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REVIEW ARTICLE

Creatine as an antioxidant

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Abstract Creatine monohydrate (Cr), the most diffuse supplement in the sports industry, is receiving greater attention because of its beneficial effects in a wide number of human degenerative diseases and conditions. These effects can be barely explained on the basis of the sole ergogenic role of the Cr/CrP system. Indeed, a wide number of research articles indicate that Cr is capable of exerting multiple, non-energy related, effects on diverse and relevant cellular targets. Among these effects, the antioxidant activity of Cr emerges as an additional mechanism which is likely to play a supportive role in the Cr-cytoprotection paradigm.

Keywords Creatine · Oxidative stress · Antioxidant · Cytotoxicity · Genotoxicity · Differentiation

Introduction

Over the past decade the use of creatine monohydrate (Cr) for therapeutic and chemopreventive purposes has received increasing attention (Gualano et al. 2009). The beneficial effects of Cr supplementation have been reported for a large number of muscular, neurological and cardiovascular diseases (Sharov et al. 1987; Gordon et al. 1995; Holtzman et al. 1999; Matthews et al. 1999; Neubauer et al. 1999; Felber et al. 2000; Vorgerd et al. 2000; Mazzini et al. 2001; Stout et al. 2001; Wyss and Schulze 2002; Tarnopolsky et al. 2004; Tarnopolsky 2007) as well as in sarcopenia and ageing (Bender et al. 2007; Tarnopolsky 2007; Tarnopolsky and Safdar 2008). The benefits of Cr have been generally attributed to the Cr-induced buffering of cellular ATP levels, a pronounced decrease of which would otherwise lead to the accumulation of intracellular Ca^{2+}, formation of reactive oxygen species (ROS) and tissue oxidative damage (Persky and Brazeau 2001). Indeed, most of these pathologies recognize multiple aetiological factors among which the detrimental role of oxidative stress has been stably recognized (Gilgun-Sherki et al. 2004; Kovacic and Thurn 2005).

In 2002 Lawler (Lawler et al. 2002) demonstrated that Cr acts as a direct scavenger of radical species in an acellular setting. Thereafter, studies from our laboratory as well as from other groups have been devoted to understanding whether Cr may exert antioxidant effects in cultured mammalian cells (a summary of the studies dealing with the antioxidant or antioxidant-like activity of Cr is presented in Table 1). The overall aim of this article was to review these studies and their biological, nutritional and therapeutic implications.

Direct antioxidant effect of Cr

The first evidence for an "antioxidant-like" activity of Cr was reported by Matthews et al. (1998). In this study, the authors provided evidence that oral Cr supplementation

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Table 1 The antioxidant or "antioxidant-like" effects of Cr

Reference	Effect
Matthews et al. (1998)	Cr supplementation protects rats from nitropropionic acid-intoxication
Lawler et al. (2002)	Direct antioxidant effect of Cr in acellular systems
Berneburg et al. (2005)	Cr protects from induction of the common deletion, a mtDNA mutation marker, generated in normal human fibroblast by repetitive UV-A irradiation through an energy-dependent mechanism
Lenz et al. (2005)	Protection of mtDNA against UV- and oxidative stress-induced damage in human keratinocytes
Meyer et al. (2006) Santiago et al. (2008)	Cr prevents mitochondrial ROS formation through the preservation of mtCK activity
Sestili et al. (2006)	Cr affords cytoprotection in oxidatively injured cultured mammalian cells *via* direct antioxidant activity
Guidi et al. (2008)	Cr protects against oxidative mtDNA damage in HUVEC cells
Rakpongsiri and Sawangkoon (2008)	The combination of Cr supplementation, oestrogen replacement and combined exercise training ameliorates the cardiac reserve function and antioxidant reservation against oxidative stress in oestrogen-deficient hamsters
Bender et al. (2007)	Improved health and survival in Cr-supplemented mice
Fimognari et al. (2009)	Creatine protects against oxidative RNA damage
Rambo et al. (2009)	Attenuation of seizures and oxidative damage in pentilentrazol-intoxicated rats
Sestili et al. (2009)	Cr supplementation prevents the inhibition of myogenic differentiation in oxidatively injured C2C12 murine myoblasts
Hosamani et al. (2010)	Attenuation of rotenone-induced mitochondrial oxidative damage and neurotoxicty in *Drosophila melanogaster* supplemented with Cr
Young et al. (2010)	Induction of the antioxidant enzymes peroxiredoxin-4, a type 2 peroxiredoxin-4 reductase and thioredoxin dependent peroxide reductase in C2C12 cells

was capable of protecting rats from nitropropionic acid-intoxication (an animal model of Huntington's disease). Similar protective effects are granted by some well-established antioxidants such as *N*-acetylcysteine. Indeed, production of ROS and reactive nitrogen species represents an aetiologically relevant event in this model of Huntington's disease. The same authors found that oral Cr supplementation was capable of significantly attenuating the hydroxyl radical and peroxynitrite generation in nitropropionic acid-intoxicated rats. However, this latter effect was not ascribed to a direct scavenging activity of Cr, but rather to an energy-related mechanism, namely the amelioration of intracellular calcium buffering in target cells ensuing the prevention of free radical production by mitochondria. Few years later, Lawler et al. (2002) was the first to test the hypothesis that Cr was capable of acting as a radical scavenger using a well-controlled acellular experimental setting. This study showed that Cr—unlike Cr phosphate (CrP)—was capable of scavenging charged radicals such as the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical, superoxide anion and peroxynitrite; by contrast, no significant quenching effect was observed on the non radical oxidants H_2O_2 and tert-butyl-hydroperoxide (tBOOH). Unfortunately, the effect of Cr on toxicologically relevant H_2O_2- or tBOOH-derived radicals (hydroxyl and alkoxyl radicals, respectively (Guidarelli et al. 1995)), was not specifically investigated. It was also suggested that the antioxidant activity might relate to some of the effects observed in vivo upon Cr supplementation in athletes, such as the diminished muscle fatigue.

