Uncoupling of oxidative phosphorylation and antioxidants affect fusion of primary human myoblasts in vitro

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Reactive oxygen species are at the origin of muscular fatigue and atrophy. They are also linked to muscular dystrophies, a group of human genetic diseases. Several studies point to the benefits of application of antioxidants and uncouplers of oxidative phosphorylation to improve the functional activity of normal and pathological muscles. Other studies point to potential dangers of these compounds. **Aim.** To study the effect of mitochondria-targeted antioxidants and uncouplers of oxidative phosphorylation on muscle differentiation. **Methods.** Muscle differentiation was induced by serum starvation and monitored by troponin T staining. **Results.** the mitochondria-targeted uncoupler of oxidative phosphorylation C12TPP, but not the mitochondria-targeted antioxidant SkQ1, inhibit fusion of primary myoblasts upon their differentiation, but do not affect the synthesis of troponin T, a protein marker of muscle differentiation. **Conclusion.** The effect of C12TPP could be at least partially mediated by inhibition of reactive oxygen species (ROS) production since antioxidant N-acetylcysteine at high doses also inhibited differentiation of myoblasts. **Keywords:** skeletal muscle, muscle differentiation, mitochondria-targeted antioxidants, uncouplers of oxidative phosphorylation

Introduction

Oxidative stress generated in human muscles increases fatigue and may even contribute to muscular atrophy (reviewed in [1]). In skeletal muscles, oxidative stress is produced in mitochondria and cytosol and expresses itself in a high level of reactive oxygen species (ROS), mostly originating from mitochondria (mtROS; reviewed in [2,3]). Oxidative stress, ROS and mtROS are linked to the pathological conditions such as muscular dystrophies, a group of heterogeneous human genetic diseases mostly affecting skeletal muscles. Indeed, oxidative stress was shown to play a role in the pathogenesis of Duchenne muscular dystrophy (DMD) [4], facioscapulo-humeral dystrophy (FSHD) [5–7] and some other muscular dystrophies (reviewed in [8]).

During muscle repair and regeneration, satellite-derived CD56+ myoblasts differentiate into myotubes. This process may be inhibited by high ROS levels, e.g. due to glutathione depletion [9]. The level of ROS may be reduced by both specific enzymes and low-molecular weight compounds, called antioxidants. ROS play an important role in...
Various cell processes such as proliferation and differentiation, in defense against infections. ROS may activate redox-sensitive transcription factors and cascades, in a number of cellular signaling systems (reviewed in [2]). At the same time, high ROS levels are detrimental for cells. Antioxidants are widely used as dietary supplements in treating muscular fatigue and atrophy [1], and they are currently studied as potential treatment of muscular dystrophies (Passerieux et al., 2015; Turki et al., 2012; Whitehead et al., 2010) (reviewed in [8]). For example, N-acetylcyesteine (NAC), a classical antioxidant was tested in the DMD murine model where it decreased inflammation and myonecrosis (de Senzi Moraes Pinto et al., 2013). At the same time, it appears that ROS are required for muscle differentiation [11, 12]; therefore a complete ROS inhibition may be deleterious for successful muscle regeneration.

In the skeletal muscle, most of the ROS production occurs in mitochondria [13], e.g. during contraction [14], or in response to the external signals such as cytokines [15]. Thus, it would be tempting to use a class of antioxidants specifically targeted to mitochondria to improve the specificity and the impact of antioxidant action. The mitochondria-targeted antioxidants are a class of molecules combining lipophilic cations capable of penetrating through the mitochondrial membranes using the energy of the transmembrane potential (reviewed in [16]) with an antioxidant part. These molecules were successfully tested for their capacity to protect muscles from senescence [17] and immobilization-induced activation of muscle proteolysis [18]. Mitochondria-targeted peptide SS-31 which protected mitochondria against an oxidative damage was shown to improve the skeletal muscle performance in aged mice [19]. At the same time, like general antioxidants, a negative effect of mitochondria-targeted compounds was observed: high doses of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL (100 nM and 100 mM, respectively) were shown to repress the myotubes fusion in H9c2 transformed rat cardiac myoblasts [12].

