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The effects of the β2-agonist drug clenbuterol on taurine levels in heart and other tissues in the rat

M. H. Doheny, C. J. Waterfield*, and J. A. Timbrell*

Department of Toxicology, School of Pharmacy, University of London, London, United Kingdom

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Summary. The administration of a single subcutaneous dose of clenbuterol to rats altered the level of taurine in certain tissues. Taurine levels in cardiac tissue were significantly decreased 3 h after the administration of 250 μg/kg of clenbuterol and remained significantly depressed at 12 h post-dose only returning to control values by 24 h. The level of taurine in the liver increased 3 h after clenbuterol administration but was lower than the control value at 24 h post dose. Lung taurine levels were significantly lower than the control value at 12 hr post dose and remained depressed until 24 h post dose. Clenbuterol caused a significant increase in taurine levels in serum and muscle at 3 and 6 hr postdosing respectively but not at other time points. Serum creatine kinase (CK), activity was slightly but significantly raised at the 12 and 24 h time point.

The effects of clenbuterol on tissue taurine content were not dose-dependent over the range studied (63–500 μg/kg). However taurine levels in the lung were significantly reduced at all doses and in the heart were significantly lower in the treated groups at all except the lowest dose, 12 h post dosing. Liver taurine levels were significantly increased at the highest dose of 500 μg/kg.

The reduction of taurine concentrations in the heart, caused by clenbuterol, is of concern as taurine has been shown to have protective properties in many tissues especially the heart.

Keywords: Amino acids – β-Adrenoceptor agonist – Clenbuterol – Taurine

Introduction

The β-amino acid taurine is believed to be important in the functioning of many mammalian tissues, especially the myocardium, where it is found in high concentrations (20 mM; Huxtable, 1992). There are various known roles for taurine including conjugation of bile acids and proposed functions of taurine include modulation of calcium levels, maintenance of osmolarity, stabilization

* Present address: Department of Pharmacy, King's College London, U.K.

of membranes (Huxtable, 1992), antiarrhythmic activity in the heart (Read and Welty, 1963; Chazov et al., 1974) and regulation of ion fluxes (Dietrich and Diacono, 1971; Guidotti et al., 1971; Dolara et al., 1978). Although the physiological role of taurine in the heart remains unclear, a relationship between cardiac pathophysiology and taurine has been observed. Taurine is acquired both via the diet and is synthesised from cysteine in most mammalian species (Huxtable, 1992). Animals who cannot synthesize their own taurine, such as cats, develop dilated cardiomyopathy as well as other adverse effects when taurine levels are low due to reduced intake of taurine in the diet. This can be prevented by supplementation of the diet with taurine (Pion et al., 1987). Taurine has also been shown to be effective in the treatment of congestive heart failure in human patients (Azuma et al., 1985) and patients suffering from congestive heart disease have been shown to have lower heart and platelet taurine levels (Paasonen et al., 1982). However more recent data indicates that depletion of myocardial taurine leads to a reduction in ischemia-induced infarct size (Allo et al., 1997).

Taurine levels in the heart are directly related to β-adrenoceptor activation and it has been reported that a high-affinity uptake system for taurine exists in the heart (Huxtable and Chubb, 1977; Huxtable, 1992). β-Adrenergic stimulation of this system increases taurine influx into the heart (Huxtable and Chubb, 1977). However studies in the isolated perfused heart found this uptake is not blocked by verapamil indicating that it is not calcium dependent and that the influx rate is responding to cellular cyclic AMP levels (Azari and Huxtable, 1980).

Lombardini (1980) used high doses of the non-selective β-agonist drug, isoproterenol and found transient but large fluctuations of taurine levels in various tissues including the heart. Recently similar changes were found after exposure to β_2-selective β-agonist drugs (Waterfield et al., 1995; Carvalho et al., 1994). Non-selective β-agonists have been shown to have cardiotoxic effects but are seldom used clinically. However β_2-agonists are widely used for the treatment of asthma, in the prevention of premature labour, and to treat respiratory disease in cattle and horses. Further clinical uses for clenbuterol in humans have been proposed, for example in the treatment of patients with inherited muscular dystrophy, muscle wasting and endotoxeamia (Martineau et al., 1992). This is because of its repartitioning effects, which results in an increase in muscle mass and a decrease in fat deposition (Reeds et al., 1986; Yang and McElligott 1989). Clenbuterol is already used illegally in livestock to improve the protein:fat ratio and is used by athletes to increase muscle growth (Yang and McElligott, 1989).