Although this study clearly demonstrated the scavenging capacity of Cr, it did not investigate whether Cr acted as an antioxidant in cellular experimental systems.

This prompted us to address this issue (Sestili et al. 2006). Following Cr pre-loading, three mammalian cell lines—U937 (human promonocytoid), C2C12 (murine mioblasts) and HUVEC (human umbilical vein endothelial cells)—all expressing Cr transporters were treated with a panel of oxidative stressors capable of generating toxic radical species in vivo, namely H_2O_2, tBOOH and peroxynitrite, and various end-points indicative of antioxidant activity were determined. The three stressors were given to cells at concentrations resulting in mild to extensive cytotoxicity. Notably, in living cells H_2O_2 and tBOOH undergo Fenton reaction and form hydroxyl and alkoxyl radicals, respectively (Guidarelli et al. 1995). Cr pre-loading (concentrations ranging from 0.1 to 10 mM) was found to be mildly but significantly cytoprotective in all the conditions tested; this effect was dose-related (Fig. 1a–c shows the effect of Cr in these three cell lines) and similar to that granted by the hydrophilic vitamin E analogue trolox, used as a reference antioxidant (Azzi et al. 2004). Morphological and ultrastructural analysis at the light and transmission electron microscope confirmed these results. Interestingly, Cr cytoprotection was fully prevented by adding the Cr uptake inhibitor *β*-guanidinopropionic acid

(GPA) (Otten et al. 1986) to the Cr pre-incubation milieu, indicating that the intracellular fraction of Cr is responsible for the observed protective effects. Cr pre-loading was unable to increase the activity of the two main antioxidant detoxifying enzymes, namely catalase and glutathione peroxidase, suggesting that the effects of Cr were not mediated by an increased activity of these two enzymes. In an opposite but converging direction, catalase and glutathione-depleted U937 cells were—as expected—sensitized to H_2O_2 toxicity but still responsive to Cr cytoprotection and to an extent identical to that found in normal, undepleted cells. Interestingly, Cr pre-loading—similarly to trolox—attenuated the decrease of non protein sulphydrils (NPSH, of which GSH represents $\geq$90%) typically induced by either H_2O_2 or tBOOH. In addition, the levels of extracellular H_2O_2 found at the end of treatments in the supernatants of U937 cells were lower in Cr pre-loaded- as compared with unsupplemented- samples. Both these effects (sparing of NPSH and reduction of the extracellular levels of H_2O_2) might depend on (1) increased availability of NPSH, (2) the overall amelioration of the antioxidant cellular systems, (3) the prevention of the generation or (4) on direct scavenging of toxic ROS derived from the oxidative stressors. As to the first two hypotheses, they seemed unlikely since Cr, as discussed above, did not increase the activity of catalase or glutathione peroxidase, nor affected the cellular constitutive levels of NPSH. Thus, it is plausible that the protective effects discussed so far depend on the direct antioxidant activity which, evidently, takes place also in cellular systems provided that intracellular free Cr reaches a "threshold" level attainable upon adequate supplementation. As to the mechanism underlying the antioxidant activity, the possibility that Cr might act as an iron chelator (Azzi et al. 2004; Sestili et al. 2007) capable of blocking the ROS-generating Fenton reaction was also ruled out. Thus, Cr seemed to act as a scavenger of radical species. The similarity between Cr and trolox or other reference radical scavengers (Sestili et al. 2006) found in our experimental setting further supports this concept. However, these reference antioxidants do not exhibit a character peculiar of Cr, i.e. its fundamental role in cellular energy status. As discussed earlier, and as proposed by various authors in studies dealing with Cr-protective effects, the energetic role of Cr might represent *per se* a mechanism capable of rendering cells more resistant to oxidative stress. To understand whether Cr cytoprotection might depend on an antioxidant or an energy-related mechanism, the intracellular levels of free Cr and of CrP following pre-loading stages identical to those used in cytotoxicity experiments were determined by means of HPLC. The three cell lines (U937, HUVEC and C2C12) accumulated different levels of Cr as well as of CrP, but the protective effects appeared only when free Cr levels significantly increased. In particular, among the three cell lines tested, U937 cells rapidly accumulated fairly high levels of free Cr in the absence of a corresponding increase in CrP. Despite this behaviour, U937 cells were rapidly and significantly protected by Cr supplementation. Furthermore, a linear correlation irrespective of cell type was found between the free Cr intracellular levels and the degree of cytoprotection (Fig. 1d), while no correlation was found in the case of CrP levels. Thus, the effects observed in this oxidative stress paradigm are clearly related to the intracellular availability of free Cr rather than of its phosphorylated form.

