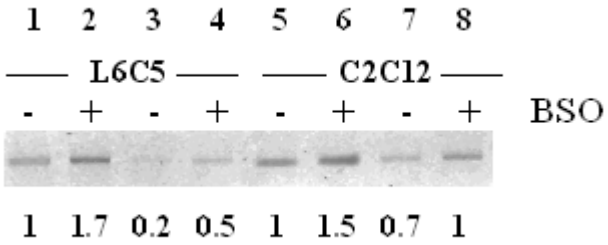

Fig. 31. Analysis by RT-PCR of TrxR expression.
L6C5 (*lanes 1-4*) and C2C12 (*lanes 5-8*) cells were grown in the absence (*lanes 1, 3, 5 and 7*) or in the presence (*lanes 2, 4, 6 and 8*) of BSO, in both proliferating (*lanes 1, 2, 5 and 6*) and differentiating (*lanes 3, 4, 7 and 8*) conditions. Total RNA was reverse-transcribed and amplified with primers specific for the cytosolic isoform of the enzyme. The blot is representative of two independent experiments. In bold is represented the densitometric analysis of autoradiography: values are reported as fold over control, arbitrarily set to 1, after normalization with 18S rRNA.

8. DISCUSSION

Vitamin C metabolism

Vitamin C not only enhances carnitine biosynthesis and facilitates glycogen storage in skeletal muscle (Russell et al., 2000), but also protects cells against ROS generation induced by physical exercise (Ashton et al., 2003). Blood and plasma levels of vitamin C decrease in those who exercise (Balakrishnan et al., 1998), probably because the increased respiration rate unbalances the RO/NS/antioxidants ratio; thus, vitamin C is largely consumed to protect against RO/NS damage (Balakrishnan et al., 1998, Gleeson et al., 1987).

It has been demonstrated that high doses of AA improve the energetic state of rat skeletal muscle during postischemic reperfusion, probably due to its antioxidant function (Lagerwall et al., 1995, Lagerwall et al., 1997). In addition, several studies documented that plasma antioxidant concentrations (especially for vitamin C) positively correlate with physical performance and skeletal muscular strength in elderly people (Cesari et al., 2004).

We found that skeletal muscle cells had specific and efficient systems to import vitamin C. Both cell lines employed in this study, were able to take up AA through specific carriers: SVCT2 was always present, whereas SVCT1 was detected at very low levels only in proliferating L6C5 cells, but not in differentiated cells. In line with the levels of SVCT2 expression, L6C5 myoblasts were more efficient in AA import than C2C12 myoblasts. When muscle cells differentiated, their ability to take up AA decreased.

Skeletal muscle cells also imported DHA through GLUT transporters, with the C2C12 cells being more efficient; the difference reflected a different expression of GLUTs in the two cell lines (Vera et al., 1995). Like AA transport, DHA uptake decreased after differentiation, in line with the reported down-modulation of GLUTs expression in differentiated skeletal muscle cells (Sarabia et al., 1990).

In both cell lines, AFR and DHA reductase activities were modulated by differentiation: AFR reductase activity increased, whereas basal DHA reductase activity decreased. Considering the singular contribution of different enzymatic systems, the NADPH-GSH and NADPH-thioredoxin dependent systems decreased. This was in agreement with the finding that thioredoxin reductase activity and expression decreased in differentiated cells (Fig.11). On the other hand, the relative contribution of the NADH-lipoic acid dependent system increased, in line with the reported increase of

mitochondrial enzymatic activities involved in energy metabolism in differentiated cells (Moyes et al., 1997). These enzymatic systems were present in the two cell lines with different abundances: in particular, C2C12 cells, besides being more efficient in DHA import, were also more efficient in vitamin C recycling.

Oxidative conditions selectively modulated the different components of vitamin C homeostasis: indeed, GSH-depletion up-regulated SVCT2 gene expression in differentiated cells and DHA trapping, as a consequence of more efficient DHA reductase activities. This adaptive response appeared to be NADPH-thioredoxin dependent, in agreement with the results showing that thioredoxin reductase activity and expression were induced by GSH loss (Fig.31).

