Green tea extract decreases muscle necrosis in mdx mice and protects against reactive oxygen species

Timo M Buetler, Mathilde Renard, Elizabeth A Offord, Heinz Schneider, and Urs T Ruegg

ABSTRACT

Background: Duchenne muscular dystrophy is a severe X-linked congenital disorder characterized by lethal muscle wasting caused by the absence of the structural protein dystrophin.

Objective: Because generation of reactive oxygen species appears to play an important role in the pathogenesis of this disease, we tested whether antioxidant green tea extract could diminish muscle necrosis in the mdx mouse dystrophy model.

Design: A diet supplemented with 0.01% or 0.05% green tea extract was fed to dams and neonates for 4 wk beginning on the day of birth. Muscle necrosis and regeneration were determined in stained cryosections of soleus and elongator digitorum longus muscles. Radical scavenging by green tea extract was determined in differentiated cultured C2C12 cells treated with tert-butyldihydroperoxide, with the use of 2',7'-dichlorofluorescin diacetate as a radical detector.

Results: This feeding regimen significantly and dose-dependently reduced necrosis in the fast-twitch muscle elongator digitorum longus but at the doses tested had no effect on the slow-twitch soleus muscle. Green tea extract concentration-dependently decreased oxidative stress induced by tert-butyldihydroperoxide treatment of cultured mouse C2C12 myotubes. The lower effective dose tested in mdx mice corresponds to ≈1.4 L (7 cups) green tea/d in humans.

Conclusion: Green tea extract may improve muscle health by reducing or delaying necrosis in mdx mice by an antioxidant mechanism.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease leading to early disability and to death, usually in early adulthood (1). The disease results from the absence of the structural protein dystrophin, and a variety of mutations and deletions in the dystrophin gene, located on chromosomal band Xp21, were identified as causing the disorder (2). The pathogenesis of DMD is frequently studied in the dystrophic mdx mouse model (3, 4). Despite a complete absence of dystrophin, mdx mice breed normally and have a normal life span. Nevertheless, the absence of dystrophin results in muscle weakness and muscle necrosis followed by regeneration from satellite cells, a process that normally starts at around the fourth week of age (5).

Muscle wasting in DMD and in mdx mice is a degenerative process that involves cycles of necrosis and regeneration. Degenerative processes are usually accompanied or even caused by alterations in calcium homeostasis and in the redox balance. We and others showed that muscle cells from dystrophic mdx mice had slightly elevated cytosolic calcium concentrations ([Ca 2+]c) that dramatically increased when the cells were stressed (high amounts of extracellular calcium, hypoosmotic shock) (6, 7). We used this stress technique to evaluate pharmacologic interventions to counteract the exaggerated increase in [Ca 2+]c or influx of Ca 2+. Both α-methylprednisolone and creatine reduced the exaggerated Ca 2+ responses in primary cultures of muscle cells from mdx mice (6, 8–10). Creatine also reduced necrosis in fast-twitch elongator digitorum longus (EDL) muscle from treated mdx mice but had little effect on slow-twitch soleus muscle (11).

Because the degenerative process in muscle wasting also involves an inflammatory response, it was suggested that oxidative stress may be involved in disease progression (12–16). Others found that dystrophic muscle cells appeared to be inherently more susceptible to oxidative stress than were normal muscle cells (12, 13, 15) and that this susceptibility inversely correlated with the amount of residual dystrophin expression (16). Several authors detected markers of oxidative stress in muscles of either DMD patients or mdx mice (14). Evidence for an involvement of reactive oxygen species (ROS) comes from observations that biological byproducts of oxidative stress were higher than...
normal (lipid peroxidation and protein carbonyls) (17), that cellular antioxidants were lower than normal (glutathione and vitamin E) (14, 18), and that concentrations of antioxidant enzymes were altered (14, 18). Results from Bornman et al (19) suggested that prevention of oxidative stress in mdx mice may protect against disease progression. Their experimental rationale was to decrease the availability of free iron, which participates in transition metal–catalyzed generation of the highly reactive hydroxyl radical (Fenton reaction). In addition, iron deprivation increased the expression of the protective heat shock protein 70 over baseline (19).