As to the identification of the oxidation product(s) of Cr, mass spectrometry analyses of solutions containing Cr and H_2O_2 or peroxynitrite as well as of lysates from Cr-supplemented cells treated with the aforementioned oxidants showed the formation of putative oxidation products with MW at 136 and 150 (Sestili et al. 2006) whose structures have still to be better defined by means of infrared spectroscopy and mass–mass spectrometry.

Protective activity of Cr on oxidatively injured mitochondrial (mt) DNA

Mitochondria and mtDNA represent important targets for oxidative damage. Indeed, mtDNA mutations have recently been reported as being an aetiological factor in oxidative stress-related disorders (Copeland 2010) including cardiovascular diseases and inherited or acquired neurodegenerative disorders, several types of tumours, mitochondrial myopathies and the normal ageing process. Mitochondrially targeted antioxidants have been proposed as valuable tools to protect mitochondria from pathologically and toxicologically relevant alterations (Reddy 2008). Due to its mitochondrial tropism (Walzel et al. 2002), we extended our studies to investigate the possible protective effects of Cr on oxidatively damaged mtDNA from HUVEC cells. Cells were pre-incubated for 24 h with or without Cr and then treated for 30 min with 200 μM H_2O_2: cells were then grown for up to 72 h and assayed at specific time points for viability (again, Cr was cytoprotective) and nuclear and mtDNA damage (Guidi et al. 2008). This latter endpoint was determined using a two-step strategy based on a Long PCR and a REAL-TIME PCR: importantly, this assay allows monitoring of the integrity of mtDNA directly from total cellular DNA avoiding processes which can increase base oxidation such as isolation of mitochondria or separate mtDNA purification steps (Guidi et al. 2008). Results obtained showed that Cr significantly protected mtDNA from oxidative damage, with respect to H_2O_2-treated, Cr-unsupplemented samples. The effect of Cr on mtDNA, as assayed immediately after oxidative challenge, was dose

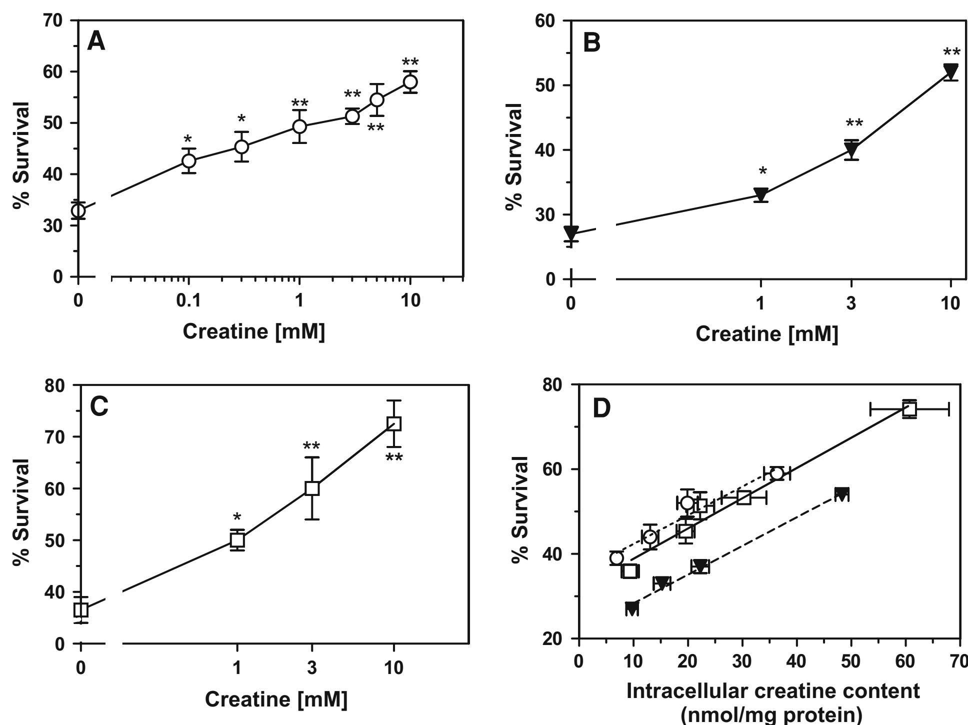

Fig. 1 Effect of Cr pre-loading on oxidant-induced cytotoxicity in cultured mammalian cells. U937 (**a**), HUVEC (**b**) or C2C12 (**c**) cells were pre-incubated for 1 h (U937) or 24 h (HUVEC and C2C12) with increasing concentrations of Cr and then treated for 1 h with 0.3 (U937), 0.075 (HUVEC) or 0.2 mM (C2C12) H_2O_2. Cell survival was determined after 48 h of growth in fresh, Cr- and H_2O_2- free culture medium using the trypan blue exclusion assay. Results represent the means ± SEM from five to eight separate experiments. *$p < 0.01$ and **$p < 0.005$ (unpaired *t* test) compared with Cr-unsupplemented cells. **d** Correlation plot between the survival data shown in panels **a**, **b** and **c** and the levels of intracellular Cr determined (HPLC) in parallel cell samples immediately before oxidative challenge. Key: U937 cells, *circles/dotted line*; HUVEC, *triangles/striped line*; C2C12, *squares/solid line*

dependent in the 3–10 mM range; concentrations lower than 3 mM were ineffective. Moreover, the protection afforded by 10 mM Cr was significant up to 24-h repair periods, while no significant difference with Cr unsupplemented cells could be seen at 48 h. It is worth noting that Cr effects were more pronounced at very early post-challenge times: this suggests that Cr prevents the induction of the lesions—possibly *via* direct antioxidant activity—rather than acting after and/or downstream their formation. This notion, i.e. the prevalence of a non-metabolic mechanism, is indirectly supported by the observation reported by Guidi et al. (2008) that in an acellular setting Cr reduced the extent of H_2O_2-induced lesions on 600 bp linear or circular DNA.

