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Plasma and Muscle Polyamine Levels in Aerobically Exercised Rats Treated with Salbutamol

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Abstract

The induction of hypertrophy of cardiac and skeletal muscles has been studied after treatment with two different salbutamol dosages, therapeutic and doping.

Treatment of rats subjected to a physical training schedule with repeated doses ($16\,\mu g\,kg^{-1}$ per day or $3\,mg\,kg^{-1}$ per day) of salbutamol, a specific β-adrenergic agonist, induced a marked increase in both skeletal and heart-muscle weight, whereas total body weight did not change significantly. Adrenergic involvement of salbutamol-linked muscle hypertrophy was demonstrated by co-administration of the non-specific β-adrenergic antagonist, propranolol ($20\,mg\,kg^{-1}$ per day). Salbutamol-induced muscle hypertrophy was associated with an increase in serum, skeletal-muscle and heart levels of the naturally occurring polyamines putrescine, spermidine and spermine.

These observations suggest the involvement of polyamines in muscle hypertrophy and the possible role of blood polyamines as exposure biomarkers in β-adrenergic-muscle hypertrophy.

Chronic treatment with β-adrenergic agonists as doping agents in elite athletes is partly because of enhancement of ventilation parameters, changes in the shape and size of skeletal muscle fibres and positive inotropic (increase in contractile strength) and chronotropic (increase in heart rate) effects that can lead an improvement in physical performance. It has been reported that chronic exposure to β-adrenergic agonists leads to increased protein deposition in muscle and removal of lipids from fat depots (re-partitional effect; Yang & McElligott 1989). Salbutamol sulphate (Ventolin) as an aerosol is widely recommended to asthmatic patients as a bronchodilator because of its relaxant properties on smooth muscle (Price & Clissold 1989). It has also been recommended for patients suffering from exercise-induced asthma before they start exercise (Inman & O' Byrne 1996). Its re-partitional effect and physical performance improvement can lead not only to fraudulent results but also to toxic side effects. These facts must be considered not only by International Olympic Committee authorities, but also from a sanitary point of view (Libretto 1994).

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The stimulation of the adrenergic system in both developmental and therapeutic processes induces physiological and biochemical alterations, some mediated by the binding of the agonists to the receptors. Unlike heart neonatal development and physical exercise, β-adrenergic-linked hypertrophy increases the amount of skeletal and heart muscle with no replication of myocytes (Slotkin et al 1987).

One biochemical change associated with β-adrenergic stimulation is a rapid increase in ornithine decarboxylase activity followed by accumulation of polyamines (Bartolome et al 1980a). The role of naturally occurring polyamines (putrescine, spermidine and spermine) in cell growth, and differentiation of skeletal and heart muscles and other tissues, is well-established (Pegg 1988). The pathway of polyamine biosynthesis is controlled by two highly regulated enzymes, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. Both enzymes have very short half-lives, circumstances that enable strict control of their activities by the mechanisms of cellular homeostasis (McCann & Pegg 1992). Rapid changes in ornithine decarboxylase activity and the consequent increase in intracellular polyamine content seem to be an early feature after several stimuli including those that

cause muscle and heart hypertrophy (Bardocz et al 1992; Cubría et al 1998a, b). Bartolome et al (1980b) showed that isoproterenol heart hypertrophy could be prevented by the irreversible ornithine decarboxylase inhibitor α-difluoromethylornithine. Other short-term effects of β-adrenergic compounds in heart myocytes mediated by polyamines include calcium homeostasis (Koenig et al 1983). Some authors regard polyamines as secondary messengers of inwards calcium current generated after β-adrenergic stimulation as influx of sugars and amino acids (Fan & Koenig 1988; Koenig et al 1988).

In this report we describe a study of the induction of hypertrophy of cardiac and skeletal muscles after treatment with two different dosages, therapeutic and doping, of salbutamol. We have also measured blood and muscle polyamine levels in control and aerobic exercised rats.

Materials and Methods

Chemicals

The β-adrenergic antagonist propranolol and high-performance liquid chromatography (HPLC)-standards of putrescine, spermidine, spermine, 2-hydroxydiaminopropane and dansyl chloride were purchased from Sigma (St Louis, MO). Salbutamol was a generous gift from Glaxo (Middlesex, UK). All the other reagents and chemicals were of standard laboratory grade.