Green tea is consumed in many parts of the world and is recognized for its antioxidative and cancer chemopreventive properties (20). Prolonged survival in Japanese cancer patients drinking ≥1.0 L (5 cups) green tea/d was reported (21). Moreover, green tea extracts (GTEs) and the tea’s main constituent, (−)-epigallocatechin gallate, significantly inhibited carcinogen-induced skin, lung, forestomach, breast, esophagus, duodenum, and colon cancers in animal models (20). Green tea polyphenols inhibited cell proliferation in various tumor cell lines in culture and stimulated apoptosis preferentially in transformed cells but not in normal cells (22). The exact mechanism of action of green tea polyphenols is not known, but they possess antioxidative capacity and can trap hydroxyl and peroxyl radicals (23). This antioxidative effect was also reported in humans, who had increased plasma antioxidative capacity after controlled green tea consumption (24, 25).

In the present study, we investigated the effect of crude GTE on muscle necrosis in the mdx dystrophic mouse model. In addition, we assessed whether GTE protects cultured C2C12 myotubes from the prooxidant effect of tert-butylhydroperoxide (BHP).

**MATERIALS AND METHODS**

**Chemicals**

GTEs (decaffeinated Sunphenon DCF-1) in powdered form were a gift from the Taiyo Kagaku Co (Yokkaichi, Japan). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Juro Supply AG, Lucerne, Switzerland), 2′,7′-Dichlorofluorescein (DCF) and DMEM were from Sigma (Buchs, Switzerland). Human recombinant insulin-like growth factor I was from R&D (Abingdon, United Kingdom). Tissue culture reagents were from Life Technologies (Basel, Switzerland). All other reagents were of analytic grade and were from various commercial sources.

**Animals and feeding protocol**

The mdx mice were originally obtained from Iffa Credo (L’Arbresle, France), but later generations were bred in-house. Mice were housed in plastic cages in a temperature-controlled environment with a 12-h light-dark cycle and free access to food and water. All animal experiments were performed in compliance with the Swiss Federal Veterinary Office’s guidelines, based on the Swiss Federal Law on Animal Welfare of 1978, and were approved by the Cantonal Veterinary Service. A control group of 21 mdx mice (4 litters) were fed a standard rat diet (Eberle NAFAG, Gossau, Switzerland), and 2 experimental groups of 4 and 10 mdx mice (1 and 2 litters, respectively) were fed a standard diet supplemented with 0.01% and 0.05% (by wt) GTE, respectively, prepared by Eberle NAFAG. The regimen started at birth by feeding the dams the control or GTE diets. The offspring also had access to the GTE diet after weaning (at ~3 wk of age).

**Histologic studies**

The EDL and soleus muscles of 4-wk-old mdx mice were removed bilaterally under anesthesia and immediately frozen in liquid nitrogen–cooled isopentane. Mice were euthanized by decapitation. Frozen muscles were cut into 10-μm-thick sections with a cryostat (Frigocut 2800; Reichert-Jung, Arnsberg, Germany) and stored at −80°C. To ensure that we would obtain a large cross-sectional area, cross sections of hematoxylin–eosin–stained whole-muscle were taken from the midpoint of the muscle body. This staining procedure distinguishes healthy myofibers, identified by the presence of peripheral nuclei, from necrotic and regenerating areas, characterized by infiltrating lymphocytes (inflammatory and necrotic areas), and areas of regenerating myofibers displaying central nuclei. The total cross-sectional areas and the necrotic and regenerated surfaces were determined by using the Neurolucida system (Microbrightfield, Colchester, VT). Results are expressed as the ratio of the area occupied by inflammatory cells and regenerating myofibers divided by the total surface area as a percentage.

**Cell culture**

C2C12 myoblasts were cultured in DMEM containing 10% fetal calf serum in a humidified atmosphere of 5% CO₂ in air at 37°C. To induce myobute differentiation, confluent cultures of C2C12 myoblasts were incubated in DMEM containing 2% horse serum and 10 μg insulin-like growth factor I/L to stimulate growth and differentiation.

