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Polyamine-mediated Heart Hypertrophy Induced by Clenbuterol in the Mouse

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Abstract

The use of β-agonists as growth-promoting agents in cattle could lead to toxic side-effects in man. One such effect is the accumulation of polyamines which seem to be implicated in muscle and heart hypertrophy. We have studied the induction of cardiac hypertrophy after treatment with clenbuterol and the role of polyamines in this effect.

Treatment of mice with repeated doses of clenbuterol, a specific β-adrenergic agonist, resulted in a marked increase in heart muscle weight whereas total body weight did not change significantly. Clenbuterol-linked cardiac hypertrophy could be prevented by co-administration of either the non-specific β-adrenergic antagonist, propranolol, or the irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine. The clenbuterol-induced cardiac hypertrophy was associated with a corresponding increase in the level of the polyamines putrescine, spermidine and spermine.

These observations are indicative of the role of polyamines in cardiac hypertrophy induced by clenbuterol.

The use of β-agonist drugs as growth-promoting agents in cattle is mainly because of the increase in protein degradation and fat removal with little or no changes in total body weight (Maltin et al 1987). Clenbuterol is one of the most potent re-partitioning agents used for this purpose both experimentally and in livestock (Reeds et al 1986; Maltin et al 1989; Yang & McElligott 1989). This re-partitioning effect has important economic consequences in industry, but consumption of large amounts of contaminated food could lead to toxic side-effects in man. The public health implications must be considered by sanitary authorities (Martínez-Navarro 1991).

The stimulation of the adrenergic system in both developmental and pathological processes induces physiological and biochemical alterations, some of which are mediated by the binding of the agonists to the receptors. However, unlike heart neonatal development, β-adrenergic-linked hypertrophy produces an increase in skeletal and heart muscle with no replication of myocytes (Slotkin et al 1987). One of the biochemical changes associated with β-adrenergic stimulation is the rapid increase in ornithine decarboxylase activity; this is followed by the accumulation of polyamines (Bartolome et al 1980a).

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The role of naturally occurring polyamines (putrescine, spermidine and spermine) in cell growth, differentiation and replication of heart muscle, and other tissues, is well-established (Pegg 1988). The pathway of polyamine biosynthesis is regulated by two highly regulated enzymes, ornithine decarboxylase and *S*-adenosylmethionine decarboxylase. Both enzymes have very short half-lives, a circumstance enabling strict control of their activity by the mechanisms of cellular homeostasis (McCann & Pegg 1992). Rapid changes in ornithine decarboxylase activity, and consequently in intracellular polyamine content, seems to be an early feature after several stimuli including those that cause muscle and heart hypertrophy (Bardocz et al 1992; Cubría et al 1997). Bartolome et al (1980b) showed that isoproterenol heart hypertrophy could be prevented by the well-known irreversible ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO). In addition, short-term effects of β-adrenergic compounds in heart myocytes mediated by polyamines include calcium homeostasis (Koenig et al 1983). Some authors have suggested that polyamines are second messengers of inwards calcium current and influx of sugars and amino acids generated after β-adre-

nergic stimulation (Fan & Koenig 1988; Koenig et al 1988).

In this study, we report the induction of cardiac hypertrophy after treatment with clenbuterol and the role of polyamines in this pathophysiological effect.

Materials and Methods

Chemicals

The β-adrenergic drugs clenbuterol and propranolol and HPLC standards putrescine, spermidine, spermine, 2-hydroxydiaminopropane and dansyl chloride were purchased from Sigma (St Louis, MO). α-Difluoromethylornithine (DFMO) was a generous gift from Merrell Dow (Cincinnati, OH). Serum enzyme kits, were purchased from Boehringer Mannheim (Germany). Other reagents and chemicals were of standard laboratory grade.