Drug treatment

Male Wistar rats, 165 ± 10 g, were divided into groups of 6–8 animals of similar body weight. They were housed homogeneously in communal cages at a constant temperature of 22°C on a 12-h light–dark cycle and fed with a solid diet Panlab A-04 (Barcelona, Spain) for rats; tap water was freely available. Rats were injected intraperitoneally either with saline solution or with an equivalent volume of saline containing propranolol hydrochloride (20 mg kg^{-1} per day). After 30 min each animal was injected intraperitoneally either with salbutamol sulphate (16 μg kg^{-1} per day for the therapeutic protocol or 3 mg kg^{-1} for the doping protocol), or with an equivalent volume of vehicle. Solutions of salbutamol and propranolol were prepared freshly with sterilized saline immediately before administration to animals. Blood samples (1 mL) were collected by puncture of the ocular plexus with a capillary tube 15, 30, 60 and 90 days after the beginning of the experiment. Rats were killed by cervical dislocation 3 months after the beginning of the experiments. Heart, the leg skeletal muscle gastrocnemius, and suprarenal fat were excised, washed with ice-cold saline, weighed, and frozen at −80°C until used for analysis of polyamine content.

Aerobic training

Rats were trained in a treadmill with variable slope and speed, throughout the experimental period. The exercise protocol to which the rats were subjected ensured aerobic training (Willis et al 1988).

Determination of polyamines

Putrescine, spermidine and spermine in tissues and blood samples were determined by means of a prederivatization method described elsewhere (Escribano & Merodio 1994). Tissues were homogenized in 5 vols distilled water and then treated with perchloric acid (6% final concn). The extracts were centrifuged (10 000 *g* for 15 min, at room temperature) and supernatant (0·2 mL) was neutralized with a saturated solution of $NaHCO_3$ (0·2 mL) before overnight dansylation with a solution of dansyl chloride in acetone (20 mg mL^{-1}; 0·4 mL). After two extractions with toluene the organic phase was evaporated under a stream of nitrogen, resuspended in acetonitrile (1 mL) and polyamines were quantified by HPLC analysis on a C_{18} reversed-phase column according to the method cited above. 2-Hydroxydiaminopropane was used as internal standard. Concentrations of polyamines were expressed as nmol mL^{-1} blood or nmol g^{-1} wet tissue.

Results

Table 1 shows the heart-to-body weight ratio (cardiosomatic index) of the rats after 90 days treatment with salbutamol (16 μg kg^{-1} per day or 3 mg kg^{-1} per day) under resting conditions or under aerobic training conditions. Chronic exposure to salbutamol led to a significant dose-dependent enlargement of the cardiac muscle in resting compared with untreated control rats ($P < 0·05$) and in aerobically trained compared with resting control rats ($P < 0·001$) or with trained control rats ($P < 0·05$). More significant differences were found in those groups exposed to the largest doses of salbutamol (3 mg kg^{-1} per day); for these the cardiosomatic index was 33% higher than that found for resting controls. The effect of training was to enhance this value to 0·036 (44% higher than resting controls and 24% higher than aerobically trained controls). Total body-weight changes throughout the experimental period were not observed (Table 2).

These significant differences were partially abolished by co-administration of propranolol

Table 1. Muscle (gastrocnemius) to total-body-weight ratio and cardiosomatic index (heart weight to total-body-weight ratio) of salbutamol-treated aerobically exercised or unexercised rats.

Treatment	(Gastrocnemius muscle weight/body weight) $\times 10^2$		(Heart weight/body weight) $\times 10^2$	
	Untrained	Trained	Untrained	Trained
Control	0·48 ± 0·09	0·53 ± 0·09*	0·25 ± 0·09	0·29 ± 0·04†
Salbutamol 16 $\mu g\,kg^{-1}$	0·52 ± 0·05†	0·56 ± 0·03*‡	0·28 ± 0·07†	0·32 ± 0·02*‡
+ propranolol 20 $mg\,kg^{-1}$	0·49 ± 0·05	0·051 ± 0·06*	0·26 ± 0·06	0·29 ± 0·03†
Salbutamol 3 $mg\,kg^{-1}$	0·55 ± 0·08†§	0·60 ± 0·04*‡	0·32 ± 0·05*	0·36 ± 0·07*‡
+ propranolol 20 $mg\,kg^{-1}$	0·51 ± 0·11†	0·53 ± 0·06*	0·28 ± 0·06†	0·31 ± 0·01†§

Animals were trained aerobically for 3 months by the method of Willis et al (1988). Propranolol was administered at a dose of 10 $mg\,kg^{-1}$ per day 30 min before injection of salbutamol (16 $\mu g\,kg^{-1}$ per day or 3 $mg\,kg^{-1}$ per day). After 3 months and the organs and tissues were excised for analysis. Values are the means ± s.d. of results from 6 to 8 animals. * $P < 0.001$, † $P < 0.005$, significantly different from results from untrained controls; ‡ $P < 0.001$, § $P < 0.005$, significantly different from results from exercised control values.