Resistance to oxidative stress

The two cell lines differently responded to ROS generation: C2C12 cells were resistant, while L6C5 myoblasts appeared quite sensitive to oxidative damage. Indeed, L6C5 cells were 10-20 times more sensitive than C2C12 cells to cytotoxic and apoptotic effects of H_2O_2. Moreover, differentiated L6C5 cells acquired an apoptosis-resistant phenotype, paralleled by changes in redox homeostasis.

Resistance to oxidative stress appeared to be correlated to levels of catalase, thioredoxin reductase, glutathione, as well as to levels and composition of redox sensitive transcription factors (see below).

The analysis of the overall antioxidant profile suggested that the glutathione system could be the main redox sensor in L6C5 myoblasts, while C2C12 cells appeared to be more strictly dependent on the thioredoxin reductase system. In L6C5 cells this dependence could change during differentiation, possibly related to the drastic fall in glutathione content.

Redox signalling

The differences in cellular redox state and susceptibility to oxidative stress are also correlated to differences in NF-kB and AP-1 signalling pathways. Indeed, the resistant cell line possess high levels of NF-kB DNA binding activity and AP-1 complexes containing inhibitory subunits. Accordingly, the apoptotic resistant phenotype observed in L6C5

differentiated cells was achieved by retaining the same levels of NF-kB activity observed in myoblasts and increasing the inhibitory Fra components in AP-1 heterodimers.

From these data, can be drawn a picture (Fig. 32 and 33) accounting for the different susceptibility of the two cell lines to oxidative imbalance. The more resistant phenotype of C2C12 cells was achieved, beside induction of NF-kB, also by high levels of the key redox sensor TrxR, which maintained high levels of thioredoxin into the nucleus, thus enhancing NF-kB transcriptional activity (Flohe et al., 1997, Harper et al., 2001). The high levels of NF-kB DNA binding activity and TrxR, together with Fra-containing AP-1 complexes, allowed increased expression of antioxidant enzymes and survival of C2C12 cells after oxidant exposure (Fig. 32). In addition, C2C12 cells, possessing endogenous protective systems (TrxR, AFR and DHA reductases), did not strictly depend on vitamin C uptake.

On the contrary, in L6C5 cells the lower levels of TrxR and the higher levels of GSSG exerted negative effects on NF-kB and AP-1 activities (Galter et al., 1994). Furthermore, L6C5 myoblasts had huge amounts of GPx, which has been shown to inhibit NF-kB activation by increasing the half-life of the inhibitory subunit I-kB alpha (Kretz-Remy et al., 2001), and AP-1 complexes containing the activating c-Fos subunit. The resulting redox state led to a sensitive phenotype characterized by H_2O_2-mediated AP-1 induction and subsequent cell death (Fig. 33 A). To overwhelm the less active defence systems, L6C5 myoblasts were more efficient in AA import than C2C12 cells. Indeed, the susceptibility of L6C5 myoblasts to oxidative stress could be abrogated by vitamin C supplementation, through induction of NF-kB activity and Fra expression, thus inhibiting the expression of AP-1 target genes.

A peculiar characteristic of L6C5 cells is that, during differentiation, the redox state underwent drastic changes. L6C5 myotubes decreased glutathione levels and GPx activity, while increasing catalase and NF-kB activities. In addition, AP-1 complexes shifted from an activating (containing c-Fos) to an inhibitory (containing Fra-2) behaviour. Thus, the presence of scavengers and survival factors allowed to overwhelm the oxidant conditions (Fig. 33 B) and myotubes were no more dependent on exogenous antioxidants. Although the difference in apoptosis sensitivity could be due to other reasons (such as the relative ratio of pro- and anti-apoptotic molecules), nonetheless the intracellular redox status may contribute to the ability of differentiated cells to counteract an oxidative insult.

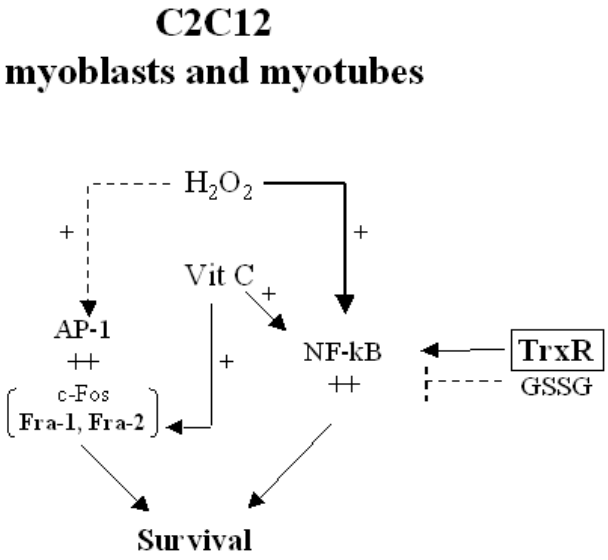

Fig. 32. Schematic model explaining the crosstalk between oxidants and antioxidants in C2C12 muscle cells and suceptibility to oxidative stress.