It was found that short term, repeated treatment of rats with the β_2-agonist drugs salbutamol and clenbuterol administered in the drinking water markedly reduced urinary excretion of taurine and depleted liver taurine (Carvalho et al., 1994; Waterfield et al., 1995).

However the selective β_2-agonists are used clinically in the treatment of asthma. Therefore, in view of the protective role taurine is thought to play in the heart, the effect of β_2-agonist drugs on levels of taurine in the liver, where it is synthesized, and in the heart is of crucial importance. Thus decreasing the

level of taurine in heart tissue by the use of β_2-agonists, even transiently, could be an important factor in the known side effects of β_2-agonists.

The present study was designed to elaborate further the effect of the β_2 selective agonist drug clenbuterol on taurine levels in different tissues including the heart, over time and with increasing doses.

Materials and methods

Materials

The following compounds were supplied by Sigma Chemical Company (Poole, Dorset, U.K.); clenbuterol, o-phthalaldehyde (OPA; HPLC grade), taurine, Dowex resins, DTNB (5,5-dithiobis-2-nitrobenzoic acid) for measurement of total non-protein sulfhydryls (TNPSH), glutathione. Mercaptoethanol, sodium hydroxide (Aristar), sulfosalicylic acid and boric acid were obtained from BDH., (Lutterworth, Leicestershire, U.K.); Methanol (HPLC grade) from Rathburn (Wakeburn, Scotland, U.K.). Water was of ultra high quality (UHQ), prepared using an Elgastat water purifier. Serum biochemistry kits for the Monarch 2,000 were supplied by Boehringer (Mannheim GmbH Diagnostica). Hypnorm was obtained from Roche and Hypnovel from Janssen Animal Health.

Methods

Female Random Hooded rats (GlaxoWellcome, outbred, 150–200 g) were used in all experiments and were acclimatized in communal cages for a period of 8 or more days on arrival. They were allowed food (rat and mouse standard diet, Bantin & Kingman Universal Ltd., Aldbrough, Hull, U.K.) and water *ad lib.*

A. Time course study

After the acclimatization period the animals were assigned to 13 groups of 4 animals, each of similar mean weights and housed in communal cages. They were allowed to acclimatize for 24 h and were provided with food and water *ad lib.* Lighting was controlled to give a regular 12 h light-dark cycle (8 am on–8 pm off) and room temperature was maintained at 21 ± 1°C. At time zero the food was removed from each cage and only water was allowed *ad lib.* for 24 h. Treated animals received clenbuterol dissolved in 0.9% phosphate buffered saline (PBS) and administered as subcutaneous (s.c.) injections (250 μg/kg). Control animals received injections of saline. After designated time periods, the animals were anaesthetized with Hypnorm : Hypnoval : water (1 : 1 : 2; 3.3 ml/kg i.p.) and 5 ml of blood exsanguinated from the abdominal aorta. Blood was collected into "Microtainer serum separators" (Beckton Dickinson and Co., Rutherford, NJ, USA) left to stand for at 45 minutes, centrifuged (13,000 rpm., 1 min, @ room temperature) to separate the serum, then frozen (−80°C). The heart, lung, liver and muscle (gastrocnemius) were rapidly removed, rinsed in cold saline, blotted to remove excess moisture, weighed and then dissected keeping half the ventricular tissue of the heart, the right lobe of the liver, the lung tissue and muscle which were then frozen in liquid nitrogen and stored (−80°C) until subsequent analysis of taurine and total non-protein sulphydryl groups (TNPSH). The remaining ventricular tissue was placed in phosphate buffered formaldehyde (10.5%) for histopathology. Tissues were processed and stained with haematoxylin and eosin.

B. Dose response study

Clenbuterol was administered as a single dose of 63, 125, 250, 375 or 500 μg/kg (s.c. in PBS) to female random hooded rats (150–200 g), housed in communal cages. Control

animals received PBS. At 12 h post dose the animals were sacrificed and again the heart, lung, liver, muscle and blood were removed and processed as described above.