(20 $mg\,kg^{-1}$ per day) 30 min before the β-adrenergic agonist. Propranolol administered by subcutaneous injection partially prevented salbutamol-induced myocardial hypertrophy in both therapeutic and doping regimes.

Similar results were obtained when skeletal muscle was analysed. Gastrocnemius weight-to-total-body-weight ratio was determined for all the experimental groups three months after the beginning of the experiments. The results in Table 1 are indicative of significant salbutamol-linked hypertrophy and enlargement as a result of the aerobic training. The extent of salbutamol-linked muscle hypertrophy was dose-dependent, the muscle-weight-to-body-weight ratio being clearly increased in both therapeutic and doping groups, the average estimates for the resting and training groups, respectively, being 0·0053 and 0·0056 in the group receiving 16 $\mu g\,kg^{-1}$ salbutamol daily and 0·0057 and 0·0060 in the group receiving 3 $mg\,kg^{-1}$ daily. All these results were significantly higher than those found in the resting untreated group. Similarly to heart hypertrophy, gastrocnemius enlargement was prevented when propranolol (20 $mg\,kg^{-1}$ per day) was administered, thus pointing at the adrenergic nature of the muscle hypertrophy.

Table 2. Body weight of salbutamol-treated rats subjected to physical training.

Treatment	Untrained	Trained
Control	411 ± 22	335 ± 17
Salbutamol 16 $\mu g\,kg^{-1}$	395 ± 31	344 ± 32
+ propranolol 20 $mg\,kg^{-1}$	384 ± 27	327 ± 26
Salbutamol 3 $mg\,kg^{-1}$	399 ± 27	348 ± 37
+ propranolol 20 $mg\,kg^{-1}$	381 ± 21	327 ± 18

Each result is the mean ± s.e. of results from 6 to 8 animals.

Heart and muscle polyamine levels were analysed in both resting and trained rats under the salbutamol doping protocol. Table 3 shows the effect of salbutamol on heart polyamine content. Large and dose-dependent increases in the levels of putrescine, spermidine and spermine were detected as a consequence of salbutamol exposure. This increase was larger for putrescine (3·3- and 7-fold in resting animals treated with 16 $\mu g\,kg^{-1}$ per day and 3 $mg\,kg^{-1}$ per day, respectively, than for untreated resting controls) than for spermidine and spermine. Training also induced increases of cardiac polyamine levels to values estimated as 250% for putrescine, 170% for spermidine and 135% for spermine compared with the resting untreated control. Polyamine pattern during physical training was also significantly increased, putrescine levels being more sensitive than those of spermidine and spermine to the β-agonist (more than ninefold induction). In all the cases shown in Table 3 the co-administration of the non-specific β-adrenergic antagonist propranolol at the doses and regimes mentioned above partially prevented heart polyamine overload.

A closely similar pattern was observed when polyamine levels were analysed in gastrocnemius muscle (Table 4). A significant dose-dependent enhancement of polyamine levels was detected as a consequence of continuous exposure to salbutamol. Unlike those in cardiac muscle, levels of spermidine and spermine in gastrocnemius muscle responded to the β-adrenergic agonist less drastically than did putrescine, the accumulation of which was estimated at > 300% in those animals exposed to the largest salbutamol doses. Similar putrescine (333%), spermidine (169%) and spermine (138%) overload levels were observed in rats subjected to aerobic physical training and treated

Table 3. Heart polyamine levels in salbutamol-treated rats subjected to physical training.

Treatment	Untrained			Trained		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Control	61·69 ± 1·46	55·31 ± 0·42	48·61 ± 0·20	154·63 ± 0·56†	94·11 ± 0·14†	66·10 ± 0·36
Salbutamol 16 $\mu g\,kg^{-1}$	206·42 ± 0·85*	164·47 ± 2·29*	153·38 ± 1·42*	235·16 ± 2·47*‡	215·01 ± 1·43*‡	204·64 ± 1·71*‡
+ propranolol 20 $mg\,kg^{-1}$	49·95 ± 0·19	92·77 ± 0·69	80·09 ± 0·72	111·27 ± 0·62	105·64 ± 0·22†	95·69 ± 0·33†
Salbutamol 3 $mg\,kg^{-1}$	437·86 ± 2·70*	247·97 ± 0·97*	233·09 ± 1·18*	521·79 ± 0·68*‡	302·85 ± 1·40*‡	292·05 ± 2·64*‡
+ propranolol 20 $mg\,kg^{-1}$	96·63 ± 1·40†	93·53 ± 0·67†	84·29 ± 0·34†	161·78 ± 0·62*	130·25 ± 1·42*	97·18 ± 0·60†

