EFFECTS OF FORMOTEROL ON PROTEIN METABOLISM IN MYOTUBES DURING HYPERTHERMIA

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Accepted 14 July 2010

ABSTRACT: Proteolysis in skeletal muscle is mainly carried out by the activity of the ubiquitin-dependent proteolytic system. For the study of protein degradation through the ubiquitin–proteasome pathway, we used a model of hyperthermia in murine myotubes. In C2C12 cells, hyperthermia (41°C) induced a significant increase in both the rate of protein synthesis (18%) and degradation (51%). Interestingly, the addition of the β_2-adrenoceptor agonist formoterol resulted in a significant decrease in protein degradation (21%) without affecting protein synthesis. The decrease in proteolytic rate was associated with decreases in gene expression of the different components of the ubiquitin-dependent proteolytic system. The effects of the β_2-agonist on protein degradation were dependent exclusively on cAMP formation, because inhibition of adenylyl cyclase completely abolished the effects of formoterol on protein degradation. It can be concluded that hyperthermia is a suitable model for studying the anti-proteolytic potential of drugs used in the treatment of muscle wasting.

Muscle Nerve **43:** 268–273, 2011

Skeletal muscle protein content, and therefore muscle composition and function, depends on the balance between the rates of protein synthesis and degradation. Proteolysis in skeletal muscle is mainly carried out by the activity of the ubiquitin-dependent proteolytic system,[1,2] whereas both the lysosomal- and calcium-dependent systems seem to have a secondary role.[3–5] An elevated temperature that accompanies sepsis and severe infections may contribute to muscle proteolysis,[6] and hyperthermia has been shown to stimulate muscle protein breakdown through the ubiquitin–proteasome pathway.[7,8]

β_2-adrenergic agonists are potent muscle growth promoters in many animal species.[9,10] They produce skeletal muscle hypertrophy[11–14] and cause a reduction in body fat content.[15,16] Formoterol is a highly potent, β_2-adrenoceptor-selective agonist that combines the clinical advantages of rapid onset of action with duration of action. This compound is already in use in humans for treatment of bronchospasm associated with asthma. In vitro, formoterol is a potent airway smooth muscle relaxant with high efficacy and very high affinity and selectivity for the β_2-adrenoceptor.[17] Moreover, formoterol relaxes bronchial smooth muscle and provides important clinical benefits in symptomatic patients with chronic obstructive pulmonary disease (COPD).[18] Formoterol, like other long-acting β_2-adrenoceptor agonists, attenuates the allergen-induced late asthmatic reaction[19] and, under certain conditions, has more effect than other β_2-adrenoceptor agonists (salbutamol and salmeterol).[20] Interestingly, results from our laboratory indicate that formoterol is a very efficient agent for prevention of muscle weight loss in tumor-bearing rats.[21] In vivo treatment can effectively reverse muscle wasting by decreasing protein degradation and increasing the rate of protein synthesis in skeletal muscle, thereby favoring protein accretion.[21]

The aim of this investigation was to study the effects of a β_2-agonist on hyperthermia in murine myotubes as a model for increased protein degradation through the ubiquitin–proteasome pathway.

METHODS

Cell Culture. C2C12 mouse skeletal muscle cells were obtained from the American Type Culture Collection. Cells were passaged in high-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 25 ng/ml amphotericin B, 110 μg/ml sodium pyruvate, and 2 mM L-glutamine, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For experimental analyses, cells were seeded at 3.7×10^4 cells/cm^2 in 10% FBS/DMEM until they reached 90–100% confluence 24 h later. At this timepoint, the medium was replaced by DMEM containing 10% horse serum (HS) for induction of differentiation. Abundant myotube formation, monitored microscopically, occurred after 4 days in 10% HS/DMEM. Fused myotube cultures were utilized for

Abbreviations: ANOVA, analysis of variance; BCA, bicinchoninic acid; COPD, chronic obstructive pulmonary disease; C, control; DDA, 2′,5′-dideoxyadenosine; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; EDTA, ethylene-diamine tetraacetic acid; F, formoterol; FBS, fetal bovine serum; H, hyperthermia; HBSS, Hanks buffered saline solution; H+R, hyperthermia and returned at 37°C; HS, horse serum; MHC, myosin heavy chain; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis

Key words: β2-agonists, C2C12 cells, hyperthermia, ubiquitin-dependent proteolytic system protein degradation

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Published online 15 January 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mus.21852

experimental analysis 5 days after transferring cells to 10% HS/DMEM.