C2C12 cells have a better redox state (high NF-kB and thioredoxin reductase activities, and AP-1 inhibitory complexes).

Vit C: vitamin C; TrxR: thioredoxin reductase; GSSG: oxidized glutathione

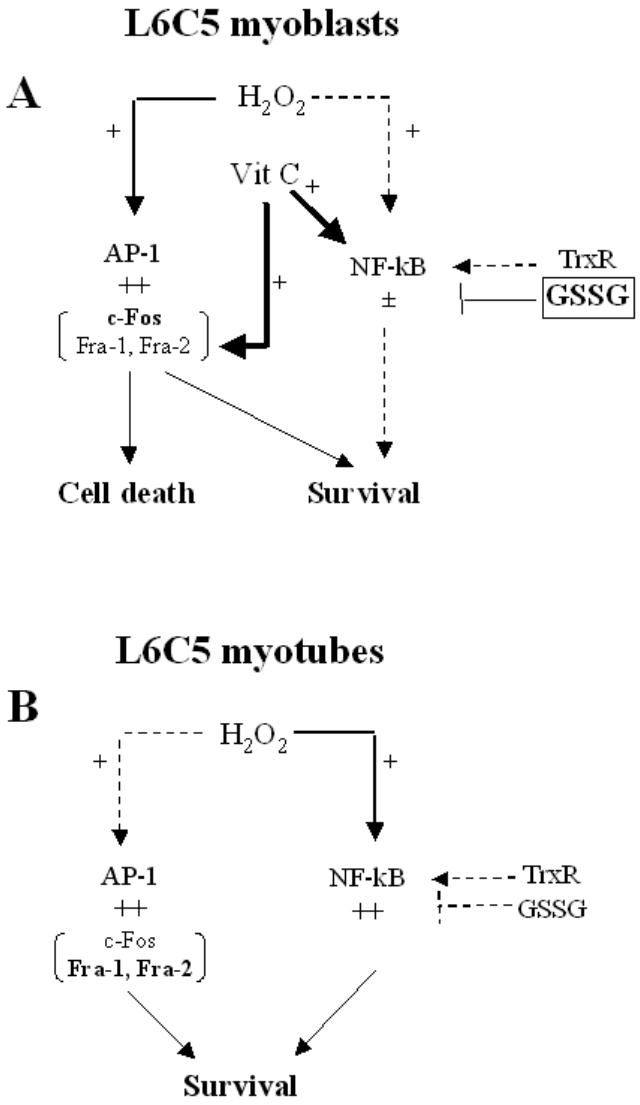

Fig. 33. Schematic model explaining the crosstalk between oxidants and antioxidants in L6C5 muscle cells and suceptibility to oxidative stress.

(A) L6C5 cells have a worst redox state (low NF-kB activity, active AP-1 complexes and high GSSG/GSH ratio).

(B) L6C5 myotubes acquire a resistant phenotype by enhancing catalase and NF-kB activities and shifting AP-1 complexes from an activeting to an inhibitory behaviour.

Vit C: vitamin C; TrxR: thioredoxin reductase; GSSG: oxidized glutathione

9. CONCLUSIONS

This study demonstrates that the requirement of exogenous antioxidants is inversely related to the ability to modulate endogenous defence mechanisms.

Our conclusion is supported by several findings: (i) C2C12 cells, possessing efficient endogenous systems, import less AA than L6C5 myoblasts, which possess less active cellular defence systems; (ii) differentiation is accompained by improved endogenous systems, resistance to oxidative stress and decreased import of exogenous vitamin; (iii) NF-kB and AP-1 are the sensors more closely related to the overall cellular redox state; (iv) oxidative conditions, such as glutathione depletion, induce SVCT2 and thioredoxin reductase as adaptative systems.