Thus, Cr supplementation may play an important role in mt genome stability in that it could normalize mitochondrial mutagenesis and prevent its functional consequences such as the decrease of oxygen consumption, mitochondrial membrane potential, ATP content and cell survival. These effects might be clinically relevant in preventing or ameliorating a wide range of pathologies where mitochondrial oxidative damage is known to play an aetiological role. Also, protection of mtDNA damage might represent one of the mechanisms contributing to the improved health and survival recently reported by Bender et al. (2007) in mice receiving a Cr-enriched diet.

Protective activity of Cr on oxidatively injured cellular RNA

RNAs are the only known molecules which possess the double property of being depository of genetic information, like DNA, and of displaying catalytic activities, like protein enzymes. RNA molecules intervene in all steps of gene expression and in many other biological activities (Hermann and Westhof 2000).

RNA and DNA are chemically similar. However, their structure and function are different. The ability of xenobiotics to target DNA has been extensively studied. RNA could have enhanced potential for attack by xenobiotics because it possesses some characteristics (largely single-stranded, more abundant than DNA, subcellular distribution in close proximity of mitochondria, etc.) making it theoretically more susceptible to chemical insults than DNA (Bregeon and Sarasin 2005). While less lethal than

mutations in the genome, such non-acutely lethal insults to cells have been recently associated with underlying mechanisms of several human diseases, especially chronic degeneration. A significant loss of RNA integrity has been demonstrated in advanced human atherosclerotic plaques (Martinet et al. 2004, 2005), several neurodegenerative diseases including Alzheimer disease, Parkinson disease, dementia with Lewy bodies, and prion diseases (Nunomura et al. 2009). Of note, RNA damage may also affect the balance between protein degradation and synthesis and repair-regeneration processes in skeletal muscles which ultimately determines total muscle mass and therefore sarcopenia (Malatesta et al. 2010; Tateyama et al. 2003; Tanaka et al. 2007; Hofer et al. 2008; Nunomura et al. 2009).

RNA damage can be related to the exposure to particular xenobiotics: different compounds able to damage RNA have been identified through an innovative automated high-throughput approach (Fimognari et al. 2008).

By using this approach, the RNA-damaging activities of different compounds have been demonstrated: H_2O_2; doxorubicin, which acts as both an alkylating and an oxidizing agent; spermine, one of the few NO-donors releasing authentic NO and ROS; *S*-nitroso-*N*-acetylpenicillamine (SNAP), a donor of NO (Fimognari et al. 2008).

In an attempt to envisage the possible use of Cr in the prevention or amelioration of a wide range of human diseases where RNA damage may play an etiological role, a study analysed the effects of Cr in counterstaining the RNA-damaging activity of the earlier reported compounds.

Interestingly, Cr was able to reduce the RNA damage induced by two of those toxic agents (H_2O_2 and doxorubicin), while it lacked activity in counterstaining the RNA damage induced by the NO donors spermine and SNAP. Its inhibitory activity against H_2O_2-induced RNA damage was dependent on its capacity to directly scavenge free radicals (Fimognari et al. 2009).

The mechanism for Cr protection against RNA damage induced by doxorubicin is more complex. The Cr kinase/CrP system is an important target of doxorubicin toxicity (Tokarska-Schlattner et al. 2002). Doxorubicin was able to oxidize creatine kinase thiols (direct effect) and generate ROS that contribute to further damage (indirect effect) (Weinstein et al. 2000). The protective effect of Cr against the RNA damaging activity of doxorubicin could be partially attributed to a build-up of CrP stores, which increase the efficiency of ATP regeneration. The radical scavenging activity of Cr also points to the role of free Cr as an antioxidant and suggests that the effect on RNA damage induced by doxorubicin might represent an additive, important mechanism contributing to its cytoprotective activity in doxorubicin-intoxicated cells (Fimognari et al. 2009). On the whole, these studies indicate that the beneficial effects of Cr supplementation may derive, beyond from its contribution to cellular energetics, also from its ability to protect a critical target such as RNA from oxidative attack.