Animals were trained for three months by the method of Willis et al (1988). The animals were killed and the hearts perfused before analysis for polyamines. Values (nmol g^{-1} wet tissue) are means ± s.e. of results from 6 to 8 animals. * $P < 0.001$, † $P < 0.005$, significantly different from results from untrained controls; ‡ $P < 0.001$, significantly different from results from exercised controls.

with 3 $mg\,kg^{-1}$ salbutamol per day. In all the groups shown in Table 4 co-administration of salbutamol and propranolol partially reduced muscle polyamine accumulation.

As a consequence of organic polyamine overload blood polyamine levels also increased in all the experimental groups. Plasma polyamine levels (Table 5) increased in line with the increase in cardiac and skeletal muscle described above. Putrescine was the polyamine which became most concentrated in plasma as a result of salbutamol exposure—levels nearly 12-fold higher than in the resting untreated group were found in rats exposed to 3 $mg\,kg^{-1}$ salbutamol per day. Spermidine and spermine concentrations were also significantly increased, although to a lesser extent (5-fold induction) than putrescine. In addition, accumulation of plasma polyamines also occurred during aerobic physical training when compared with the resting untreated group. Similar to the other results propranolol only partially prevented the plasma polyamine burden.

Discussion

In this work we have studied the effect of two salbutamol regimes, one resembling a therapeutic schedule, the other of higher dosage resembling that used for doping purposes, in both resting and aerobically trained rats. Similarly to other workers with ageing rats (Carter et al 1991; Carter & Lynch 1994) and mice (Cubría et al 1998a) we observed no significant changes in total body weight throughout the experimental period, but cardiac and skeletal (gastrocnemius) muscle size and weight were significantly increased. Several studies have demonstrated that muscle-mass gain induced by β-agonists is a result of modification of muscle-protein turnover (Reeds et al 1986; Maltin et al 1987; Benson et al 1991). Unlike true anabolic hormones (Bates & Pell 1990), experiments performed in the presence of various β-adrenergic antagonists have shown that enlargement of the skeletal muscle cannot be attributed to an increase in protein biosynthesis but to a decrease in protein degradation (Maltin et al 1993).

Continuous administration of salbutamol during a prolonged period of time induced a significant gain in muscle mass estimated as 10–20% more than in untreated controls. It has been shown that clenbuterol treatment facilitates muscle-mass gain after different traumas, e.g. burns after gamma-ray exposure (Zeman et al 1994) or muscle atrophy induced by denervation (Zeman et al 1987; Maltin

Table 4. Skeletal muscle polyamine levels in salbutamol-treated rats subjected to physical training.

Treatment	Untrained			Trained		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Control	71·00 ± 0·42	43·13 ± 0·20	125·97 ± 0·20	122·17 ± 0·15	60·79 ± 0·14	169·80 ± 0·36
Salbutamol 16 $\mu g\,kg^{-1}$	129·56 ± 2·29†	55·62 ± 1·42	132·50 ± 1·18	153·81 ± 1·44*	91·15 ± 1·71§	189·30 ± 2·64
+ propranolol 20 $mg\,kg^{-1}$	85·56 ± 0·69	27·56 ± 0·72	99·60 ± 0·34	106·47 ± 0·22	34·24 ± 0·34‡	127·66 ± 0·60
Salbutamol 3 $mg\,kg^{-1}$	217·63 ± 0·97*	63·19 ± 2·70	189·18 ± 1·42†	406·69 ± 0·15*‡	102·89 ± 1·68*§	233·83 ± 1·71*§
+ propranolol 20 $mg\,kg^{-1}$	92·48 ± 0·67	46·86 ± 1·40	160·67 ± 0·72	153·06 ± 1·42	58·70 ± 0·62	183·90 ± 0·34

Animals were trained for three months by the method of Willis et al (1988). Animals were killed and gastrocnemius muscles excised, weighed and analysed for polyamines. Values (nmol g^{-1} wet tissue) are means ± s.e. of results from 6 to 8 animals. * $P < 0.001$, † $P < 0.005$, significantly different from results from untrained controls; ‡ $P < 0.001$, § $P < 0.005$, significantly different from results from exercised controls.