Serum enzymes

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and other serum parameters were measured at 37°C using the appropriate kits (Boehringer) with an automatic centrifugal analyser (IL Monarch 2,000, Instrumentation Laboratory (UK) Ltd.). Isoenzymes of creatine kinase (CK) and lactate dehydrogenase (LDH) were separated using electrophoresis and quantified by scanning densitometry using the REP automated electrophoresis system (Helena Laboratories, UK).

Taurine and total nonprotein sulphydryls (TNPSH)

Tissue taurine and TNPSH were measured using the methods of Waterfield (1994) and Ellman (1959) as previously described (Waterfield et al., 1995). Sections of frozen tissues were weighed (0.15–0.55 g) into sulphosalicylic acid (2 ml, 0.2 M, 4°C) and homogenized over ice using a "Polytron" tissue homogenizer. The homogenate was centrifuged (4,000 rpm, 15 min, 4°C), and aliquots of supernatant were taken for analysis. For taurine analysis the supernatant (25 μl) was extracted on dual bed ion – exchange columns by elution with 4 ml UHQ water (4 × 1 ml). The internal standard, homoserine, was added to the eluant (100 μl, 100 μM). For TNPSH 0.25 ml was assayed by the method of Ellman (1959). As reduced glutathione (GSH) constitutes >95% of liver and heart TNPSH, this was used as a measure of GSH (DeMaster and Redfern, 1986; Potter and Tran, 1993).

Statistical analysis

Where the mean of a single treated group was being compared with the mean of a control group the Student "t" test was used to measure significance. Where the mean of one control group was being compared to the mean of two or more treated groups Dunnett's test for multiple comparisons with a single control was used.

Results

Clinical effects of clenbuterol

Organ weights

Clenbuterol administration caused an increase in liver weights 9 h post dose (expressed as a percentage of body weight; Fig. 1). There was no change in heart or lung weights in treated animals compared to controls.

Histology

Sections taken from the hearts of animals, treated with the highest dose of clenbuterol (500 $\mu g \cdot kg^{-1}$) showed some abnormal tissue and some areas showed vacuolation. A minimal inflammatory reaction was also seen in two animals and the affected areas had pyknotic nuclei.

Biochemical effects of clenbuterol

Serum clinical chemistry

Serum cholesterol at 1 h after dosing was significantly lower in the treated animals but by 9 and 12 h after dosing, levels were significantly increased

compared to those seen in controls (Table 1). Triglyceride levels were significantly increased in the treated animals at 1, 3, 6 and 12 h after dosing (Table 1). The creatine phosphokinase (CK) activity in the serum of treated animals was significantly increased at 24 h post clenbuterol administration. The CK-isoenzymes were also measured in order to investigate further the increase seen in CK activity. It was found that CK-BB ("brain – type" isoenzyme) was increased significantly at the 24 h time point in the treated animals. CK-MM, ("muscle" – type isoenzyme) was not effected and the CK-MB, ("myocardial type isoenzyme) was undetectable in the serum. However this isozyme has a short half-life in serum and therefore any elevation in levels could have been missed. Clenbuterol caused a significant increase in serum hydroxybutyrate dehydrogenase (HBDH; Table 2) 12 h after administration but not in alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase

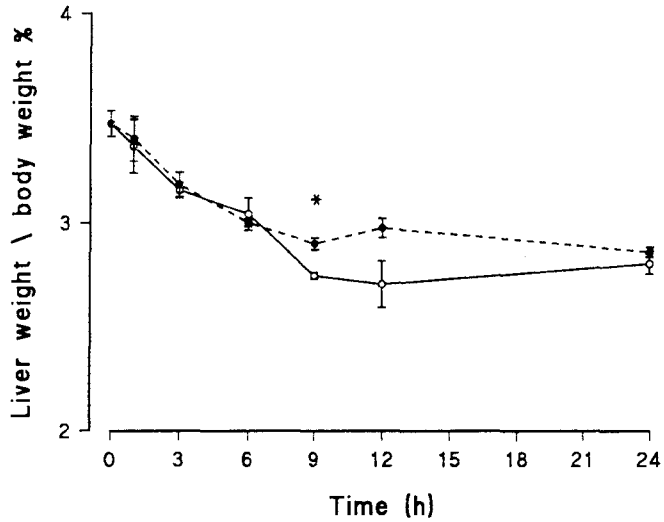

Fig. 1. Effects of clenbuterol on liver weight. Values are means ± S.EM. P value were calculated by the Students "t" test for paired data (*$p < 0.05$). Control animals ○; treated animals ●. Data are means ± SEM, N = 4

Table 1. Serum cholesterol and triglyceride levels in rats measured at various times after dosing with clenbuterol (250 $\mu g \cdot kg^{-1}$ s.c.)