Antioxidant activity of Cr in differentiating murine myoblasts and chick embryo neuroblasts

It is well known that ROS—when their level exceeds the cellular antioxidant capacity—not only induce cell death in target tissues, but also affect/impair biologically and physiologically relevant processes. With regard to skeletal muscle, oxidative stress profoundly affects the proliferation/differentiation balance of satellite-derived cells and myoblasts (Langen et al. 2002) and promotes the loss of mature myofibers (Buck and Chojkier 1996). Notably, oxidative stress is known to play a concausal and detrimental role in a variety of multifactorial muscular pathologies characterized by proliferation/differentiation imbalance such as Duchenne dystrophy (Messina et al. 2006), myotonic dystrophy (Toscano et al. 2005), sarcopenia (Fulle et al. 2004) and muscle repair. Counteracting the deleterious effects of ROS in these situations is a clinically advisable goal which in principle could be attained administrating specific antioxidants through the diet. However, not all the antioxidants are characterized by favourable pharmacokinetics with regard to muscle tissues; on the contrary, Cr fulfils the requisites of a "nutritargeted" ("nutritargeting" means targeting nutrients to specific "target" tissues (Biesalski and Tinz 2008)) muscular antioxidant since it is highly taken up, concentrated and durably retained by muscle cells (Wyss and Kaddurah-Daouk 2000). At this level, Cr exerts also a number of positive effects such as the increased expression of selected myogenic transcriptional regulators (MRFs)- and of IGF-1-mRNAs (Louis et al. 2004; Deldicque et al. 2007) and augmentation of the CrP stores (Alfieri et al. 2006). Based on these premises, we investigated whether Cr prevented, in comparison with established antioxidants, the arrest of the myogenic process in C2C12 myoblasts exposed to oxidative stress at an early differentiation time (Sestili et al. 2009). Differentiating cultures were pre-loaded with Cr for 24 h, treated with H_2O_2 given as a bolus for 1 h and then grown for up to 5 days to allow progression and completion of the myogenic program. In keeping with previous observations, H_2O_2 was mildly cytotoxic to differentiating C2C12 and markedly hampered the progression of the differentiative task. Interestingly, Cr significantly and dose-dependently attenuated the cytotoxic impact of H_2O_2, as assessed by means of MTT, trypan blue and total protein determination assays; pre-loading with trolox behaved similarly. Oxidative stress caused a significant decrease of

Cr and CrP levels in Cr-unsupplemented cultures at 48 h post-treatment, as compared with unintoxicated controls. On the contrary, Cr-supplementation resulted in a three to fourfold increase in Cr and CrP levels at the H_2O_2 treatment stage: notably, these levels, although decreasing over time, were still higher at 48 h post-oxidative challenge, as compared with untreated, unsupplemented cells. Replacing Cr with trolox did not affect the basal Cr or CrP levels, while slightly preventing their decrease in Cr-unsupplemented cells 48 h post H_2O_2 challenge. As to ATP and NPSH levels, both Cr and trolox attenuated the depletion observed at 48 and 0 h after H_2O_2 treatment, respectively. Addition of GPA to Cr-enriched media during the pre-loading stage prevented either the intracellular accumulation of Cr or its protective effects. Thus, both Cr and trolox were equally capable of rescuing cells from the cytotoxic activity of the oxidant and, at the concentrations used, seemed to exert a similar antioxidant activity. However, Cr effects were markedly different as compared with those of trolox in terms of the rescue of the differentiative capacity of intoxicated cultures. H_2O_2 treatment abolished the progression of C2C12 myogenic differentiation as assessed by means of biochemical, morphological (Fig. 2), ultrastructural and molecular analyses; a negligible rescue was afforded by trolox while Cr pre-loading restored almost completely the ability of C2C12 to execute the myogenic program. Indeed, Cr-enriched cultures, unlike trolox-enriched ones, were characterized by the presence of a percentage of developing or mature myotubes (as determined by means of light, transmission and scanning electron microscopy 2 and 4 days post-challenge) similar to that of controls; proteomic analysis of Cr cultures in the course of differentiation showed electropherograms indistinguishable from those of control, unintoxicated cells: in particular, annexin 1, gelsolin and ATP synthase D-chain, whose levels were low to undetectable in H_2O_2-treated cells and that increased in control cultures according to their differentiation (Scholz and Hinssen 1995; Tannu et al. 2004), were normally expressed in Cr pre-loaded, H_2O_2-treated cells. Similar results were obtained with regard to myosin heavy chain, a reliable and typical marker of muscle differentiation (Ardite et al. 2004). Transmission electron microscopy also indicated that—48 h after treatment—H_2O_2-intoxicated cells showed marked degeneration and loss of mitochondria, suggesting that these organelles might represent a particularly relevant target in this toxicity paradigm: again, this effect was prevented by Cr-priming, and not by trolox. Protection of mitochondria is of utmost importance with regard to muscle differentiation since it is well known that this process requires sustained mitochondrial activity and mitochondriogenesis (Rochard et al. 2000). It is likely that the rescue of mitochondria might also depend on the ability of Cr to reduce the extent of