Drug treatment

Male Balb C mice were divided into groups of ten animals of similar body weight (24 ± 2 g). Animals 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 for mice. Both food and water were freely available. Mice were injected intraperitoneally either with saline solution or with an equivalent volume of saline containing propranolol hydrochloride ($1{\cdot}5\,mg\,kg^{-1}$). Thirty minutes later each animal was injected intraperitoneally either with clenbuterol ($1{\cdot}5\,mg\,kg^{-1}$) or with an equivalent volume of vehicle; these injections were then repeated twice a day. Solutions of clenbuterol and propranolol were freshly prepared with sterilized tap-water immediately before administration to the animals. DFMO was administered in drinking water at a final concentration of $15\,g\,L^{-1}$ (w/v). The mice were killed by cervical dislocation 12 days post-treatment. The hearts were excised, washed with ice-cold saline, weighed, and frozen at −80°C until used for analysis of polyamine content.

Polyamine determination

Putrescine, spermidine and spermine in heart extracts were determined by use of a pre-derivatization method described elsewhere (Escribano & Merodio 1994). Tissues were homogenized in 5 vol distilled water and then treated with 4% perchloric acid (final concentration). The extracts were centrifuged (10 000 *g* for 15 min at room temperature) and 0·2 mL supernatant was neutralized with 0·2 mL saturated $NaHCO_3$ solution, before dansylation overnight with a solution containing dansyl chloride in acetone ($20\,mg\,mL^{-1}$; 0·4 mL). After two extractions with toluene the combined extract was dried under a stream of nitrogen, resuspended in acetonitrile (1 mL) and analysed by HPLC on a C_{18} reverse-phase column. 2-Hydroxydiaminopropane was used as internal standard for the quantification. Concentrations of polyamines were expressed as nmol (mg total protein)$^{-1}$ (Bradford 1976).

Serum enzymes

Serum enzymes were determined in blood samples (about 1 mL) freshly collected by intra-cardiac puncture of ether-anaesthetized mice. Creatine phosphokinase (CK), its myocardial isozyme (CKmb) and lactate dehydrogenase were measured spectrophotometrically according to instructions given by Boehringer Mannheim.

Results

Figure 1 shows the cardiosomatic index (ratio of heart weight to body weight) for the twelve days of continuous treatment with clenbuterol. The experiment was performed in the presence and absence of the non-specific β-adrenergic antagonist propranolol (Figure 1A) or the irreversible ornithine decarboxylase inhibitor DFMO, to establish the role of an adrenergic response and the involvement of polyamines, respectively. Continuous administration of clenbuterol under the regimes described above induced a significant ($P \leq 0{\cdot}001$) increase in cardiosomatic index after the 2nd day post-treatment. The involvement of adrenergic response in this process was assessed by co-administration of propranolol 30 min before clenbuterol injection. Propranolol significantly reduced heart growth from the 4th day onwards in the clenbuterol-treated animals. No differences in cardiosomatic index was observed between propranolol-treated and control groups. Propranolol did not prevent initial cardiac hypertrophy when it was injected with clenbuterol but attenuated the hypertrophy after four days of treatment.

Heart growth was also assessed in DFMO-treated animals. Figure 1B shows that from the beginning of the treatment DFMO completely prevented the development of clenbuterol-linked heart hypertrophy. There was also a significant reduction in cardiosomatic index in animals treated solely with DFMO, compared with controls.

Figure 2 shows that after the 2nd day post-treatment levels of putrescine, spermidine and spermine were significantly ($P \leq 0{\cdot}001$) higher in clenbuterol-treated animals than in control animals, and remained at the higher levels throughout the period of analysis. Involvement of the adrenergic system