Time (h)	Cholesterol ($mmol \cdot l^{-1}$)		Triglyceride ($mmol \cdot l^{-1}$)	
	Control	Treated	Control	Treated
1	2.69 ± 0.15	2.32 ± 0.10 **	0.49 ± 0.15	1.29 ± 0.14***
3	2.43 ± 0.18	2.23 ± 0.14	0.40 ± 0.07	0.70 ± 0.09**
6	2.60 ± 0.15	2.68 ± 0.27	0.33 ± 0.03	0.44 ± 0.06*
9	2.75 ± 0.11	3.00 ± 0.03*	0.38 ± 0.11	0.45 ± 0.05
12	2.55 ± 0.15	3.00 ± 0.03***	0.32 ± 0.07	0.52 ± 0.04**

Values, mean ± SEM; N = 3–4, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, using Student's t test.

(AST) or bilirubin (data not shown). Serum total protein was significantly decreased in the treated animals at 3, 6, 9 and 12 h post clenbuterol administration (Table 2). Serum creatinine and urea levels were significantly increased in treated animals at 1 and 3 h post dose and at 1, 3 and 6 h post dose respectively (Table 3).

Taurine levels

The level of taurine in heart tissue was reduced to a minimum value 6 h after administration of clenbuterol (250 μg/kg) and returned to levels similar to those seen in the control animal by 24 h after dosing. At 3 h post clenbuterol administration, the taurine content of the heart decreased significantly and by 12 h post dose the levels of taurine in the treated animals were approximately 28.1% lower than those seen in the control animals (Fig. 2A). In the dose response study, where tissues were removed 12 h after clenbuterol administration, taurine levels in the heart were reduced in all groups administered clenbuterol (Fig. 2B). This reduction was significant for all doses except 63 μg/kg.

Table 2. Serum creatinine and urea levels in rats measured at various times after dosing rats with clenbuterol (250 $\mu g \cdot kg^{-1}$ s.c.)

Time h	Creatinine ($mmol \cdot l^{-1}$)		Urea ($mmol \cdot l^{-1}$)	
	Control	Treated	Control	Treated
1	62.25 ± 4.27	97.50 ± 7.23***	6.22 ± 0.67	10.28 ± 0.80***
3	58.25 ± 2.99	73.00 ± 11.11*	5.88 ± 0.49	11.67 ± 0.21***
6	62.25 ± 3.40	59.75 ± 2.75	6.22 ± 0.50	9.78 ± 0.95***
9	58.00 ± 4.83	60.67 ± 5.30	7.21 ± 1.50	6.30 ± 0.93
12	63.00 ± 3.16	60.00 ± 7.62	7.81 ± 0.77	7.87 ± 1.83

Values, mean ± SEM; N = 3–4, *$p < 0.05$; ***$p < 0.001$, using Student's t test.

Table 3. Serum creatine kinase (CK), hydroxybutyrate dehydrogenase (HBDH) and total protein levels in rats measured at various times after dosing with clenbuterol (250 $\mu g \cdot kg^{-1}$ s.c.)

Time (h)	CK ($iu \cdot l^{-1}$)		HBDH ($iu \cdot l^{-1}$)		Total protein ($g \cdot l^{-1}$)	
	Control	Treated	Control	Treated	Control	Treated
1	275 ± 63	332 ± 141	152 ± 17	258 ± 66	–	–
3	224 ± 43	280 ± 111	130 ± 40	218 ± 54	61 ± 3	55 ± 2.73*
6	371 ± 160	352 ± 171	221 ± 103	211 ± 126	60 ± 2	57 ± 0.67*
9	217 ± 131	186 ± 26	116 ± 85	122 ± 34	61 ± 1	59 ± 0.45*
12	188 ± 6	267 ± 12*	55 ± 25	126 ± 26*	62 ± 1	57 ± 0.25***
24	138 ± 17	221 ± 97*	–	–	–	–

Values, mean ± SEM; N = 3–4, *$p < 0.05$; ***$p < 0.001$, using Student's t test.