Thus, cellular adaptation can be achieved by:
(i) endogenous defence mechanisms
(ii) uptake and maintenance of exogenous nutritional antioxidants.

These adaptative responses may be important to prevent injury of skeletal muscle during exercise. Whether a different sensitivity to oxiditave stress between myoblasts and myotubes exists *in vivo*, remains to be established. However, myoblasts health should be important for its destiny: the different sensitivity to oxidative imbalance may represent an additional regulatory mechanism controlling whether these precursor cells undergo fusion into myotubes or death before fusion. Since oxidative stress could be related with decreased physical performance and muscle cell leakage, one can speculate that improvement of the antioxidant state in differentiated muscle cells should be a compensatory mechanism to counteract the detrimental effects of exercise-induced muscle damage.

EXPERIMENTAL PROCEDURES

REAGENTS

Dehydroascorbic acid was obtained from ICN (Aurora, OH, USA). Cell culture media were obtained from Invitrogen, Carlsbad, CA, USA; heat-inactivated fetal bovine serum FBS and trypsin were obtained from HyClone, Oud-Beijerland, Holland. Tris (hydroxymethyl)-aminomethane, HCl, choline chloride, trichloroacetic acid, iodonitrotetrazolium chloride, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate were obtained from Fluka (Buchs, Switzerland). Ascorbate oxidase was obtained from Boehringer Mannheim (Mannheim, Germany). Magnesium ascorbic acid-2 phosphate (AA-2P), a stable derivative of vitamin C, was obtained from Wako Pure Chemical Industries (Neuss, Germany). [γ-^{33}P] ATP was purchased from Amersham (Arlington Heights, IL). The inhibitor of JNK activity (L-JNKI1) was from Alexis (Carlsbad, CA, USA) and the inhibitor of NF-kB nuclear migration (Anti-NF-kB Inhibitory Ligand) was from Upstate (Lake Placid, NY, USA). All the other reagents, unless otherwise specified, were from Sigma Chemical (St. Louis, MO, USA)

CELL CULTURES

The mouse C2C12 (ATCC, Manassas, VA, USA) and rat L6C5 (a clone derived from the L6 muscle cell line; ICLC, Genova, Italy) myoblasts were grown in DMEM supplemented with Glutamax-I (L-alanyl-L-glutamine), 4500 mg/l glucose (Invitrogen, Carlsbad, CA, USA) and 10 % (v/v) heat-inactivated foetal bovine serum (FBS; HyClone, Oud-Beijerland, Holland), at 37°C with 5% (v/v) CO_2 in a humidified atmosphere. No antibiotics were used. Cells were split 1:6 twice weekly and fed 24 hours before each experiment.

Differentiation into myotubes was achieved by culturing preconfluent cells (85% confluency) in medium containing 2% FBS: reduction of serum allowed cell to cell fusion and formation of myotubes (Fig. 12 A-D) expressing myogenin, a marker of muscle differentiation (Fig. 12 E).

CaCo2 cells were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum.

INTRACELLULAR ROS

Intracellular ROS generation was measured by using the fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes Inc., Eugene, OR). After different treatments, cells were incubated with 10 μM CM-H_2DCFDA for 20 min at 37°C in the dark. Radical formation was assessed by flow cytometry in a FACSCalibur Flow Cytometer (Becton Dickinson, CA, USA). CM-H_2DCFDA mean fluorescence was registered at 530 nm (bandwidth 30 nm) exciting at 488 nm using a 15 mW Argon laser. Ten thousand events were evaluated for every analysis.

CYTOTOXICITY ANALYSIS

Cells were treated with different concentrations of H_2O_2 (50-300 μM) for 1 (myoblasts) or 24 hrs (myotubes). After treatment, cells were rinsed twice with PBS and then cultured in conditioned medium for additional 24 hours. When indicated, 100 μM AA-2P was added 24 hrs before the oxidative treatment, and maintained until the end of treatments. To analyse apoptosis induction, cells were fixed with paraformaldehyde (4% in PBS) and the condensed/fragmented nuclei were evaluated by staining with Hoechst 33258 (Sigma). Apoptotic cell frequency was calculated as the percentage of condensed cells/1000 cells scored. The results were additionally confirmed by the measure of caspase-3-like activity (CaspACETM Assay Kit, Promega), following the manufacturer's instructions.