**In vitro tests**

The antioxidative capacity of GTE was determined with the use of C2C12 myotubes in suspension. After trypsinization, myotubes were centrifuged at 300 × g for 5 min at room temperature and resuspended in Hanks buffered salt solution (HBSS: 1.26 mmol CaCl₂/L, 0.5 mmol MgSO₄/L, 5.36 mmol KCl/L, 150 mmol NaCl/L, 0.44 mmol KH₂PO₄/L, 0.27 mmol Na₂HPO₄/L, 4.2 mmol NaHCO₃/L, and 5 mmol glucose/L; pH 7.4). Cells were treated with GTE at room temperature for 20 min in HBSS, centrifuged as above, resuspended in HBSS, and transferred to a quartz cuvette in a Fluorolog 3000 spectrofluorimeter (Yobin Yvon Ltd, Stanmore, United Kingdom) at 37°C. The formation of ROS was monitored with the use of DCFH. DCFH was applied to cells as the nonreactive DCFH-DA. During cellular uptake DCFH-DA is deacetylated by membrane-associated esterases to DCFH, which is not fluorescent. Oxidation by ROS then yields the highly fluorescent DCF. After a 2-min equilibration at 37°C, DCFH-DA was added to the final concentration of 5 μmol/L and the development of basal DCF fluorescence at excitation and emission wavelengths of 501 and 521 nm, respectively, was followed for another 2 min. Oxidative stress was induced by adding 0.5 mmol BHP/L, and the formation of DCF was followed for 18 min. The rate of DCF formation during the last minute of incubation was used to determine the specific ROS formation, normalized for protein content determined by the Bradford assay (26). Results are expressed as the amount of DCF formed/min ng protein⁻¹. To ensure that the antioxidative activity of GTE was caused by intracellular localization of the active components, extracellular GTE was removed by centrifugation at 300 × g for 5 min at room temperature before the BHP stress protocol.
**RESULTS**

Total food consumption was monitored during the 4 wk of treatment, and animals consuming the GTE-enriched diets consumed slightly but not significantly less food than the controls. This lower consumption, however, did not result in a lower body weight at 4 wk in the GTE-supplemented mice than in the controls (Figure 1). The weights of soleus and EDL muscle excised from treated mice were not significantly different from those of untreated mdx mice. The daily GTE intake in the 0.05% GTE–supplemented mice corresponds to ≈140 mg GTE/kg body wt for an adult mouse or ≈4 mL brewed green tea (20).

The results of quantitative evaluation of the necrotic and regenerating areas of stained cryosections of soleus and EDL muscle from control and GTE-treated animals are shown in Figure 2. In slow-twitch soleus muscle of untreated mdx mice, ≈77% of the surface was occupied by necrosis or regenerating cells or both, whereas ≈47% of EDL muscle had necrotic or regenerating surfaces. GTE treatment had no detectable effect on soleus muscle, but in fast-twitch EDL muscle the area occupied by necrotic and regenerating fibers was 30 ± 13% and 26 ± 7% after treatment with 0.01% GTE and 0.05% GTE, respectively.

Control experiments in which differentiated C2C12 myotubes were treated with BHP showed that neither DCFH nor DCFH-DA reacted significantly with BHP. Because neither DCFH-DA nor DCFH reacted with BHP, the fluorescence detected in whole cells must stem from cell-mediated, BHP-derived ROS [ie, OH· (27)]. The rate of BHP-induced DCF formation in control myotubes was ≈55 pmol·min⁻¹·mg protein⁻¹. Other prooxidants were tested for their capacity to generate cell-dependent fluorescence (data not shown). Of these, N-ethylmaleimide (1 mmol/L) and 2,3-dimethoxy-1,4-naphthoquinone (10 μmol/L) strongly reacted with DCFH in the absence of cells and could not be used. Cisplatin (10 μmol/L), paraquat (10 μmol/L), bacterial lipopolysaccharide (10 mg/L), and potassium cyanide (0.1 mmol/L) were relatively inert toward DCFH, but their cell-mediated generation of fluorescence was marginal compared with BHP. The concentration of BHP used (0.5 mmol/L) was nontoxic to the cells during the 20 min of treatment as determined by visual evaluation (floating cells, membrane blebbing) and protein quantification. The use of a combination of DCFH-DA and BHP ensured that the fluorescent signal detected was representative of ROS generated within myotubes.