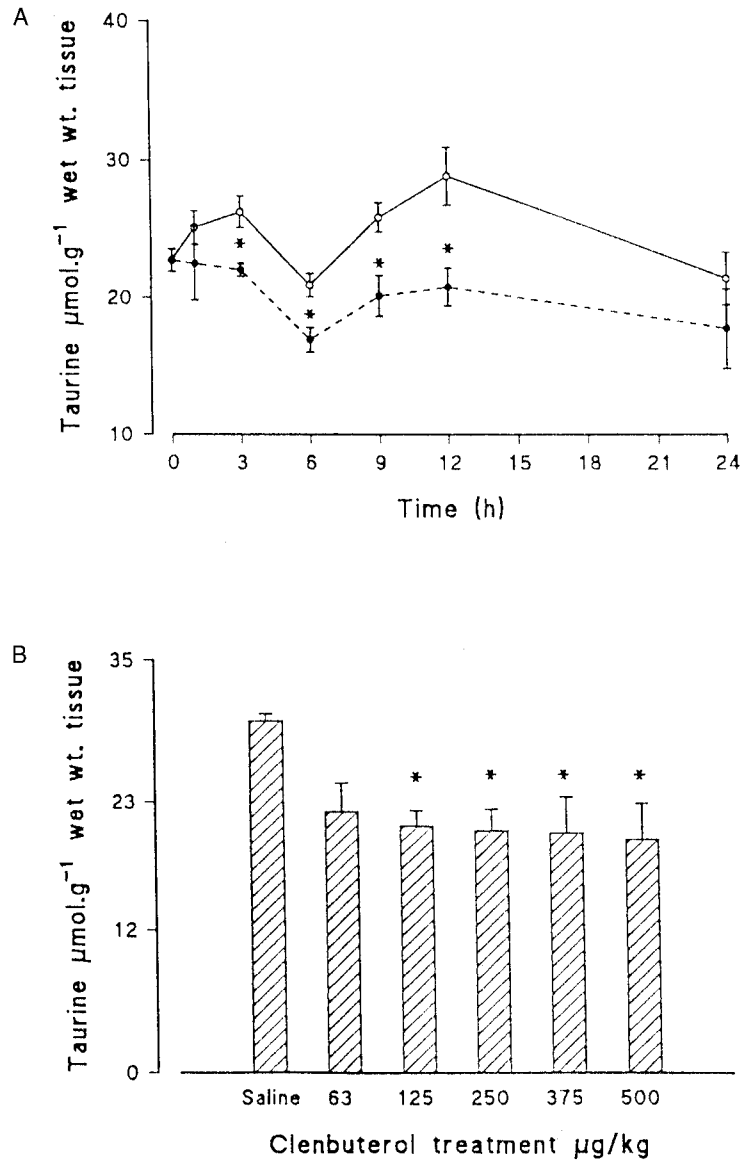

Fig. 2. A Effects of clenbuterol on levels of taurine in rat heart. Values are means ± S.EM. P value were calculated by the Students "t" test for paired data (*$p < 0.05$). Control animals ○; treated animals ●. Data are means ± SEM, N = 4. **B** Effects of varying doses of clenbuterol on levels of taurine in rat heart measured at 12 hr postdose. The values are reported as means ± S.E.M. P value was calculated using Dunnett's test. Data are means ± SEM, N = 4

The content of taurine in the lung seemed initially increased although not significantly, at 3, 6 and 9 h after clenbuterol administration (Fig. 3A). The levels of taurine then decreased significantly at 12 h after clenbuterol administration and remained significantly depressed until 24 h after dosing. In the dose response study the content of taurine in the lung was significantly decreased at all doses studied except 375 μg/kg (Fig. 3B).