Another class of mitochondria-targeted molecules includes uncouplers of oxidative phosphorylation. At low doses these molecules reduce the mtROS production by mildly decreasing the mitochondrial transmembrane potential without repressing ATP synthesis [20]. For example, 2,4-dinitrophenol (DNP) has multiple tissue-protective effects in different animal and in vitro models due to the suppression of oxidative stress [21]. Unfortunately, DNP has some toxic effects and a narrow window of therapeutic activity. The other possible therapeutic strategy exploiting the effect of mild uncoupling is based on the expression of the uncoupling protein specific for brown fat (UCP1); however, the overexpression of UCP1 in muscles led to a significant delay in the re-establishment of neuro-muscular junctions after injury exacerbated the pathology in amyotrophic lateral sclerosis animal model [22].

Recently, a new class of uncouplers, lipophilic cations, has been described. One of the representatives of this new generation of uncouplers, a lipophilic membrane-penetrating cation dodecyltriphenylphosphonium (C_{12}TPP), was found to uncouple oxidative phosphorylation via the stimulation of transmembrane cycling of endogenous free fatty acids [23]. Later the uncoupling effect of other lipophilic cations was described and some of them (derivatives of Rhodamine19) catalyzed the transmembrane transport of protons in the absence of free fatty acids due to the protonation/deprotonation reactions of the cation [24]. These compounds have a wider effective concentration range because of their self-limiting mechanism of uncoupling and their therapeutic effects were demonstrated in the animal models of ischemic brain [25,26] and kidney [25] injury. The effect of lipophilic cations has not been tested on muscles yet.

Here we analyzed the effect of different concentrations of the C_{12}TPP lipophilic cation on the differentiation of human CD56^+ primary myoblasts in vitro and compared it to the antioxidants: mitochondria-targeted antioxidant SkQ1 and NAC (a general antioxidant).
Materials and Methods

Cell culture conditions and transfection. Primary myoblasts (a kind gift of Prof. Dalila Laoudj-Chenivesse) were isolated from skeletal muscle biopsies, purified using CD56/NCAM magnetic beads (Miltenyi Biotec) and cultured as described elsewhere [27].

Myogenic differentiation and myotubes quantification were carried out essentially as described in [28]. Primary myoblasts were seeded at a low density and cultured in a proliferation medium, 10 ml of which supplemented or not with the indicated concentrations of antioxidants or uncouplers. 48 h later the cells were collected, washed and seeded in the 35 mm collagen-coated petri dishes at 80–100% confluence. Myogenic differentiation was induced in the differentiation medium, supplemented or not with the indicated concentrations of antioxidants or uncouplers. The cells were kept 6 days under differentiation conditions and were then fixed and immuno-stained with anti-Troponin T primary antibody, as described below.

Analysis of myotube fusion. Fusion was analyzed after 6 days of differentiation using the Myotube Fusion Index (MFI) determined by dividing the number of nuclei in multi-nucleated myotubes by the total number of nuclei in a given microscopic field. Three fields per culture were counted using the ImageJ software.

Immunofluorescence staining and quantification. Differentiated myotubes were stained with mouse monoclonal anti-Troponin T (Sigma # T6277, 1:50) and observed under a fluorescent microscope as described elsewhere [28]. To create a full image of the specimen, the images of adjacent fields were stitched together using Cartograph software (Microvision).

Results and Discussion

Satellite-derived myoblasts from skeletal muscles differentiate into myotubes. This process is essential for the muscle repair and regeneration. The conflicting data exist about a role of oxidative stress in this process. ROS produced by NADPH oxidase are considered to be an essential signaling components for the embryonic cardiomyogenesis [29]. Scavenging of mtROS using mitochondria-targeted antioxidants (MitoQ and MitoTEMPOL) and mitochondria-targeted catalase prevents the murine the cardiac myoblasts differentiation (Lee et al., 2011). On the other hand, a severe oxidative stress induced by glutathione depletion inhibits the differentiation of murine skeletal muscle cells [9]. Most of these studies were carried on the immortalized animal cells. We decided to test the effect of cationic mitochondria-targeted uncoupler of oxidative phosphorylation C12TPP on the differentiation of primary human CD56+ myoblasts and compare it to the effects of mitochondria-targeted and general antioxidants. The cells were isolated from the skeletal muscle biopsies of two different individuals and induced to differentiate in vitro using serum starvation as described elsewhere [30]. Different concentrations of C12TPP, mitochondria-targeted antioxidant SkQ1 or NAC, were added to myoblasts 48h prior to the onset of differentiation, then the same amount of the tested compound was added to the differentiation medium. The results of the differentiation were assayed 6 days after its onset.