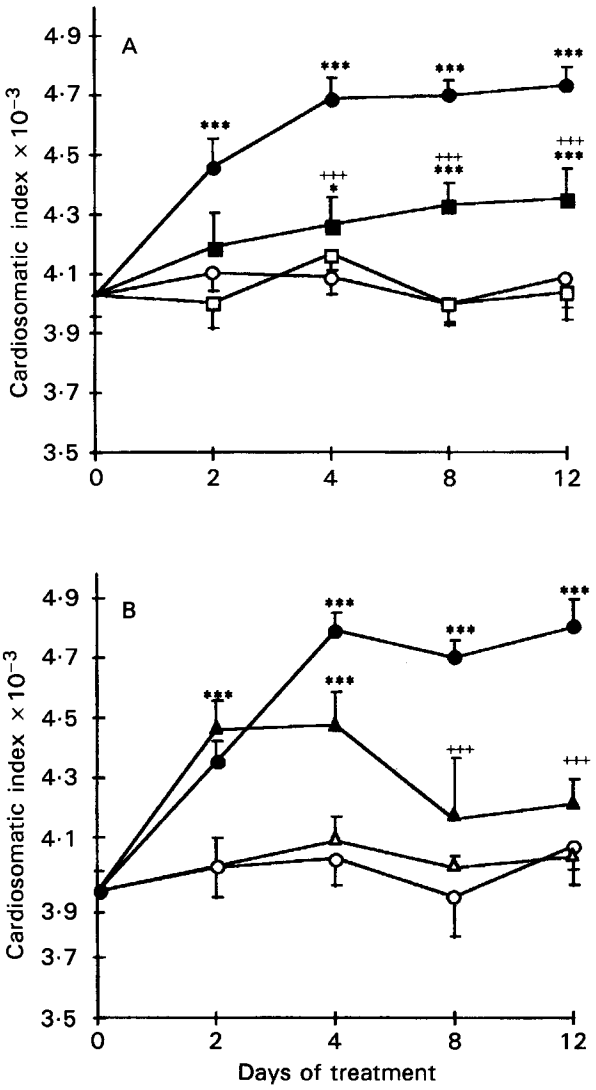

Figure 1. Cardiosomatic index (ratio of heart weight to body weight) during repeated daily treatment with clenbuterol in mice co-treated with propranolol (A) or DFMO (1·5% w/v in water) (B). Clenbuterol (1·5 mg kg^{-1} in saline) was injected intraperitoneally twice a day. Animals were killed and freshly dissected on the days indicated on the plots. ○, Control; ●, clenbuterol; □, propranolol; △, DFMO; ■, clenbuterol plus propranolol; ▲, clenbuterol plus DFMO. Mice were killed 4 h after injection. Data are the means ± s.d. of results from at least nine animals. $^{***}P \leq 0{\cdot}001$, $^{*}P \leq 0{\cdot}01$, significantly different from control; $^{+++}P \leq 0{\cdot}001$, significantly different from result for clenbuterol alone.