Punctual Hyperthermia and Measurement of Protein Degradation and Protein Synthesis. Punctual hyperthermia was carried out as previously described by Smith and colleagues.[8] C2C12 cells were exposed for 6 hours to 41°C (H), or they were exposed for 6 hours to 41°C and then returned to 37°C (H+R). C2C12 myotubes were prelabeled with L-(2,6-^{3}H)phenylalanine (Amersham, Bucks, UK) as previously described[22] for a 24-h period, after which they were washed extensively in phosphate-buffered saline (PBS), and incubated in fresh DMEM for a 2-h period at 37°C until no more radioactivity appeared in the medium. Protein degradation was measured by the release of [2,6-^{3}H]phenylalanine into the medium (DMEM-supplemented with 1% glutamine, 1% penicillin–streptomycin–amphotericin B and 10% HS) after 6-h incubation at 37°C or 41°C in the presence of 2 mM cold L-phenylalanine to prevent reincorporation of the radiolabel.

After the incubation, the cells were rinsed twice in PBS. The release of radioactivity during the incubation was linear. Values for protein degradation are presented as the percentage of the respective control value at 37°C of the amino acid radioactivity in the medium versus the total radioactivity incorporated into protein. To determine the rates of protein synthesis, myotubes in six-well plates were incubated in non-radioactive experimental medium (DMEM supplemented with 10% HS) for various periods of time. The culture medium was replaced with experimental medium containing 1 μCi of [2,6-^{3}H]phenylalanine/ml for the final hour of incubation. Cells were rinsed twice in ice-cold Hanks buffered saline solution (HBSS)–L-phenylalanine (2 mM). Monolayers were precipitated with 1 ml 10% trichloroacetic for at least 1 h at 4°C and, after washing once with ice-cold 10% trichloroacetic, the precipitated protein was dissolved by incubation for at least 1 h at 37°C in 1 ml of 0.5 M NaOH containing 0.1% Triton X-100.

Radioactivity was determined using a liquid scintillation counter, and protein content was determined using the bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Waltham, Massachusetts). The rate of incorporation of radioactivity was constant during the period of measurement. Values for protein synthesis are presented as the percentage of the respective control value at 37°C and were assessed as radioactivity incorporated into protein per milligram of total protein. Cultures were incubated with or without formoterol (0.1 μM).

Measurement of Intracellular Cyclic AMP Level. To measure the intracellular cAMP level, C2C12 myoblasts were cultured in 35-mm culture dishes and allowed to differentiate into myotubes in DMEM containing 10% HS. Cell cultures were treated with 0.1 M HCl after first removing the medium. Culture cells were incubated for 10 minutes and visually inspected in order to verify cell lysis. The medium was centrifuged at 3600 *g* at room temperature, and the supernatant was assayed for cAMP using a cAMP enzyme immunoassay kit (Sigma Co., St. Louis, Missouri), according to the manufacturer's instructions. 2′,5′-Dideoxyadenosine (DDA; Sigma Co.) was used as an inhibitor of adenylyl cyclase (50 μM).