Table 5. Plasma polyamine levels in salbutamol-treated rats subjected to physical training.

Treatment	Untrained			Trained		
	Putrescine	Spermidine	Spermine	Putrescine	Spermidine	Spermine
Control	8·20 ± 1·47	4·90 ± 0·42	2·41 ± 0·20	12·45 ± 0·56	7·20 ± 0·14	4·77 ± 0·36
Salbutamol 16 $\mu g\,kg^{-1}$	60·93 ± 0·85*	14·76 ± 2·28*	7·62 ± 1·41*	95·21 ± 2·47*‡	23·67 ± 1·44*‡	11·45 ± 1·71*‡
+ propranolol 20 $mg\,kg^{-1}$	12·59 ± 0·19	5·55 ± 0·69	1·17 ± 0·72	15·26 ± 0·61†	9·17 ± 0·22†	3·05 ± 0·33
Salbutamol 3 $mg\,kg^{-1}$	96·39 ± 2·70*	25·53 ± 0·97*	12·13 ± 1·18*	124·96 ± 1·68*‡	30·15 ± 1·40*‡	18·98 ± 2·64*‡
+ propranolol 20 $mg\,kg^{-1}$	21·02 ± 1·40†	4·04 ± 0·67	1·08 ± 0·34	29·24 ± 0·62†§	9·05 ± 1·42†	4·09 ± 0·60*

Animals were trained for three months by the method of Willis et al (1988). Before the animals were killed, blood was collected and analysed for polyamines. Values (nmol mL^{-1} plasma) are means ± s.e. of results from 6 to 8 animals. * $P < 0·001$, † $P < 0·005$, significantly different from results from untrained controls; ‡ $P < 0·001$, § $P < 0·005$, significantly different from results from exercised controls.

et al 1989). These effects start early after the first doses, reaching maximum 8 days later and being reduced as result of desensitization of the receptors after 14 days (Stadel et al 1983).

Unlike physical exercise, β-adrenergic-induced hypertrophy occurs with no replication of DNA in the muscle fibres (Rehfeldt et al 1994). Differences in fibrilar width and shape but none in nucleus number or DNA content were apparent in β-adrenergic-treated animals. This growth process was efficiently prevented by co-administration of the β-adrenergic antagonist propranolol, as reported by Cubría et al (1998b) in mice treated continuously with clenbuterol in a subacute trial.

Polyamines have been implicated as secondary messengers in heart short-term effects induced by β-adrenergic drugs. Reported outcomes include positive inothropic effects (increase in the contractile strength of muscular fibres), positive chronotropic effects (increase in heart rate) and increase in systolic pressure followed by a complete diastolic void volume. An increase in polyamine levels involves morphological and biochemical alterations in myocytes such as changes in calcium homeostasis and nutrient (sugar and amino acid) influx from the extracellular medium (Fan & Koenig 1988). Most of these changes are mediated by polyamines as secondary messengers (Koenig et al 1983).

Besides these short-term effects, the involvement of polyamine metabolism in β-adrenergic heart and muscle hypertrophy has been well-documented. Previous studies with the non-specific β-adrenergic agonist isoproterenol have shown that polyamine biosynthesis preceded myocardial mass gain (Caldarera et al 1974; Bartolome et al 1980a, b; Pegg & Hibasami 1980). β-Adrenergic stimulation induces production of ornithine decarboxylase, the key enzyme of polyamine metabolism, by post-translational mechanisms that involve an increase in protein half-life with no changes in mRNA content (Cubría et al 1998a).

Although it has been reported that adrenergic stimulus in rats induced increments in putrescine and spermidine content only, spermine remaining unaffected (Caldarera et al 1974; Tipnis et al 1989), studies of freshly isolated rat myocytes have demonstrated that treatment with isoproterenol increased the levels of all three polyamines (Koenig et al 1988).

Polyamine metabolism in heart and muscle hypertrophy in mice has been studied by Cubría et al (1998b); they administered a solution of the irreversible ornithine decarboxylase inhibitor α-difluoromethylornithine in tap water to the animals and observed that both polyamine content and heart hypertrophy were effectively prevented. Unlike during physical training, plasma polyamine levels responded significantly and dose-dependently to administration of salbutamol during the experimental period and plasma levels of putrescine, spermidine and spermine were significantly and substantially increased in response of salbutamol but not to physical exercise. These increases were abolished by co-administration of propranolol. These results suggest an exclusive effect of the β-adrenergic agonist independent of the exercise, thus implicating polyamines as a possible secondary biomarker of doping with β-adrenergic drugs.

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