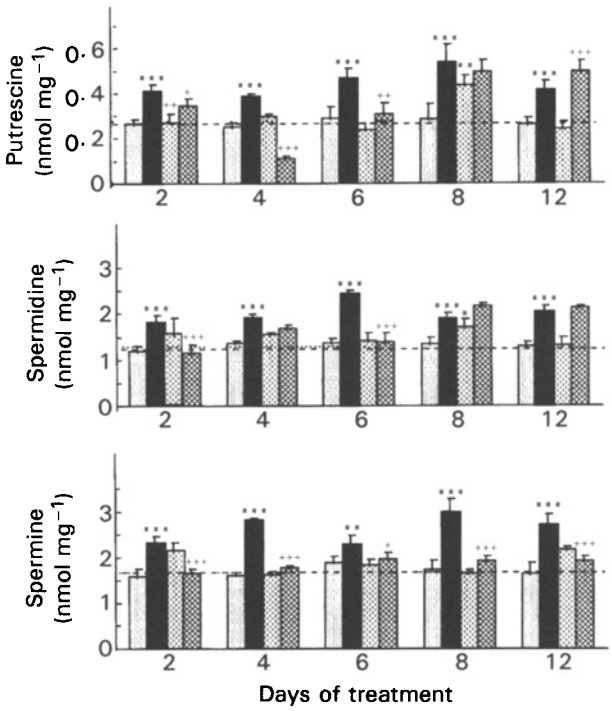

Figure 2. Adrenergic effect of continuous administration of clenbuterol on mouse-heart polyamines. Animals were injected daily with propranolol (1·5 mg kg^{-1} day^{-1}) then with saline or clenbuterol (1·5 mg kg^{-1} day^{-1}) or both. Animals were killed 4 h after administration of clenbuterol or at 4-h periods thereafter and heart polyamine content was determined. Data are the means ± s.d. of results from at least nine animals. $^{***}P \leq 0{\cdot}001$, $^{**}P < 0{\cdot}005$, $^{*}P < 0{\cdot}01$, significantly different from control; $^{+++}P \leq 0{\cdot}001$, $^{+}P \leq 0{\cdot}01$, significantly different from result for clenbuterol alone. ▨, Control; ■, clenbuterol; ▩, propranolol, ▩, clenbuterol + propranolol.

in the increase in heart polyamine levels was studied in animals treated with propranolol, either solely or co-administered with clenbuterol. Although treatment with propranolol prevented putrescine and spermidine accumulation during the first stage of the experimental period (days 2 to 6), spermine levels in clenbuterol-treated mice remained higher during the experiment and propranolol significantly ($P \leq 0{\cdot}001$) blocked the accumulation of this polyamine to concentrations near those observed for the control animals.

Figure 3 shows the effect of DFMO on polyamine levels in the mouse heart during clenbuterol-linked heart hypertrophy. DFMO alone was effective at depleting putrescine pools from day 1 ($P \leq 0{\cdot}001$) to day 4 ($P \leq 0{\cdot}05$) after the beginning of the experiments whereas in the later period the level of putrescine recovered. Spermidine and spermine levels remained unchanged. Nevertheless, DFMO significantly prevented accumulation of putrescine and, to a lesser extent, that of spermidine and spermine, throughout the experimental period in clenbuterol-treated animals.

Cellular injury after clenbuterol treatment was analysed using the serum enzymes lactate dehydrogenase and CK, and the cardiac isozyme of CK, CKmb, as exposure biomarkers of muscle damage. Figure 4A shows the large increase ($P \leq 0{\cdot}001$) in serum CK in clenbuterol-treated mice in the early stages of the experimental period (day 4). The serum CK value increased by as much as ca. 15-fold. Clenbuterol-induced release of CK diminished