Real-Time Polymerase Chain Reaction. Total RNA from C2C12 cells was then extracted (TriPureTM kit; Roche, Barcelona, Spain) by a commercial modification of the acid guanidinium isothiocyanate/phenol/chloroform method.[23] First-strand cDNA was synthesized from total RNA with oligo-dT15 primers and random primers pdN6 by using a cDNA synthesis kit (Transcriptor Reverse Transcriptase; Roche, Barcelona, Spain). Analysis of mRNA levels for the genes from the different proteolytic systems was performed with primers designed to detect the following gene products: ubiquitin (forward: 5′ GAT CCA GGA CAA GGA GGG C 3′; reverse: 5′ CAT CTT CCA GCT GCT TGC CT3′); E2 (forward: 5′ AGG CGA AGA TGG CGG T 3′; reverse 5′ TCA TGC CTG TCC ACC TTG TA 3′); C8 proteasome subunit (forward: 5′ CAA CCA TGA CAA CCT TCG TG 3′; reverse: 5′ GCC TCA CAG CCT TCT CTT TG 3′); MuRF-1 (forward: 5′ ATC ACT CAG GAG CAG GAG GA 3′; reverse 5′ CTT GGC ACT CAA GAG GAA GG 3′); atrogin-1 (forward: 5′ GTT TCC ATT GGG ATT GTT GG 3′; reverse: 5′ TGT TCC ATT GGT TCA TCA GC 3′); m-calpain (forward: 5′ TTG AGC TGC AGA CCA TC 3′; reverse 5′ GCA GCT TGA AAC CTG CTT CT 3′); cathepsin B (forward 5′ CTG CTG AGG ACC TGC TTA C 3′; reverse: 5′ CAC AGG GAG GGA TGG TGT A3′); and 18S (forward: 5′ GCGAATGGCTCATTAAATCAGTTA 3′; reverse: 5′ TGGTTTTGATCTGATAAATGCACG 3′). To avoid the detection of possible contamination by genomic DNA, primers were designed in different exons. The real-time polymerase chain reaction (PCR) was performed using a commercial kit (LightCycler FastStart DNA Master^{PLUS} SYBR Green I; Roche, Barcelona, Spain). 18S mRNA was used as the invariant control for all studies.

Myofibrillar Proteins. For myofibrillar protein analysis, C2C12 cell cultures were homogenized in 10 mmol/L HEPES (pH 7.5), containing 10 mmol/L $MgCl_2$, 5 mmol/L KCl, 0.1 mmol/L ethylene-

diamine tetraacetic acid (EDTA), 0.1% Triton X-100, 1 mmol/L dithiothreitol (DTT), and 5 μl of a protease inhibitor cocktail per milliliter (Sigma Co.) of buffer. The cells were then centrifuged at 7000 rpm for 5 min at 4°C, and the supernatants were collected. Protein concentrations were determined according to the bicinchoninic acid method (Pierce, Thermo Fisher Scientific, Waltham, Massachusetts).

Equal amounts of protein (50 or 100 μg) were heat denatured in sample loading buffer [50 mmol/L Tris-HCl (pH 6.8), 100 mmol/L DTT, 2% sodium dodecylsulfate, 0.1% bromophenol blue, and 10% glycerol], resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide, 0.1% SDS), and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore). The filters were blocked with 5% PBS–nonfat dry milk and then incubated with polyclonal antibodies anti-MHC (Oncogene, Cambridge, Massachusetts), anti-actin (Sigma Co.), and anti-tubulin (Sigma Co.). A polyclonal antibody to anti-Na^+/K^+ ATPase α-subunit (Developmental Studies Hybridoma Bank, Iowa City, Iowa) was used as control for the different studies. Donkey anti-mouse peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania), rabbit anti-goat peroxidase-conjugated IgG (Acris Antibodies GmbH, Germany), and goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad Laboratories, Madrid, Spain) were used as secondary antibodies. The membrane-bound immune complexes were detected by an enhanced chemiluminescence system (EZ-ECL; Biological Industries, Kibbutz Beit Haemek, Israel).

Biochemicals. Biochemicals were all reagent grade and obtained either from Roche S.A. (Barcelona, Spain) or from Sigma Co. Radiochemicals were purchased from Amersham International (Amersham, Bucks, UK). Formoterol fumarate micronized was kindly provided by Industriale Chimica (Saronno, Italy).

Statistical Analysis. Statistical analyses of the data were performed by one- and two-way analyses of variance (ANOVAs).

RESULTS

The results shown in Figure 1 indicate that, at 6 hours after hyperthermia (a rise in culture temperature from 37°C to 41°C), there was an increase in both protein degradation (51%; Fig. 1A) and synthesis (18%; Fig. 1B). Similarly, 24 hours following thermal injury (recovery phase H+R), both protein degradation and synthesis were increased, but here the increase in synthesis was more elevated and the ratio of synthesis versus degradation increased.

