PROJECT REVIEW

"Prevalence of the exercise-induced bronchoconstriction using the mannitol test, insight into potential ergogenic and deleterious effects of beta-2 agonists."

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Beta2-agonist use under medical control is in constant increase in the elite sportsmen, particularly in the endurance sports. However, sensitivity of the exercise-induced bronchoconstriction (EIB) detection test is still under debate, partly due to the fact that several hypothesis exist concerning the underlying action mechanisms leading to EIB. In the first part of our multicentre project, we will re-evaluate the EIB prevalence by a new and more specific test, the mannitol test developed by S. Anderson *et al.*, in order to limit the cases of false positive and thus, both the acute and chronic use of beta2-agonists by athletes. In a second part, we will investigate the potential deleterious effect of chronic use of beta2-agonists in pulmonary function, myocardic contractile function and bone metabolism. If these two hypotesis are confirmed, it would provide a strong argument for prevention against doping.

Punishment is not the only way for the anti-doping fight. Prevention and education represent also fundamental aspects. The belief in wonderful effects of substance plays a significant role among the processes leading to doping. The exact knowledge of ergogenic effect and mechanisms involved is necessary to demystify drug effects. There are conflicting results as for the effects of acute or chronic intake of beta2-agonist intake on athletic performance. We propose to contribute to 1°) a more precise evaluation of EIB in elite population of athletes and 2°) a larger knowledge of beta2-agonist action mechanisms on different human systems which both should limit the misuse of beta2-agonists in sports.

Results and Conclusions

“Prevalence of the exercise-induced bronchoconstriction using mannitol test: insights into potential ergogenic and deleterious effects of beta-2-agonists”

This project dealing with β2-agonist focused on human performance, skeletal, cardiac, bone tissues and involved several laboratories of different countries. Nine separate studies were conducted.

In the first one, acute therapeutic oral intake of salbutamol appears to induce, an ergogenic effect during supramaximal exercise whatever the subjects' gender. An increase ($P<0.05$) in peak power and mean power output was observed during sprint exercise. This ergogenic effect could be due a positive impact of salbutamol on excitation-contraction coupling.

A second study was conducted to test this hypothesis in healthy athletes with similar therapeutic acute intake salbutamol (6 mg) under electrical stimulations in isometric condition. The peak torque of stimuli train of 1s duration at a frequency of 20 Hz was significantly modified ($P<0.05$) in low frequency fatigue condition compared to placebo situation while no change could be evidenced in resting condition suggesting a protective effect of salbutamol in fatigue state. An advantage could be anticipated in sports including maximal intermittent exercise (e.g. tennis, collective sport events). Moreover, in the twitch characteristics, the half time of relaxation was in most cases shortened under salbutamol condition compared to placebo. These protective effects against muscular fatigue elicited by acute salbutamol intake could be due to alterations in Ca^{2+} release and Ca^{2+} uptake by the sarcoplasmic reticulum.

The next studies focused on skeletal muscle adaptations. Obviously, the invasive investigations required were performed on animal. Three weeks of high doses of clenbuterol treatment induced anabolic effects ($P<0.05$) especially in EDL muscle and a greater isometric force was observed in both fast ($P<0.001$) and slow ($P<0.001$) twitch muscles (EDL and soleus, respectively) compared to control groups. Nevertheless, when maximal tetanic force was corrected for muscle cross-sectional area, specific tension was unchanged and even depressed in soleus ($P<0.001$) muscle. The increase in myofibrilar ATPase activities observed both in relaxed ($P<0.001$) and activated conditions ($P<0.001$) in soleus muscle suggests that the depressed specific tension is not due the contractile machinery it self. Another step in the muscle shortening process must be involved (see Ca2+ transient study below). The unchanged specific tension observed during clenbuterol treatment in EDL was associated with an unchanged myosin ATPase activity in fast twitch muscle. In the same line, the shortening duration of myofibrils in unloading condition was not significantly modified suggesting a lack of effect on maximal shortening velocity. Finally, significant reduction of fatigue resistance with clenbuterol treatment was observed in EDL suggesting changes within the muscle: MHC shift to fast and more fatigable isoforms, alteration in muscle calcium-handling ability and/or