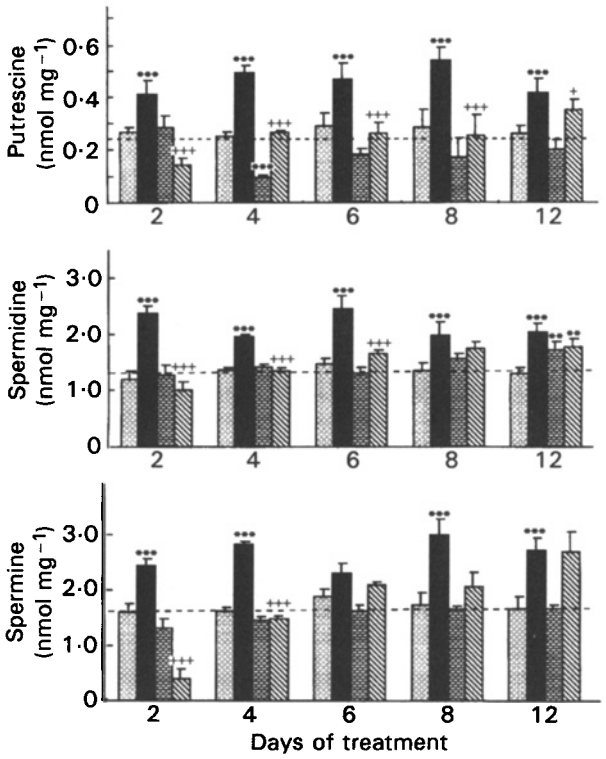

Figure 3. Polyamine content during clenbuterol heart hypertrophy in the presence of the ornithine decarboxylase inhibitor DFMO. Mice were injected with clenbuterol twice in a day and had free access to DFMO (1·5% in drinking water). Animals were killed 4 h after injection of clenbuterol or at 4-h periods thereafter and heart polyamine content was determined. Data are the means ± s.d. of results from at least nine animals. $^{***}P \leq 0{\cdot}001$, $^{**}P \leq 0{\cdot}005$, significantly different from control; $^{+++}P \leq 0{\cdot}001$, $^{+}P \leq 0{\cdot}01$, significantly different from result for clenbuterol alone. ▨, Control; ■, clenbuterol; ▩, DFMO; ▧ clenbuterol + DFMO.

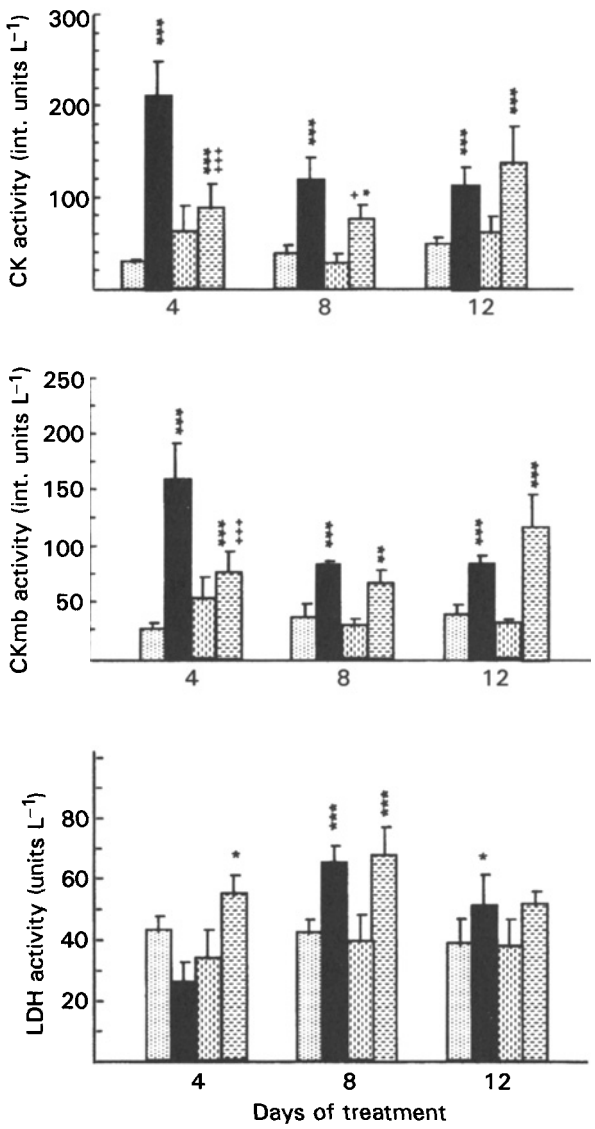

Figure 4. Activities of serum CK, CKmb and lactate dehydrogenase after continuous exposure to clenbuterol in the presence or absence of propranolol. Animals were bled by intracardiac puncture on days 4, 6 and 12 after the beginning of the experiment. Data are the means ± s.d. of results from at least nine animals. $^{***}P \leq 0{\cdot}001$, $^{**}P \leq 0{\cdot}005$, $^{*}P \leq 0{\cdot}01$, significantly different from control; $^{+++}P \leq 0{\cdot}001$, $^{+}P \leq 0{\cdot}01$, significantly different from result for clenbuterol alone. ▨, Control; ■, clenbuterol; ▨, propranolol; ▨, clenbuterol + proprandol.

during the later period of treatment. Similarly, serum CKmb (Figure 4B) increased substantially after four days of clenbuterol exposure, to levels at least six times those in control, untreated animals. However, the serum lactate dehydrogenase activity pattern was more variable than those of the enzymes mentioned above, being significantly ($P \leq 0{\cdot}001$) higher than in the control on day 8 after treatment (Figure 4C). Propranolol barely prevented lactate dehydrogenase release at all stages of the experiment.