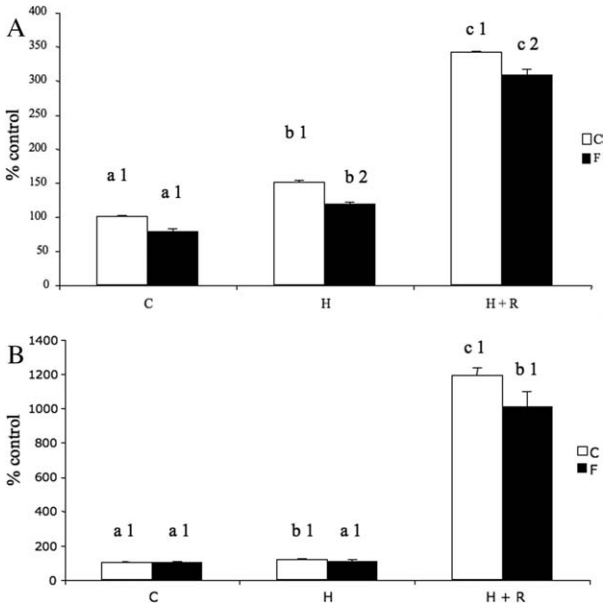

FIGURE 1. Rates of protein synthesis and degradation in C2C12 cells after hyperthermia. **(A)** Values for protein degradation are shown as percentage of the respective control value at 37°C of the amino acid radioactivity released in the medium versus the total accumulated radioactivity incorporated into the protein. **(B)** Values for protein synthesis are shown as the percentage of the respective control value at 37°C and were assessed as radioactivity incorporated into protein per milligram of total protein. C, control; F, formoterol; H, hyperthermia; C2C12 cells exposed for 6 h to 41°C; H+R, hyperthermia and return at 37°C; C2C12 cells exposed for 6 h to 41°C and then returned to 37°C. All data are expressed as mean ± SEM (n = 5). Between columns, groups having the same superscript (for temperature effect: a, b or c; for treatment effect: 1 or 2) are significantly different from each other at $P < 0.05$ using one-way ANOVA followed by post hoc Duncan test.

For this reason, although protein content was unaltered by hyperthermia, it was increased during the recovery phase (Table 1). Individual myofibrillar protein concentration was also kept in the case of tubulin and actin, but myosin heavy chain (MHC) seemed to be significantly increased after 6 hours of hyperthermia, possibly as a consequence of the increase in protein synthesis.

Some data have indicated that the increase in protein synthesis at elevated temperatures may be partially due to an increase in heat-shock proteins, which could protect the cell against further damage.[24–26] In the hyperthermia model used in this study, protein degradation was associated with increased mRNA levels for different components of the ubiquitin-dependent proteolytic system [ubiquitin (103%), C8 proteasome subunit (89%), MuRF-1 (93%), and atrogin-1 (52%)] without influencing the gene expression of either the calcium- or lysosomal-dependent proteolytic systems (Table 2). Treatment of the murine myotubes with formoterol (0.1 μM) resulted in significant decreases in the rate of protein degradation in

Table 1. Total protein and myofibrillar protein content.

					ANOVA	
		C	H	H+R	T[a]	Treatment
Protein content	Control	2.02 ± 0.18 (6)[a]	2.2 ± 0.07 (6)[a]	2.6 ± 0.08 (6)[b]	0.0025	NS
	F 0.1 μM	2.1 ± 0.11 (6)[a]	2.1 ± 0.05 (6)[a]	2.8 ± 0.13 (6)[b]		
Myofibrillar proteins						
Tubulin	Control	100 ± 8 (5)	85 ± 10 (5)	97 ± 6 (5)	NS	NS
	F 0.1 μM	104 ± 6 (4)	89 ± 6 (5)	89 ± 5 (5)		
Actin	Control	100 ± 9 (5)	108 ± 6 (5)	103 ± 8 (4)	NS	NS
	F 0.1 μM	102 ± 8 (5)	97 ± 7 (5)	103 ± 9 (4)		
MHC	Control	100 ± 5 (5)[a]	122 ± 10 (5)[b]	131 ± 10 (5)[b]	0.0054	NS
	F 0.1 μM	104 ± 6 (5)[a]	129 ± 7 (5)[b]	133 ± 11 (5)[b]		