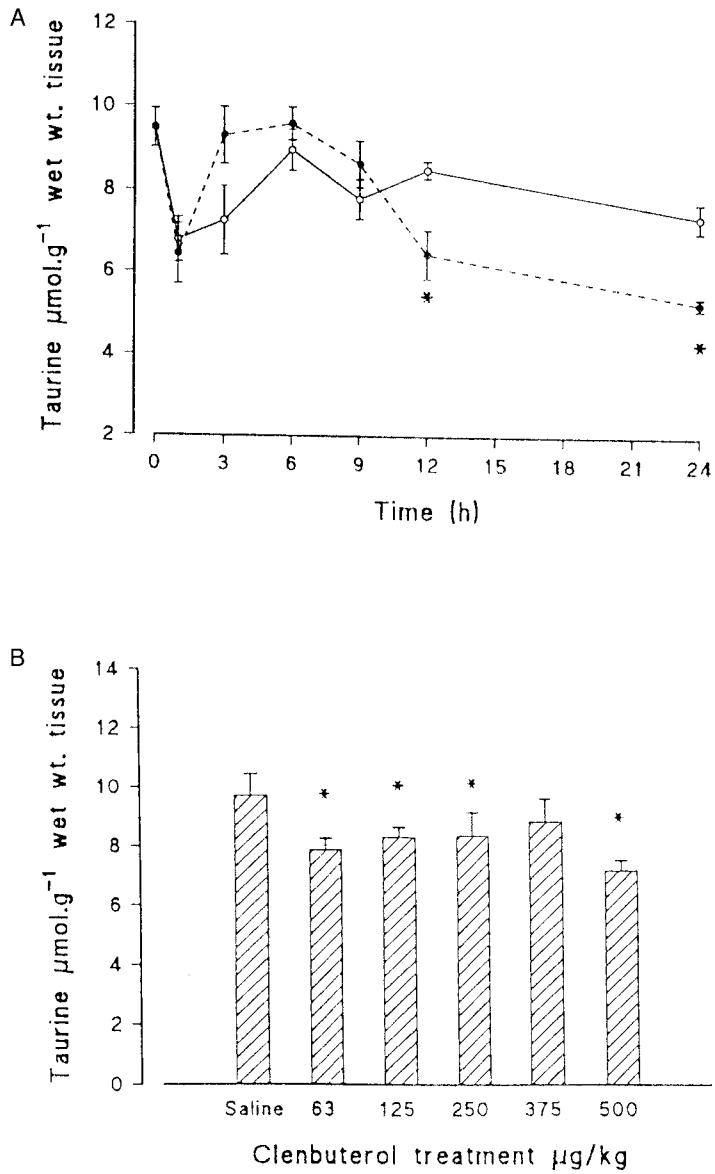

Fig. 3. **A** Effects of clenbuterol on levels of taurine in rat lung. Values are means ± S.EM. P value were calculated by the Students "t" test for paired data (*$p < 0.05$). Control animals ○; treated animals ●. Data are means ± SEM, N = 4. **B** Effects of varying doses of clenbuterol on levels of taurine in rat lung measured 12hr postdose. P value was calculated using Dunnett's test. Data are means ± SEM, N = 4

The content of taurine in the liver was significantly increased at 3h post clenbuterol administration, (Fig. 4A), and remained elevated until the 6h time point although this was not significantly different from the control value. By 24h after clenbuterol administration taurine levels were below those seen in controls. In the dose response study the content of taurine in the liver was increased 12h after clenbuterol administration and was significantly different from the control at the highest dose of 500μg/kg (Fig. 4B).