alteration in muscle energetic pathways. Concerning practical implications in doping prevention, one could argue that high dosage of clenbuterol would induce depressed specific isometric force especially in slow twitch muscle. This negative effect is of particular importance in sport events where performance depends on body mass. Another potential negative effect for slow twitch muscle lies in the increased ATP cost of shortening suggesting an altered efficiency in mechanochemico transduction and energy cost of locomotion. The most marked adverse effect concerning sport performance is probably the lesser resistance to fatigue observed with such high doses of clenbuterol especially for sport events with exercise durations around 1 min. Since these depressive effects could be linked with alteration in Ca^{2+} homeostasis the goal of next study was to characterise clenbuterol effects on calcium transients in fast twitch muscle. A decreased amplitude of global Ca2+ transients ($P<0.01$), associated with a reduced sarcoplasmic reticulum Ca2+ load ($P<0.01$) were observed after 14 days of clenbuterol administration. Local Ca^{2+} release events were also impaired ($P<0.01$) after only 9 days of clenbuterol administration. Ca^{2+} sparks in EDL muscle from clenbuterol-treated animals exhibit a reduced frequency, a smaller amplitude and a shorter spatial spread ($P<0.01$) compared to controls. The Ca^{2+} disturbances observed in the present study could explain firstly the negative effect observed in specific tension and secondly the skeletal muscle remodelling usually evidenced in the preceding study.

In this context, the two next works focused on hypertrophy and Ca^{2+} signalling pathways. At doping doses, clenbuterol provoked an hypertrophy ($P<0.05$) more marked in fast twitch muscles (EDL) compared to slow twitch muscle (soleus). In EDL muscle, phenotypic conversion became significant earlier (9 days of treatment) than hypertrophy (14 days) suggesting that the phenotype fiber shift may be a prerequisite for hypertrophy. The rate constants that described phenotypic shift during treatment were similar between soleus and EDL suggesting a similar kinetics of phenotypic remodelling between slow and fast twitch fibers. Calcineurin protein expression is often used in the literature as a marker of muscle hypertrophy. Nevertheless, the implication of this protein in muscle anabolism seems less dependent of protein expression than its activity level. In our study, hypertrophy was significant ($P<0.05$) after 14 days of treatment in EDL muscle but calcineurin expression was increased ($P<0.05$) only after 21 days. The myogenic factor MyoD is usually associated with a fast phenotype of muscle fibers. Although in our study, both soleus and EDL underwent a phenotypic shift toward a greater proportion of fast fibers, MyoD protein expression was increased ($P<0.05$) only in soleus. In EDL, the proportion of type IIb fibers increased whereas type IIa decreased. In contrast, in soleus, the main alteration lies in the increased proportion of type IIa fibers. One can expect that MyoD protein expression correlates with the proportion of type IIb fibers. The mechanisms underlying the enhancement in cross sectional area seems to implicate both the calcineurin/NFAT pathway as well as the PI3/Akt/mTOR/p70SK6 pathway. The increase in soleus MyoD expression suggests that hypertrophy may come from the stimulation of satellite cells. However in this muscle, a necro/apoptotic phase appeared in the early stage