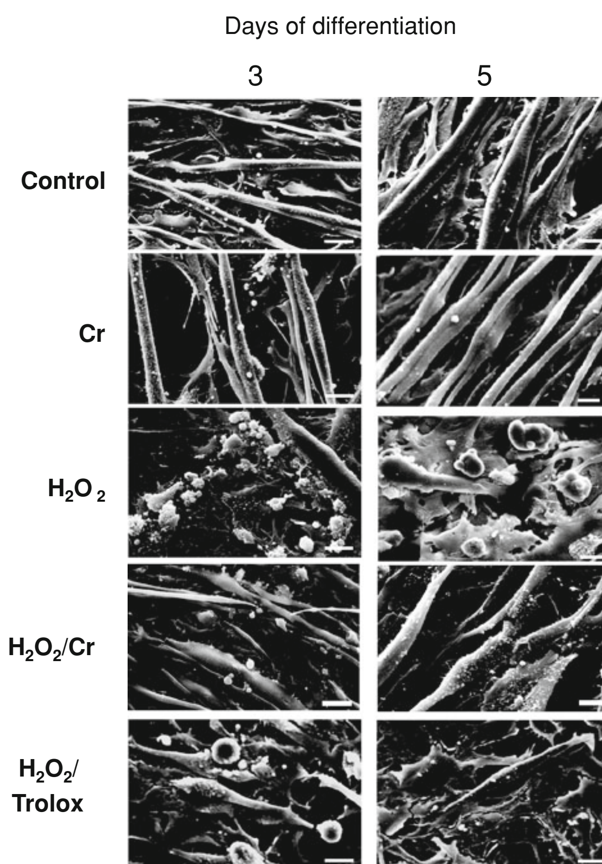

Fig. 2 Scanning electron micrographs of differentiating C2C12 myoblasts: the effect of oxidative challenge and of Cr supplementation. Cells were maintained for 24 h in differentiation medium in the absence or in the presence of 3 mM Cr; the medium was changed with fresh Cr-free medium and cultures were then treated for 1 h with 0.3 mM H_2O_2. Finally, cells were cultured for further 4 days in fresh Cr and H_2O_2-free medium. Micrographs were taken on the third and fifth day of differentiation. *Bars* 10 µm

H_2O_2-induced mtDNA damage, an effect which has been demonstrated in HUVEC cells and that is also being investigated in oxidatively injured, differentiating C2C12 cells. In this regard we found that, 48 h after the oxidative insult, differentiating C2C12 cells pre-loaded with Cr showed levels of mtDNA copies (mtDNA/nDNA ratio) higher than those from H_2O_2-treated, Cr-unsupplemented cells and similar to control cultures (unpublished observations).

Other Cr-induced effects independent from antioxidant mechanisms but relevant to the myogenic ability and its resistance to oxidative stress have been identified. Cr pre-loading up-regulated the mRNA expression of IGF-1 and of several MRFs such as MRF4 and myogenin 24 and 48 h after serum deprivation, supporting the hypothesis that a threshold level of MRF expression is reached earlier in the presence of Cr (Louis et al. 2004). Up-regulation of these targets was quantitatively similar to that observed previously (Louis

et al. 2004; Deldicque et al. 2007) and is likely to play a pivotal role in the observed hypertrophic response of Cr-primed differentiating C2C12 cells. H_2O_2 treatment significantly reduced the expression level of IGF-1, MRF4 and myogenin, as compared with untreated cells; IGF-1 seems to be particularly sensitive to oxidative challenge. Interestingly, Cr pre-loading prevented these effects: indeed, 3 days after serum deprivation and 2 after oxidative challenge Cr-primed H_2O_2-injured cells expressed levels identical (MyoD and myogenin) to, or even higher (IGF-1 and MRF4) than those of control, Cr-unsupplemented cells. Again, the effects induced by trolox were far different than those elicited by Cr: indeed, it lacked the capacity of stimulating the expression of either IGF-1 or of the other MRFs.

It is also worth noting that it has been recently reported that IGF-1 induces a strong oxidant-resistant phenotype, which reflects the inhibition of ROS production in the cytosolic and mitochondrial compartments in mesangial cells (Kang et al. 2003; Yang et al. 2005). This additional mechanism might contribute to render cells more resistant to oxidative stress and its consequences. Further studies in this direction will be needed to fully establish this hypothesis.

As to non muscular tissues, recent experimental reports suggest that Cr might play an important role in correct CNS neuron differentiation and function. As an example, Cr can be exocytotically released by neurons as a cotransmitter (Almeida et al. 2006) and modulate $GABA_A$ receptors exerting either inhibitory (De Deyn and Macdonald 1990) or excitatory (Koga et al. 2005) actions. It is worth considering that GABA receptor activity plays important roles during brain development participating in neuron differentiation, migration, dendritic maturation and modelling of neuronal circuitry (for an extensive review, see Represa and Ben-Ari 2005). Moreover, in vitro Cr supplementation was seen to readdress neuron differentiation toward GABA-ergic phenotype in striatal (Andres et al. 2005) and spinal cord cell cultures (Ducray et al. 2007). In spite of these evidences, during—and up to the later stages of—pregnancy, endogenous Cr synthesis in the developing brain is very limited in such a way that maternal Cr is the main source for most of foetal life (Braissant et al. 2007; Ireland et al. 2009). As a consequence, developing CNS is seriously exposed to the risk of Cr deficiency in case of premature birth or placental insufficiency, a fact that suggests that Cr supplementation could be important in these situations. Also, pro-differentiative and antioxidant activities of Cr on CNS could assume particular interest because there is mounting evidence that oxidative damage to vital cellular targets contributes to the pathogenesis of brain injury in both the immature and mature nervous system (McQuillen and Ferriero 2004). After perinatal ischemic injury from various stimuli, it is believed that the infant brain experiences a cascade of toxic events involving energy failure, glutamate release, activation of NMDA receptors, calcium influx and formation of NO by nNOS (Vexler and Ferriero 2001). These events result in mitochondrial dysfunction and leakage of O_2, which is dismutated to more toxic reactive species such as hydrogen peroxide and hydroxyl radical.