For more details see Methods. Results expressed as mean ± SEM for the number indicated in parentheses. C, control; F, formoterol; H, hyperthermia; C2C12 cells exposed 6 h at 41°C; H+R, hyperthermia and return at 37°C; C2C12 cells exposed 6 h at 41°C and then returned at 37°C; MHC, myosin heavy chain. Statistical significance of results determined by one-way and two-way analyses of variance (ANOVAs); NS, non-significant differences. In the same row, groups having the same superscript (a or b) are significantly different from each other (temperature effect) at $P < 0.05$ using two-way ANOVA followed by post hoc Duncan test.

both the hyperthermia- and recovery-phase groups (Table 1). Interestingly, formoterol did not affect protein synthesis (Table 1). As can be seen in Table 1, formoterol did not influence either total protein content or specific myofibrillar protein content. In spite of these results, formoterol influenced protein degradation (Fig. 2). Indeed, as can be seen in Table 2, formoterol treatment resulted in decreases in the expression of different components of the ubiquitin-dependent proteolytic system [ubiquitin (14%), C8 proteasome subunit (27%), and atrogin-1 (28%) in hyperthermia (H), and ubiquitin (18%), C8 proteasome subunit (29%), and atrogin-1 (28%) in the recovery group (H+R)]. However, formoterol did not affect the expression of either calcium- or lysosomal-dependent proteolysis.

Finally, we investigated the intracellular mechanisms of action of formoterol on protein degradation, in addition to changes in cAMP, which characterize the action of all β_2-agonists. Bearing this in mind, we used DDA (2′,5′-dideoxyadenosine, an inhibitor of adenylyl cyclase) in the hyperthermia-induced proteolysis experiments. The results

Table 2. Gene expression components of the different proteolytic systems in C2C12 myotubes after hyperthermia.

					ANOVA	
		C	H	H+R	T[a]	Treatment
Proteolytic system						
Ubiquitin-dependent						
Ubiquitin	Control	100 ± 11 (5)[a]	203 ± 9 (5)[b]	310 ± 15 (5)[c]	0.0000	0.0107
	F 0.1 μM	109 ± 7 (5)[a]	175 ± 8 (5)[b]	255 ± 16 (5)[c]		
Proteasome subunit C8	Control[a]	100 ± 17 (5)	189 ± 12 (5)[b]	304 ± 27 (5)[c]	0.0000	0.0005
	F 0.1 μM	108 ± 4 (5)[a]	138 ± 15 (5)[b]	216 ± 24 (6)[c]		
E2	Control	100 ± 21 (4)	112 ± 5 (5)	140 ± 4 (5)	NS	NS
	F 0.1 μM	108 ± 5 (5)	102 ± 10 (5)	127 ± 27(5)		
MuRF-1	Control	100 ± 17 (4)[a]	193 ± 12 (5)[b]	344 ± 13 (5)[c]	0.0000	NS
	F 0.1 μM	112 ± 8 (5)	175 ± 39 (6)	226 ± 14(6)		
Atrogin-1	Control	100 ± 18 (5)[a]	152 ± 18 (5)[b]	174 ± 4 (6)[c]	0.0000	0.0002
	F 0.1 μM	83 ± 5 (6)[a]	110 ± 8 (6)[b]	125 ± 5 (4)[b]		
Calcium-dependent						
m-calpain	Control	100 ± 17 (5)	104 ± 8 (5)	102 ± 2 (5)	NS	NS
	F 0.1 μM	105 ± 10 (6)	101 ± 8 (4)	107 ± 9 (5)		
Lysosomal						
cathepsin B	Control	100 ± 19 (4)	99 ± 7 (6)	97 ± 8 (5)	NS	NS
	F 0.1 μM	108 ± 9 (6)	93 ± 8 (6)	108 ± 3 (5)		

For details see Methods. Results expressed as mean ± SEM for the number indicated in parentheses. Effect of hyperthermia on the expression of mRNA for ubiquitin C8 protease subunit, E2, MuRF-1, atrogin-1, cathepsin B, m-calpain, in the absence or presence of formoterol (0.1 μM). The results are expressed as a percentage of controls. C, control; F, formoterol; H, hyperthermia; C2C12 cells exposed 6 h at 41°C; H+R, hyperthermia and return at 37°C; C2C12 cells exposed 6 h at 41°C and then returned at 37°C. Statistical significance of the results by one- and two-way analyses of variance (ANOVAs); NS, non-significant difference. In the same row, groups having the same superscript (a, b, or c) are significantly different from each other (temperature effect) at $P < 0.05$ using two-way ANOVA followed by post hoc Duncan test.