ENZYMATIC ACTIVITIES

Catalase activity was measured by monitoring the decomposition of H_2O_2 at 240 nm (Aebi 1984). Briefly cells were lysated with 0.05 M Tris-HCl, 0.5% deoxycolic acid, 150 mM NaCl, 1 mM PMSF. After sonication and centrifugation at 8700 rpm for 10 minutes, 0.6 mg of supernatant were tested spectrophotometrically. 900 μl of phosphate buffer (6.81 g/l H_2PO_4, 8.9 g/l Na_2HPO_4-$2H_2O$ mixed 1:1.5 at pH 7.2) 100 μl H_2O_2 (100 μM) and 100 μl of sample were added to the reaction mixture.

Total SOD activity (manganese + copper/zinc SOD) was determined according to the method of Crapo (Crapo et al., 1978), by monitoring the inhibition of reduction of cytochrome C in a coupled system with xanthine

and xanthine oxidase. 100 µl (0,2-0,4 mg of proteins) of the supernatant obtained as for catalase activity was tested spectrophotometrically by adding 100 µl cytocrome C 1 mM (in phosphate buffer), 10 µl of xantine 5 mM (in NaOH 0.1 N), 10 µl KCN 1 mM for the total SOD or 10 µl KCN 300 mM for the Mn-SOD in 750 µl of phosphate buffer (6.81 g/l H_2PO_4, 8.9 g/l Na_2HPO_4-$2H_2O$ mixed 1:1.5 at pH 7.2). 30 µl of xantine oxidase (final 0.6 U/ml) was added to the reaction mixture and the change in absorbance at 550 nm was recorded at regular intervals over 4 min.

GPx activity was measured according to Del Maestro and McDonald (Del Maestro et al., 1985) using tert-butyl hydroperoxide as the substrate. Briefly, cells were lysated with Tris-HCl 0.05 M, EDTA 1 mM, DTT 0.5 mM, PMSF 1 mM. After sonication and centrifugation at 8700 rpm for 10 minutes, 0.6 mg of supernatant were tested spectrofotometrically. 10 µl GSH (200 mM), 10 µl GSH-reductase (100 U/ml), 200 µl (0,3-0,5 mg of proteins) of sample and 780 µl of phosphate buffer (6.81 g/l H_2PO_4, 8.9 g/l Na_2HPO_4-$2H_2O$ mixed 1:1.5 at pH 7.2) were added to the reaction mixture. After 30 minutes at 37°C, 10 µl NADPH (15.5 mM) and tert-butyl hydroperoxide were added. GPx activity was evaluated by the decrease in NADPH concentration at 340 nm.

AA AND DHA UPTAKE

Intracellular AA content was measured by using HPLC with UV detection at 265_6 nm wavelength, as described (Savini I. et al., 2000). Briefly, 2.5 x 10^6 cells in a 25 cm^2 were incubated in 600 µl of transport medium (5 mM KCl, 1.9 mM KH_2PO_4, 5.5 mM glucose, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM Hepes, 147 mM NaCl, 1.1 mM Na_2HPO_4 pH 7.4) containing DHA or AA plus 1mM dithiotreitol (DTT) to prevent AA oxidation. After incubation at 37°C, the reaction was stopped by washing with 10 ml PBS, and the cells were directly extracted with 600 µl ice-cold 70% methanol containing 1 mM EDTA, and analysed. DHA was calculated as the difference between the total vitamin (measured in cell extracts incubated with 10 mM DTT for 10 minutes) and AA content (samples without DTT). To analyse the effect of Na^+ on AA or DHA transport, NaCl and Na_2HPO_4 were replaced with equimolar choline chloride and K_2HPO_4, respectively. AA or DHA transport experiments were also performed in the presence of 10 µM Cytochalasin B, a specific inhibitor of GLUT transporters.

Deoxyglucose (DOG) uptake was carried out by scintillation

spectrometry measurements. 8 x 10^5 cells in 9 cm^2 dishes were incubated in 300 µl of transport medium with 6 µCi [1,2-^{3}H]-2-DOG (30 Ci/mmol) and adequate concentrations (0-5 mM) of the respective unlabelled compound. After 30 minutes, cells were harvested, lysed and assayed by liquid scintillation spectrometry, as described (Savini et al., 2000).