Since, to the best of our knowledge, there is no study directly aimed at investigating whether Cr protects differentiating neuroblasts from oxidative attack, we set out a study similar to that carried out in differentiating C2C12 myoblasts (unpublished observations). For this purpose, primary cultures of neuroblasts from spinal cords of 7-day-old chick embryos were prepared and grown for 24 h; cultures were then maintained in the absence or in the presence of 5, 10 or 20 mM Cr for 24 h, acutely challenged with a bolus of H_2O_2 (5–30 μM for 40 min) and then cultured for up to further 4 days to monitor their viability and NPSH intracellular levels; neurite length and voltage gated ion currents (by whole-cell patch-clamp) were parallelly monitored and taken as indexes of morphological or functional differentiation, respectively.

As expected, oxidative treatment caused an acute reduction of NPSH levels and a marked, dose-related loss of viability (as assessed by means of MTT and cell counting). These effects, which are typically induced by H_2O_2 could be prevented either by the reference antioxidant trolox or by 10 mM Cr. At the differentiative level, neuroblasts surviving the H_2O_2 insult displayed profound neurite structural damages which reflect their difficulty to continue a normal differentiative task while notably, in the case of Cr pre-loaded cells, neurites were morphologically indistinguishable from those of unintoxicated controls. Trolox afforded a slightly lower protection to neurite elongation and branching, as compared with Cr. As to the assessment of the functional competence of differentiating neuroblasts, a more complex interpretation of the electrophysiological results has to be drawn. Indeed, only cells with a well-preserved membrane integrity are eligible for whole-cell patch clamp assays: as a consequence, among oxidative-stressed cells only those capable of maintaining their integrity during the procedure could be scored in such a way that the results obtained might represent some underestimation of the overall and actual suffering of H_2O_2-injured cultures. According to this latter notion, H_2O_2-treated cells did not show significant electrophysiological differences (Na^+ and K^+ voltage-gated currents) as compared with controls. On the contrary Cr-enriched cells, independently of H_2O_2 intoxication showed, with respect to controls, a dose/time-dependent increase of Na^+ and K^+ voltage-gated current intensities during differentiation. Again, all the effects elicited by Cr were prevented by co-exposure with GPA.

Other indirect effects possibly contributing to Cr antioxidant activity

Other non-scavenging effects of Cr that may anyhow contribute to increase cellular resistance to oxidative stress—or to critical situations where ROS are involved—have been reported. A recent study by Young et al. (2010), using an explorative NMR-based metabolomic and proteomic approach, demonstrates that in mature murine C2C12 myotubes Cr promotes an additional, indirect antioxidant effect, i.e. the up-regulation of one of the cellular antioxidative systems, namely the thiol redox system, which consists of the glutathione and thioredoxin pathways. Two thioredoxin reductases situated in the mitochondria and cytoplasm, respectively, were increased in Cr-treated cells: peroxiredoxin-4, a type 2 peroxiredoxin, and thioredoxin-dependent peroxide reductase. Up-regulation of these enzymes might effectively contribute to some of the protective effects discussed throughout this article. Extending this investigation to other cell types will be important in order to understand the relative contribution of direct and indirect scavenging in Cr protection from oxidative stress.

Meyer et al. (2006) reported that in hyperglicemic conditions, mt creatine kinase (mtCK) activity—supported by an availability of Cr resulting in adequate CrP/Cr ratios—prevents mitochondrial ROS formation through the ADP-cycling system and thus identified an antioxidant-like role for mtCK unrelated to the direct scavenging capacity of Cr. Beneficial effects of Cr have been observed in keratinocytes exposed to UV rays (whose toxicity depends in part on intracellular ROS formation) and H_2O_2. In this study, the authors (Lenz et al. 2005) ascribed this activity to a protective effect of Cr on CK oxidant-induced inactivation *via* a non-antioxidant mechanism, but rather *via* an energy related one. Accordingly, Berneburg et al. (2005) had previously demonstrated that 1 mM Cr, by normalizing the cell's energy status, completely abolished induction of the common deletion, a mtDNA mutation marker, generated in normal human fibroblast by repetitive UV-A irradiation.

Conclusion

Cr is capable of exerting mild direct antioxidant activity in cultured mammalian cells acutely injured with an array of different ROS generating compounds. This activity, on a molar basis, is far lower than that of many other antioxidants of natural origin: for example, many polyphenols from plants act as antioxidants in the nanomolar to micromolar range, while Cr scavenging activity becomes apparent above a threshold level which, importantly, is similar to that attainable in plasma upon oral Cr supplementation in humans (1–2 mM). However, Cr is a "physiologically on-board" ubiquitous molecule and, as a consequence, its pharmacokinetic—especially with regard to specific tissues—is far better than that of many plant antioxidants and may greatly improve its actual efficacy. Of note, Cr also protects from oxidative damage two distinct and critical cellular targets, namely mtDNA and RNA. Furthermore and differently from established antioxidants, Cr has been shown to promote other relevant effects which contribute to rescue cell viability and function under oxidative stressing conditions, particularly in the case of differentiating myoblasts (Fig. 3). In this situation Cr (1) preserves the integrity of mitochondria allowing adequate mitochondriogenesis, probably *via* its organelle-directed antioxidant activity (Guidi et al. 2008) (2) affords a significant sparing of intracellular thiols content (3) protects RNA from oxidative damage in a condition requiring a robust mRNA utilization (4) increases the expression of selected MRFs and of IGF-1 mRNAs and (5) augments the CrP stores, thus ameliorating cellular energy charge. Only