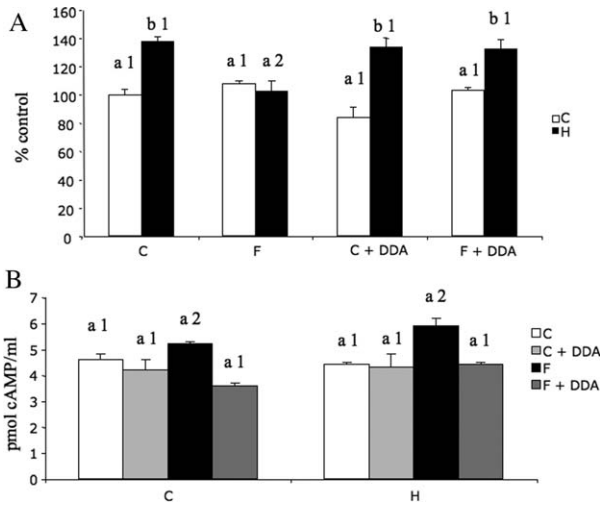

FIGURE 2. Intracellular mechanisms of action of formoterol: cAMP studies. **(A)** Cells were exposed for 6 h to 41°C and treated with 0.1 μM formoterol and the adenylyl cyclase inhibitor DDA (50 μM). The values of protein degradation are calculated as the percentage of radioactivity released into the medium of the total cell-incorporated radioactivity. **(B)** Cells were previously treated with DDA (50 μM) for 2 hours; they were then exposed 6 hours to 41°C and treated with a formoterol 0.1 mM. The values of cAMP are expressed as picomoles cAMP per milliliter. C, control; F, formoterol; H, hyperthermia; C2C12 cells exposed for 6 h to 41°C; H+R, hyperthermia and returned to 37°C; C2C12 cells exposed for 6 h to 41°C and then returned to 37°C. All data are expressed as mean ± SEM ($n = 5$). Between columns, groups having the same superscript (for temperature effect: a or b; and for treatment effect: 1 or 2) are significantly different from each other at $P < 0.05$ using one-way ANOVA followed by post hoc Duncan test.

presented in Figure 2A show that no effect of DDA on protein degradation was observed, suggesting that the effects of formoterol depend exclusively on cAMP levels. The results presented in Figure 2B show that the inhibitor did indeed work as such, because the levels of cAMP were decreased following the treatment.

DISCUSSION

Previous studies have shown that raising the culture temperature from 37° to 41°C results in a profound alteration in protein metabolism in cultured muscle cells.[8] Apparently, hyperthermia in murine myotubes can serve as a model for increased protein metabolism. Our objective in this investigation was to determine whether the β_2-adrenergic agonist formoterol could block the hypercatabolism response associated with hyperthermia. The data agree with a previous report from our laboratory, where it was suggested that the antiproteolytic effect of formoterol is based on inhibition of the ubiquitin-dependent proteolytic system.[21] From the results, it can be concluded that hyperthermia is a suitable model for studying the anti-proteolytic potential of drugs used in the treatment of muscle wasting. These studies may provide a new tool for the study of new therapeutic strategies to fight muscle wasting—and therefore cachexia—during neoplastic diseases.

The authors thank Industriale Chimica (Saronno, Italy) for kindly providing the formoterol fumarate micronized. This sudy was supported by the Instituto de Salud Carlos III (Grant 03/0100 to E.A.), the Dirección General de Investigación Científica y Técnica (Grant BFI2002-02186 to G.F.) of the Ministerio de Educación y Ciencia, Programme Alssan of the European Union Programme of High Level Scholarships for Latin America (Scholarship E05D059293BR to C.O.). Contract grant support was further provided by the Instituto de Salud Carlos III of the Ministerio de Sanidad y Consumo (06/0907), Ministerio de Ciencia y Tecnología (SAF 4744-2005), and Generalitat de Catalunya (SGR/00108).

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