VITAMIN C RECYCLING ACTIVITIES

DHA reductase activities were measured at 37°C after 20 min incubation of cell homogenates (0.6 mg proteins) with 1 mM DHA in 50 mM Tris-HCl pH 7.5 containing 1 mM EDTA. The 20 min period was chosen as DHA degradation was minimal and DHA reduction rate was time-linear. After incubation and precipitation of proteins with methanol (500 µl), samples were centrifuged at 15000 rpm for 10 minutes and the supernatant was evaluated for the AA content by HPLC. The basal DHA reductase activity (i.e. measured in the absence of exogenous cofactors) was calculated after correction for non-enzymatic reduction of DHA by a concentration of GSH corresponding to its cellular content. The contribution of single DHA reductase activities was measured in cell homogenates after overnight dialysis to remove endogenous cofactors, such as glutathione and pyridine nucleotides. To test different DHA reductase activities, the single cofactors (NADPH plus GSH; NADPH plus thioredoxin and NADH plus lipoic acid) were added to the reaction mixture. Corrections were made for direct reduction of DHA by NADPH plus GSH, NADPH plus thioredoxin and NADH plus lipoic acid.

Intracellular AFR reductase activity was measured spectrophotometrically, through the rate of ascorbate free radical-dependent oxidation of NADH, by monitoring the decrease in 340 nm absorbance (E = 6.2 mM^{-1} cm^{-1}) at 25°C; corrections were made for direct oxidation of NADH by homogenates. The assay mixture contained 0.05 mM Tris-HCl buffer pH 7.8, 1 mM EDTA, 0.1 mM NADH, 1 mM ascorbate and aliquots of each sample. The reaction was started by adding 0.28 U of ascorbate oxidase to generate ascorbate free radical (Arrigoni O. et al., 1981).

Transmembrane AFR reductase activity was monitored at 265 nm by analyzing the prevention of AA autoxidation in the presence or in the absence of cells (Savini et al., 1998).

WESTERN BLOT

10-20 μg of cellular proteins (in 25 mM Tris-HCl pH 8, 0.5% SDS, 0.05% mercaptoethanol, 2.5% glycerol and 0.001% bromephenol blue) were denaturated at 100°C for 5 minutes, subjected to SDS-PAGE on a 10% polyacrylamide gel and then electroblotted onto a PVDF membrane at 130 V for 1 hour. Blots were blocked with 5% non-fat dry milk (Biorad, Hercules, CA) and then incubated with anti-c-Fos (Oncogene Research Products, San Diego, CA), anti-Fra1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-c-Jun (Sigma Chemical) and anti-Fra2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies. After washings and incubation with the horseradish peroxidase-conjugated secondary antibody, detection was carried out with ECL (Amersham).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts preparation and DNA binding experiments were carried out as previously described (Schreiber et al., 1989, Lee et al., 1996).

Briefly, nuclear extracts were incubated with γ-^{33}P-labelled oligonucleotides containig either AP-1 or NF-kB consensus sequences. After 10 minutes incubation at 37°C, the reaction was stopped by adding TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

The sequence of AP-1 double-stranded probe was CGCTTGATGAGTCAGCCGGAA and the sequence of NF-kB double-stranded probe was AGTTGAGGGGACTTTCCCAGGC. Labeling was performed by using T4 polynucleotide kinase and (γ-^{33}P)-ATP. The complexes were resolved on nondenaturing 6% (w/v) polyacrylamide gels in 0.2x TBE (45 mM Tris-borate, 1 mM EDTA) buffer for 1 hour at 14 V/cm. The gel was then dried and subjected to autoradiography.

For supershift analysis, nuclear extracts were pre-incubated with 3 μg of specific antiserum for 2 hours at 4°C before addition of labelled oligonucleotide. Complexes were then processed as described above. Anti-Fra-1 and anti-Fra-2 antibodies were from Santa Cruz Biotechnology.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated by Trizol (Invitrogen). Briefly, cells were lysed

in a guanidine isothiocianate-phenol solution and total RNA was purified and concentrated by ethanol precipitation.