The control non-treated cells produced the branched myotubes with bright troponin T staining (Figure 1A). Similar results were obtained with the cells, treated with 2–200 nM SkQ1, 2–20 nM C12TPP and 1 mM NAC (Figure 1). In contrast, the myoblasts pre-treated with 200 nM C12TPP and 5 mM NAC did not form prolonged myotubes, although the individual cells had a prolonged shape and demonstrated troponin T staining (Figure 1C, G).

Next we have determined the myotube fusion index (MFI) for all experimental conditions. This parameter was obtained by dividing the number of nuclei in multi-nucleated myotubes, containing more than 3 nuclei by the total number of nuclei. The MFI was 72% for the control cells. In cells treated with 1 mM NAC and 200 nM SkQ1, the MFI slightly increased, although this was not statistically significant, in the cells treated with 5 mM NAC, the MFI was significantly lower than in the control cells.
21±10 %, and 200 nM and C_{12}TPP completely abolished cell fusion (Figure 2).

Thus, we have shown that C_{12}TPP lipophilic cation interferes with the human muscle differentiation by inhibiting the fusion of myoblasts into polynucleated myotubes at a concentration of 200 nM. The differentiation process is not completely inhibited, as the cells acquire an elongated shape (Figure 1G) and express troponin T, a marker of the muscular differentiation. Previously, the high doses of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL (100 nM and 100 mM, respectively) were shown to repress the myotubes fusion in the H9c2 transformed rat cardiac myoblasts cell line [12]. We have next compared the effect of C_{12}TPP with that of NAC, the classical antioxidant and SkQ1, the mitochondria-targeted antioxidant whose lipophilic part is identical to that of C_{12}TPP [31].

The treatment of myoblasts with 5 mM NAC significantly inhibited the myoblast fusion while a lower dose of NAC (1 mM) did not affect the MFI. This raises the question of the safety of this compound, as the opposite effects can be obtained in a very small range of concentrations. Comparing the effects of C_{12}TPP and SkQ1, one can notice that their effects are similar at the concentration of 2–20 nM, but opposite at 200 nM. This may be due to the fact that the uncoupling effect of SkQ1 is significantly less pro-
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nounced than that of C12TPP, and its mitochondria-targeted antioxidant effect is not detrimental to the cell differentiation. Moreover, the mild uncoupling effect of C12TPP may be also linked to the activation of AMP-activated protein kinase (AMPK) [32], an enzyme known to inhibit the differentiation of C2C12 myoblasts [33].

Another conclusion from this study concerns SkQ1. Its analog MitoQ was shown to repress differentiation of murine cardiac myoblasts cell lines at a concentration of 100 nM [12]. In our hands, a similar dose of SkQ1 did not affect the myoblast fusion, suggesting that SkQ1 has a better range of effective and safe concentrations as compared to MitoQ, although we cannot completely exclude that the differentiation of human myoblasts does not depend on mtROS as dramatically as in case of the murine cardiac myoblasts.

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Роз'єднання окисного фосфорилювання і антиоксидантів впливають на злиття первинних людських міобластів in vitro

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Активні форми кисню (АФК) можуть викликати м'язову втому і атрофію м'язів. АФК також пов'язані з м'язовими дистрофіями. Множество исследований указывает на положительное влияние антиоксидантов и разобщителей окислительного fosфорилирования на функциональную активность мышц в норме и патологии. Цель. Изучить влияние митохондриально-направленных антиоксидантов и разобщителей окислительного фосфорилирования на дифференцировку первичных миобластов человека. Методы. Мышечную дифференцировку вызывали инкубацией миобластов в среде, содержащей низкую концентрацию сыворотки. Миотрубочки окрашивали антителами к Тропонину T. Результаты. Митохондриально-направленный разобщитель окислительного fosфорилирования C_{12}TPP, но не митохондриально-направленный антиоксидант SkQ1, ингибирует слияние миобластов при дифференцировке, при этом не влияя на экспрессию тропонина T, белкового маркера мышечной дифференцировки. Выводы: Влияние C_{12}TPP может быть частично вызвано ингибированием АФК, так как высокие концентрации классического антиоксиданта N-ацетилцистеин также ингибировали дифференцировку миобластов человека.

Ключевые слова: скелетные мышцы, мышечная дифференцировка, митохондриально-направленные антиоксиданты, разобщители окислительного фосфорилирования.

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