Pretreatment of C2C12 myotubes with GTE for 20 min showed that GTE dose-dependently decreased the BHP-induced DCF signal with a median inhibitory concentration of 18 ± 3 mg/L (Figure 3).

**DISCUSSION**

In the present study, we showed that dietary GTE supplementation preferentially protected the EDL muscle of mdx mice from necrosis. The levels of protection are similar to results recently described for creatine (10, 11). The mechanism for GTE protection is not clear but may be mediated by its antioxidative activity because GTE dose-dependently protected cultured C2C12 myotubes from BHP-induced oxidative stress. It was shown that muscles from mdx mice are more susceptible to oxidative stress (13, 15, 16);...
thus, diminishing oxidative stress may protect against disease progression. Necrosis and regeneration were consistently found to be higher in soleus than in EDL muscle (11). Type I muscle (soleus) contains more mitochondria and may therefore have a higher capacity to generate radicals. Therefore, it could be argued that soleus muscle is exposed to higher levels of oxidative stress, necrosis, and inflammation. In turn, disease-related inflammation could exacerbate oxidative stress preferentially in soleus muscle. It could then be argued that the antioxidative capacity of GTE at the doses applied was not sufficiently great to scavenge the larger amounts of free radicals produced in soleus muscle, whereas in the less-affected EDL muscle GTE’s antioxidative capacity sufficed to reduce free radical–induced cellular damage. Because EDL muscle is composed of both slow- and fast-twitch fibers, the partial protection of EDL muscle afforded by GTE may be explained by a selective or preferential effect on fast-twitch fibers in EDL muscle with little effect on slow-twitch fibers. However, because we did not try to discriminate between slow- and fast-twitch fibers, this hypothesis needs to be tested in future experiments.

Whether green tea polyphenols are transferred from mothers to offspring is unknown, and we did not attempt to determine serum polyphenol concentrations in the offspring. Therefore, it is unclear whether the protective effect on EDL necrosis was afforded by exposure to polyphenols from birth on (via mother’s milk) or only via dietary exposure starting at weaning. Note that even if offspring received green tea polyphenols via mother’s milk, the amounts ingested most likely increased after weaning because of direct exposure. Thus, the GTE effect might be mostly caused by the GTE ingested with food after weaning.

It was suggested that oxidative stress might contribute to muscular dystrophy (19), so antioxidants might protect against disease onset or progression. Green tea polyphenols have antioxidative properties and appear to be potent scavengers of hydroxyl radicals (OH·) known for their damaging effects on cellular macromolecules (28). Takabayashi et al (29) showed that a single dose of GTE significantly decreased the amount of 8-hydroxyguanosine, a marker of OH· attack on DNA, in pancreatic and hepatic DNA in hamsters. A second protective effect of GTE polyphenols may be that they increase the expression of detoxifying enzymes (eg, glutathione transferase) and glutathione synthesis (30, 31). By this mechanism GTE might boost the endogenous antioxidative capacity and indirectly increase the detoxification of ROS. GTE and its major polyphenolic constituents are also recognized for their anticarcinogenic properties, mediated, at least in part, by their antioxidative capacity (20).

The hypothesis that GTE is chemoprotective was supported by the finding that regular intake of green tea [>1.0 L (5 cups)/d] significantly reduced the incidence of certain cancers in the Japanese population (32). In the present study, the lower effective dose of 0.01% GTE, which significantly reduced necrosis and regeneration in EDL muscle, corresponds to ≈1.4 L (7 cups) green tea/d in humans; thus, dietary intervention in DMD patients is feasible. However, because this small-scale study used laboratory animals, further studies in both animals and humans are necessary to confirm a protective effect of GTE in DMD. Nevertheless, green tea may offer an inexpensive, effective, and non-toxic intervention strategy for persons with DMD.

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