For amplification of sodium-dependent ascorbate transporters (SVCT1 and SVCT2), 1 μg of total RNA was reverse transcribed using the Superscript Preamplification System and oligo-dT primer (Invitrogen), following the manufacturer's instructions; 10% (20% in the case of SVCT1) of the first strand reaction was then PCR amplified. Control reactions were performed in order to ensure complete removal of DNA and exponential amplification of mRNA.. Amplification parameters were as follows: 94°C 30 s, 55°C 30 s, 72°C 2 min. Linear amplification was observed after 40 and 35 cycles for SVCT1 and SVCT2, respectively. The primers were: SVCT1 (+) 5'-GCCCCTGAACACCTCTCATA-3' and SVCT1 (-) 5'-ATGGCCAGCATGATAGGAAA-3'; SVCT2 (+) 5'-TTCTGTGTGGGAATCACTAC-3' and SVCT2 (-) 5'-ACCAGAGAGGCCAATTAGGG-3'. The SVCT2 primers were specific for the functional full-lenght isoform (Lutsenko et al., 2004). Twenty μl of the reaction were electrophoresed on a 4% (w/v) agarose gel, and the amplified products were visualized by ethidium bromide staining.

For amplification of cytosolic thioredoxin reductase (TrxR), 1 μg of total RNA was reverse-transcribed; 10% of the first strand reaction was then PCR-amplified. The amplification parameters were as follows: 94°C 30 s, 56°C 30 s, and 68°C 2 min. Linear amplification was observed after 20 cycles. Twenty microliters of the reaction was electrophoresed on a 1.4% (w/v) agarose gel, and the amplified product was visualized by ethidium bromide staining. The primers, which did not cross-react with the mitochondrial isoform of TrxR, were as follows: mouse TrxR (+) 5'-TGGATTTTGTCACACCGACTCC-3' and TrxR (-) 5'-CGATGGCGTAGATGTAAGGCAC-3'; rat TrxR (+) 5'-TGGACTTCGTCACACCAACTCC-3' and TrxR (-) 5'-GAATGTCACCAATGGCGTAGATG-3'.

Amplification of 18S rRNA was performed as described (Savini et al., 2002). Expression levels were evaluated by densitometric analysis after normalization with 18S.

GLUTATHIONE ANALYSIS

Intracellular reduced (GSH) and oxidized (GSSG) glutathione content was quantified by a 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB]-glutathione reductase recycling assay, spectrofotometrically at 412 nm according to the

method of Anderson (Anderson 1985). Briefly, 5 x 10^6 cells were collected and resuspended in 200 µl 5% trichloroacetic acid (TCA). Lysates were centrifuged at 14000 for 2 minutes and the supernatant analyzed for GSH/GSSG content.

The reaction buffer was 700 µl NADPH (0.3 mM), 100 µl DTNB (6 mM), 190 µl H2O, 10 µl sample. The reaction was started by adding 2.66 U/ml glutathione reductase. The total amount (reduced and oxidized glutathione) was followed at 412 nm by the TNB stechiometrical formation.

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG Reductase}} 2GSH + NADP^+$$

$$2GSH + DTNB \longrightarrow GSSG + TNB \text{ (colour)}$$

GSSG was selectively measured in samples where GSH was masked by pretreatment with 2-vinylpyridine (2%).

For glutathione depletion studies, cells were incubated with buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamyl-cysteine synthase. In order to reach the same level of GSH depletion (corresponding to 10% of GSH starting levels), we incubated L6C5 and C2C12 cells for 15 hours with 200 µM and 50 µM BSO, respectively. Different BSO concentrations have been used, as the two cell lines differ in their ability to retain reduced glutathione. The glutathione content was then measured to quantitate depletion efficiency.

KINETICS CALCULATIONS AND STATISTICS

The transport kinetic parameters were calculated by the Michaelis-Menten equation and the linear transformation of Eadie-Hofstee. Statistical analysis was conducted with the program Stat View 4.02 per Macintosh (Abacus Concept Inc, Berkley, CA, USA). Analysis of variance followed by Bonferroni *post-hoc* comparisons was performed to evaluate differences on vitamin C recycling systems, between C2C12 and L6C5 cells. Comparisons of NADH-lipoic acid, NADPH-GSH and NADPH-thioredoxin dependent systems between proliferating and differentiated cells, and between untreated and BSO-treated cells, were evaluated by using a paired *t*-test. Significant differences were accepted at $p<0.05$.

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