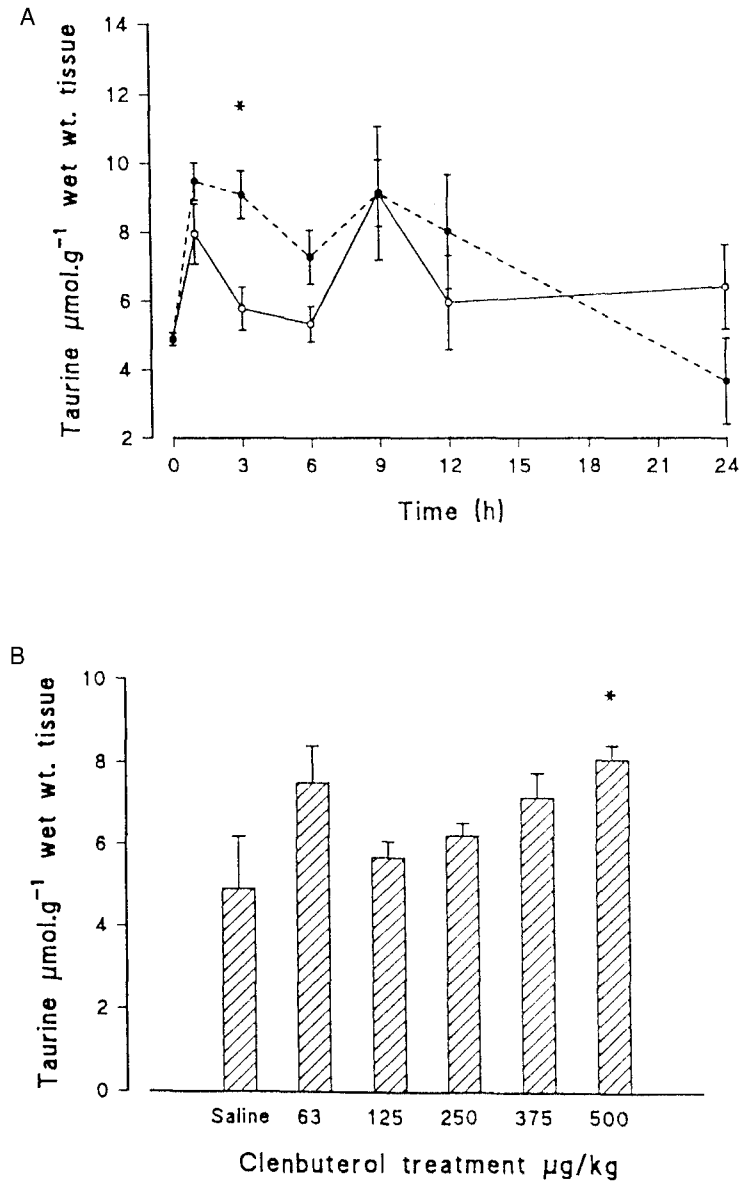

Fig. 4. **A** Effects of clenbuterol on levels of taurine in rat liver. Values are means ± S.EM. P value were calculated by the Students "t" test for paired data (*$p < 0.05$). Control animals ○; treated animals ●. Data are means ± SEM, N = 4. **B** Effects of varying doses of clenbuterol on levels of taurine in rat liver measured 12hr postdose. P value was calculated using Dunnett's test. Data are means ± SEM, N = 4

Clenbuterol did not alter muscle taurine except at 6h after dosing where there was a significant increase in levels. Increasing doses of clenbuterol did not have any effect on muscle taurine levels 12h after dosing (data not shown).

The concentration of taurine in the serum increased at 3h (at the same time the decrease in heart tissue levels of taurine occurred) but returned to control values by 6h post clenbuterol administration (Fig. 5).

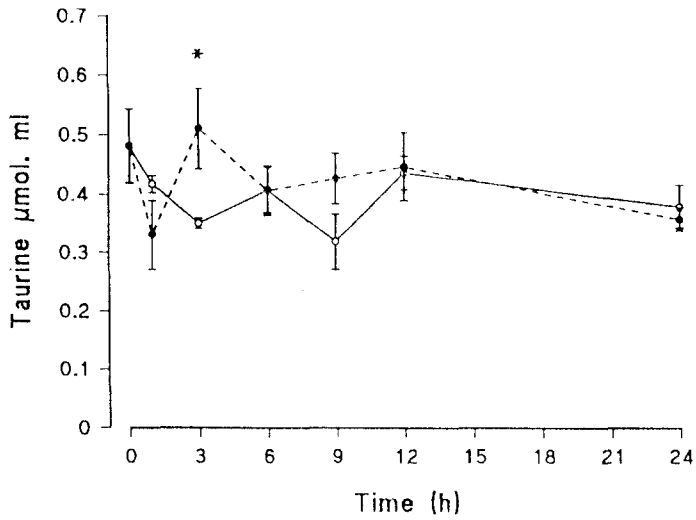

Fig. 5. Effects of clenbuterol on levels of taurine in rat serum. P value were calculated by the Students "t" test for paired data (*$p < 0.05$). Control animals ○; treated animals ●. Data are means ± SEM, N = 4

Total non-protein sulfhydryls (TNPSH)

Levels of TNPSH in liver and heart were not affected by treatment with clenbuterol (data not shown)