of the treatment, quickly followed by regeneration. Here we show that the ubiquitous calcium dependant proteolysis system, the calpains 1 & 2, are differentially activated ($P<0.05$). Soleus, characterized by a high density of !2-adreno-receptors, responded to doping doses of clenbuterol by a transient decrease ($p<0.05$) in calpain 1 activation and by a rapid calpain 2 activation. EDL, a rapid muscle responds by a progressive increase ($p<0.05$) in both calpain 1 & 2. The main difference between these two muscles is that soleus muscle underwent a rapid necro/apoptotic transient phase followed by regeneration, EDL did not. Thus, rapid muscles, with low β2-adreno-receptors density, seem to be protected against a transient necro/apoptotic phase. Calpain 1 may participate to the hypertrophy/ phenotypic process by cleaving the protein of cytoskeleton to insure the addition/replacement of the protein of the myofilaments. The precise role of calpain 2 is less clear. The calpain 2-induced partial proteolysis and activation of intermediates in the signalling cascades could participate to hypertrophy and/or the phenotypic shift, like protein kinase C. Here, the main practical implications in doping prevention lie in the two following findings: 1°) high doses of clenbuterol determine a transient deleterious impact on slow twitch muscle, rich in !2-adrenoreceptors, 2°) the ubiquitous calcium dependent proteolysis system is differentially activated in oxidative and glycolytic muscles and their activation may explain the disorganization/organization of the cytoskeleton for the addition/replacement of myofilaments required in hypertrophy. It is not excluded that cardiac muscle, very rich in !2-adrenoreceptors, could display a similar transient deleterious impact to soleus.

To further investigate the mechanisms involved in clenbuterol induced hypertrophy, another study was conducted with primary human muscle cell culture. To this end we compared the action of clenbuterol with the action of DAPT, a pharmacological inhibitor of Notch signalling and a prohibited substance in athletes. The results obtained here showed that clenbuterol and DAPT act as hypertrophic agents in vitro. They also indicate that these “anabolic” agents possess the ability to down-regulate the myostatin pathway. Indeed, myostatin could be a major player in the anabolic action of these compounds. These results raise the possibility that myostatin level might be a predictor of the use of anabolic agents. Therefore screening manipulations of myostatin signalling could be in the future a way in the fight against doping. Based on significant positive effects of chronic β-2 treatment on both muscle mass and force, we further investigate bone micro architecture because bone tissue is in principle closely related to mechanical loading and obviously to muscle force. For the first time, we observed deleterious effects of salbutamol on bone in trained rats, despite an increase in muscle mass. The alteration of bone tissue due to increased bone resorption occurred mainly in trabecular bone micro architecture ($P<0.05$) and was also observed in biomechanical measurements of cortical and trabecular bone ($P<0.05$). The doses used in our study are not so different to those delivered during the chronic administration of such drugs, particularly by young athletes under 20 years of age. This population is using more and more of these substances because of their lypolytic and anabolic effects on skeletal muscles. Unfortunately, the effects of these substances at doping

doses have never been tested in sedentary humans or in athletes. We believe that testing salbutamol and clenbuterol on the rat bones has provided a valuable initial assessment of the skeletal phenotype of some athletes who take chronic salbutamol or clenbuterol for doping purposes. Of course an increased risk of fracture and especially risk of osteoporosis in senior female athlete could be anticipated.

Finally, we studied the effect of clenbuterol administration on cardiac performance and calcium transients in cardiomyocyte. Chronic treatment of animals with clenbuterol, for three weeks induced a positive inotropism at the cellular cardiac level ($P<0.05$). This is characterized by an increase ($P<0.05$) in cellular shortening mostly due to an increase ($P<0.05$) in calcium release from the reticulum at each contraction. These modifications increase with time of treatment ($P<0.05$) suggesting a lack of β-receptors desensitization between 9 and 21 days of treatment. The clenbuterol treatment was insufficient to modify the contractile properties of the myofilaments while at least of its component, titin, is phosphorylated by the clenbuterol treatment. Three weeks of treatment in our conditions were insufficient to induce deleterious effects on cellular contractility. However considering the large increase in the calcium transient (by 30%, $P<0.05$), it is possible that a maintained hyper stimulation of the myocytes at this level or to higher levels of calcium transient will have potential arythmogenic effects.