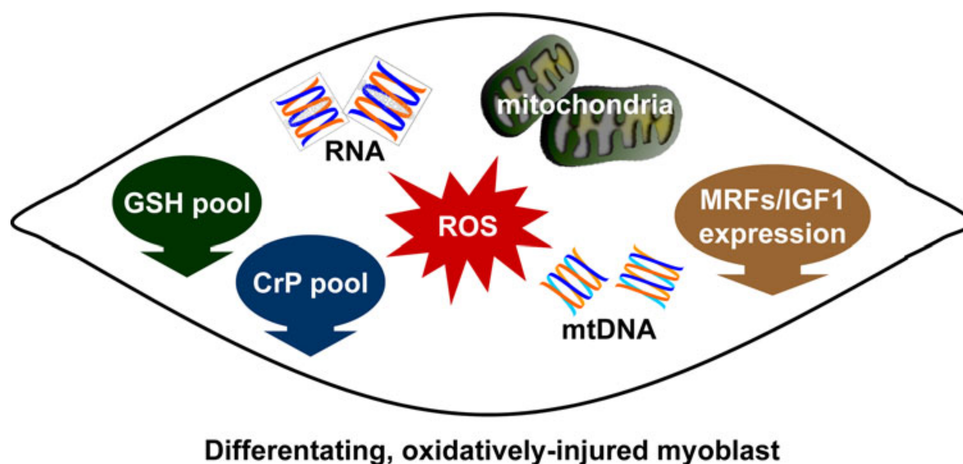

Fig. 3 Toxicologically relevant targets triggered by oxidative stress and preserved or ameliorated by Cr supplementation in differentiating C2C12 myoblasts. *MRF* muscle regulatory factors

the first three of these effects are likely to be mediated by the antioxidant capacity of Cr; the other two effects are a peculiar to Cr and independent of its antioxidant capacity, as indirectly suggested by the fact that trolox—which only acts as a ROS scavenger—increases neither MRFs and IGF-1 nor CrP pools.

These observations lend further support to the concept that the beneficial effects of Cr that are being observed at the therapeutic and preventive levels (Sipila et al. 1981; Osbakken et al. 1992; Gordon et al. 1995; Heinanen et al. 1999; Matthews et al. 1999; Vorgerd et al. 2000; Hespel et al. 2001; Mazzini et al. 2001; Stout et al. 2001; Witte et al. 2001; Tarnopolsky et al. 2004; Hansen et al. 2006; Kuethe et al. 2006) may not depend exclusively on the role of Cr in cellular energetics, but rather on multiple positive interactions with diverse cellular targets (Lawler et al. 2002; Louis et al. 2004; Lenz et al. 2005; Alfieri et al. 2006; Olsen et al. 2006; Sestili et al. 2006; Bender et al. 2007; Brosnan et al. 2007; Ducray et al. 2007; Rodriguez et al. 2007; Guidi et al. 2008; Oliveira et al. 2008). The comprehension of Cr pleiotropism is an emerging issue in research literature, and the results in this direction might be important to rationally interpret the role and exploit the therapeutic/preventive potential of Cr supplementation in specific situations.

The implications of this concept are manyfold and raise clinical, nutritional and future research issues. As discussed previously, muscle inflammation and a variety of muscular disorders are complicated by both persisting oxidative stress and impairment of the tissue repair capacity (Vorgerd et al. 2000; Hespel et al. 2001; Mazzini et al. 2001; Stout et al. 2001; Fulle et al. 2004; Tarnopolsky et al. 2004; Hansen et al. 2006). The complex antioxidant capacity of Cr might then help to better understand the mechanisms whereby its oral supplementation is somehow beneficial in the above muscle and heart diseases as well as to lend support to its use in these situations.

In a different clinical perspective, the outcome of myoblast transplantation, a promising strategy for the therapy of muscular dystrophies and heart diseases, is affected by the poor survival of implanted myoblasts (Vilquin 2005). Since it has been proposed that oxidative stress contributes to this problem, Cr pre-implant loading of myoblasts might result in a better survival and differentiative efficiency of implanted cells, and better clinical outcomes of this procedure. Finally, the use of supplementary oral Cr intakes—as well as directly and rapidly driving Cr into injured muscles by means of medical devices such as hydroelectrophoresis (Brizzi et al. 2004)—should deserve consideration in post-trauma physical rehabilitation, i.e. a situation where better muscle regeneration is obviously advisable.

From a nutritional point of view, the antioxidant activity of Cr might help to understand and reappraise the benefits arising from long-term Cr dietary supplementation, and support the advisability of elevating Cr levels not only in situations where meat consumption is obligatorily reduced (hypercholesterolemia, cardiovascular diseases, ageing, vegetarian diet), but also in specific groups (elderly, patients undergoing post-trauma rehabilitation, see also below) of omnivorous diet population. Indeed, plasma-Cr concentrations similar to those capable of eliciting the effects described herein (1–2 mM) can be achieved only through the adoption of specific oral supplementation regimens (Schedel et al. 1999). In this light, data discussed herein strengthen the rationale of "long-term dietary Cr supplementations" to gain a longer and healthier lifetime, as suggested by Bender et al. (2007) in a recent report showing that Cr improves health and survival of mice.

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