Discussion

The aim of this study was to examine the toxic effects of acute administration of the β_2-agonist, clenbuterol, and its affect on tissue taurine levels in rats. The taurine content of the heart decreased by 16 to 28% after administration of 250μg/kg of clenbuterol and by approximately 30% after all but the lowest dose used in the dose response study. Taurine is important in the cardiovascular system so a reduction of up to 30% of taurine content could have adverse effects on the heart although recent studies showed depletion of myocardial taurine lead to protection against ischemia-induced infarction (Allo et al., 1997). Indeed histological sections from the hearts of some of the treated animals showed vacuolation, inflammation and other pathological signs. Although there was also a significant increase in serum creatine kinase at 12 and 24h post dose in the treated animals, which would be consistent with heart tissue damage, the CK-MB isoenzyme (heart isozyme) was not detected but has a short half-life in plasma. The isoenzyme CK-BB (brain isozyme) is mainly responsible for this increase. Hydroxybutyrate dehydrogenase (HBDH) was also significantly increased 12h after clenbuterol administration. Variation in the levels of taurine measured over time were observed in heart, lung, liver and serum. However this was not obviously circadian and was not similar in the different tissues analysed. Therefore the underlying cause of this variation is currently unknown.

The decrease in heart taurine levels observed in this study are similar to those seen by Lombardini (1980) following the administration of a cardiotoxic doses of DL-isoproterenol (80 mg/kg). The decrease in heart taurine cannot be attributed to a reduction in taurine synthesis by the liver as taurine levels in this tissue were significantly increased by clenbuterol 3 h post dose and similar to control levels at all other time points. One explanation could involve the fact that at high doses, clenbuterol can act as a β_1-antagonist (Main, 1990). Another possible explanation could be related to the fact that clenbuterol, a repartitioning agent, causes a change in the ratio of protein to fat (Yang and McElligott, 1989). In this study serum triglyceride levels were significantly increased at the 12 and 24 h time point and serum total protein was decreased indicating changes in lipid and protein metabolism. Consequently protein synthesis and/or degradation are altered as well as fat metabolism. Either an increase in protein synthesis or a decrease in degradation may reduce the pool of amino acids available for taurine synthesis especially in the liver. As a consequence, cysteine and methionine, precursors for taurine may be reduced, therefore taurine content could be depleted with no immediate source of precursors for restoration of pool size (Waterfield et al., 1995). However taurine levels in liver were actually increased in this study at 3 hours postdosing. It therefore seems unlikely that increased protein synthesis is the cause of the acute effect unless bile flow was being affected (Waterfield et al., 1993a).

The significant increase in liver taurine levels at 3 h following clenbuterol administration is similar to that reported by Lombardini (1980) after administration of isoproterenol, where the liver taurine concentration was significantly increased at 4 h after administration. However this effect was reported to last for 14 h whereas in the clenbuterol treated animals in this study taurine concentrations were similar to controls by 6 h post clenbuterol administration. The results are contrary to those found after the continuous administration of clenbuterol, in the drinking water, for 4 days when a significant decrease in liver taurine levels was observed (Waterfield et al., 1995). The rise in liver taurine seen at 3 h after a single dose of clenbuterol was administered in this study coincided with an increase in taurine levels in the serum. This could be due to a reduction in blood flow to the kidneys which was indicated by the increased serum urea and creatinine which occurred at a similar time point after clenbuterol administration. Therefore reduced excretion of taurine via the kidneys may occur. The rise in taurine content of the liver is possibly due to a reduction of bile acid production. Whether clenbuterol affects *de novo* taurine synthesis in the liver is unknown at present but the increased liver taurine levels would be consistent with this as the liver is the major site of synthesis (Zelikovic and Chesney, 1989). It may be that clenbuterol increased liver taurine in some other way. Cholestasis tends to increase liver taurine levels and in this study serum cholesterol was significantly increased at the 12 and 24 h time point (Waterfield et al., 1993a). However bilirubin was not raised (data not shown). TNPSH levels were measured in this study as any changes in glutathione (GSH) will result in altered cysteine levels which will affect taurine synthesis (Waterfield et al., 1993b). However tissue levels of

TNPSH were not affected by clenbuterol suggesting that if depletion had occurred either levels were replenished quickly or no reduction of the GSH pool occurred and was therefore not responsible for lowering cysteine and hence taurine levels.

The effects seen in the lung and muscle were not obviously due to changes in blood taurine concentration (Fig. 5). Also these changes are different from those in the heart or liver suggesting a different mechanism.

This study has shown that in rats a single dose of clenbuterol changes taurine levels in several tissues including the heart. This effect has not been shown to be dose related and the mechanism is currently unclear. However these findings may have implications for the cardiotoxicity of β-agonist drugs.

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Authors' address: Prof. John A. Timbrell, Department of Pharmacy, King's College London, Manresa Rd., London SW3 6LX, U.K.

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