Discussion

Continuous treatment of animals with β-adrenergic agonists induces serious physiopathological changes in skeletal and heart muscles. Previous work performed with the non-specific β-agonist isoproterenol, showed that heart hypertrophy was preceded by an increase in polyamine biosynthesis. This elevation of polyamine levels involves morphological and biochemical changes in myocites leading to short- and long-term alterations. Isoproterenol-linked short-term changes involves changes in calcium homeostasis and influx of nutrients (sugars and amino acids) from the extracellular medium (Fan & Koenig 1988). These changes cause myocardial contractibility shifts that can be prevented by the calcium-channel blocker verapamil (Koenig et al 1983). Polyamines act as second messengers of these processes, and their

accumulation is strictly prevented by DFMO. Long-term changes induced by β-adrenergic agents include muscle hypertrophy; the role of polyamines in this process is related to cell-growth (Bartolome et al 1980a). Clenbuterol induced an increase in heart mass with no changes in total body weight, thus significantly increasing the cardiosomatic index (Maltin et al 1987). This effect was similar to that reported after treatment with isoproterenol, a non-specific β-agonist.

Adrenergic involvement and the role of polyamine metabolism in clenbuterol-induced hypertrophy were revealed by co-administration of propranolol and DFMO to mice (Bartolome et al 1980a). Propranolol prevented the rise of cardiosomatic index by 70 to 80% during the late stages (days 8 to 12) of the treatment. Propranolol, however, failed to prevent clenbuterol-induced cardiac hypertrophy or the increase in polyamine levels during the early phase of this treatment. These effects might by explained by pharmacodynamic and pharmacokinetic features of this agonist and antagonist.

Co-administration of DFMO (1·5% in drinking water, daily) significantly blocked the rate of heart hypertrophy caused by clenbuterol throughout the experiment. This was true both for absolute values and for the cardiosomatic index. It is known that polyamines are involved in hyperplasic processes, where increases in putrescine, spermidine and spermine levels precede DNA synthesis and cell replication, but their role in hypertrophy is not clear. Bartolome et al (1980a) showed that DFMO administered 5 days before isoproterenol, prevented ornithine decarboxylase induction and polyamine accumulation in rat heart. It is probable that isoproterenol- or clenbuterol-induced hypertrophy was inhibited by DFMO, and polyamine levels were significantly reduced, unlike those of clenbuterol-treated mice, during the early stages of the experiment. This was more noticeable when spermidine/spermine ratios were compared. DFMO not only prevented accumulation of putrescine from ornithine but also blocked its transformation to the two higher polyamines.

Finally, blood analysis also revealed that animals treated continuously with clenbuterol suffered cardiac damage. Serum CK, CKmb and lactate dehydrogenase levels were significantly higher in clenbuterol-treated mice than in animals injected with saline. Astancolle et al (1991) showed that isoproterenol caused injury and calcium overload in the perfused heart. Ole (1983) showed that this effect was reversed by propranolol, DFMO and verapamil in freshly isolated myocytes. However, induction of muscle biomarkers in blood was maximum during early experimental stages, diminishing throughout continuous injection of clenbuterol, responding to a possible desensitization process.

In conclusion, repeated doses of clenbuterol induce heart muscle growth in mice which is accompanied with an increase in polyamine concentrations. This effect could be prevented by co-administration of propranolol or DFMO. The increase in heart polyamines was associated with cardiac hypertrophy and damage.

Acknowledgements

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