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LIPOPHILIC β-ADRENOCEPTOR ANTAGONISTS STIMULATE CHOLESTEROL BIOSYNTHESIS IN HUMAN SKIN FIBROBLASTS

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Abstract—The effect of a series of β-adrenoceptor antagonists on cholesterol biosynthesis was studied *in vitro* in normal human skin fibroblasts. Some, but not all, of the drugs studied stimulated the incorporation of [2-^{14}C]-acetate into cell sterols in a dose-dependent manner. This effect was unrelated to β-blocking potency, selectivity for β_1 or β_2 adrenoceptors and partial agonistic activity of the drugs, thus ruling out a β-receptor mediated mechanism. A positive, statistically significant correlation was found, however, between the drug lipophilicity and the stimulation of sterol biosynthesis. Propranolol, the most effective agent in increasing [2-^{14}C]-acetate incorporation into cellular sterols, also enhanced the conversion of 3-hydroxy-3-methylglutaryl CoA (HMGCoA) into mevalonic acid, suggesting an interference of lipophilic β-adrenoceptor antagonists with HMHCoA-reductase, the feed-back regulated rate limiting step of cholesterol biosynthesis.

Cells in culture meet their cholesterol needs either by acquirement from an exogenous source, i.e. the low density lipoprotein (LDL) pathway, or through a *de novo* synthesis [1]. Beta-adrenoceptor antagonists, drugs that are widely used in cardiovascular pathology, seem to interfere with lipoprotein metabolism, and their effects on plasma lipid concentrations are now of some concern [2]. Propranolol was reported to stimulate receptor-mediated high affinity binding, internalization and degradation of LDL [3] and to increase [2-^{14}C]-acetate incorporation into cholesterol in human skin fibroblasts [4]. These effects could be due either to interaction with β-adrenoceptors or to non-specific membrane affinity which is related to the physicochemical nature of the drugs. [5]. The latter mechanism(s) may have pharmacological significance: the binding of these drugs to plasma proteins [6], their capacity to cross the blood–brain barrier [7], their extent of biotransformation [8], the inhibition of oxidative drug metabolism [9], their local anaesthetic properties [5] and the inhibition of human plasma lecithin-cholesterol acyltransferase [10] relate directly to their lipophilicity. The aim of this work was to investigate the nature of the enhancing effect of β-adrenoceptor antagonists on cholesterol biosynthesis in human skin fibroblasts. Drugs differing in potency, selectivity for β_1 or β_2 adrenoceptors, partial agonistic activity and lipophilicity were used. Labetalol, a mixture of α- or β-blocking enantiomers [11], was also studied.

MATERIALS AND METHODS

Racemic, (+) and (−) propranolol HCl, (±) practolol base, and (±) ICI 118,551 were from ICI (Macclesfield, U.K.); (±) metoprolol tartrate, (±) pindolol base, racemic labetalol HCl and (±) atenolol base were from Sigma (St. Louis, MO); (±) sotalol HCl was from Bristol (Roma, Italy). Eagle's minimum essential medium (F-11), fetal calf serum (FCS), trypsin-EDTA (1 X), penicillin (10,000 U/ml), streptomycin (10 mg/ml), tricine buffer (1 M, pH 7.4), HEPES (1 M, pH 7.4) and non-essential aminoacids (100 X), were purchased from GIBCO (Madison, WI); disposable culture flasks and petri dishes were from Corning Glassworks (Corning, NY), filters from Millipore (Bedford, MA). Kyro EOB was a generous gift from Procter & Gamble (Cincinnati, OH); HMGCoA was purchased from Sigma (St. Louis, MO); [$^2H_4$]mevalonolactone was prepared as previously described [12]. [2-^{14}C]-acetate, sodium salt (58.9 mCi/mmole) and [1α, 2α (n)-^{3}H] cholesterol, 47.7 Ci/mmole, were from Amersham (Amersham, U.K.).

Cells. Human skin fibroblasts were grown from explants of skin biopsies obtained from normolipidemic clinically healthy individuals. Cells were grown in monolayers and maintained in 75 cm^2 plastic flasks at 37° in a humidified atmosphere of 95% air, 5% CO_2 in F-11 medium supplemented with 10% FCS, non-essential aminoacid solution (1%, v:v), penicillin (100 U/ml), streptomycin (100 μg/ml), tricine-buffer (20 mM, pH 7.4), $NaHCO_3$ (24 mM). For all experiments, cells from the stock flasks were dissociated with 0.05% trypsin – 0.02% EDTA at confluency and seeded in 60 mm plastic petri dishes (2.5×10^5 cells). The medium was changed every 3rd day. Cells were used within the 5th and 15th passage, and utilized for experiments between the 4th and 11th day after seeding. Twenty-four hours before experiments, the medium was changed to one containing 5% human lipoprotein-deficient serum (LPDS).

Cell viability was routinely assessed by trypan blue exclusion method, and was found >90% under all experimental conditions.

Preparation of LDL. LDL were prepared from plasma of clinically healthy normolipidemic volunteers by sequential preparative ultracentrifugation [13]. Lipoproteins were used within three days from preparation and sterilized by passage through Millipore filters (0.45 μm pore size). LPDS was prepared by ultracentrifugation of pooled human sera according to Brown *et al.* [14].

Synthesis of cholesterol. It was determined by measuring the incorporation of radioactive acetate into the unsaponifiable fraction of cells [15] or into cellular sterols [16]. Six days after plating, fibroblasts were incubated in the medium containing 5% LPDS in the presence or absence of drugs and lipoproteins as reported in tables and figures; [2-^{14}C] acetate was then added (1 μCi/ml, sp. act. 0.9 μCi/μmole). All β-adrenoceptor antagonists were added to the incubation medium dissolved in H_2O except pindolol and practolol bases, which were dissolved in ethanol (1% final concentration in the incubation medium); control cell dishes were added with the same volume of the corresponding solvent. Twenty-four hours later the medium was removed, the cells were washed with phosphate-buffered saline (PBS), harvested with a rubber policeman and suspended in PBS. Aliquots were saponified at 60° for 1 hr in alcoholic NaOH after the addition of [1α, 2α(n)-^{3}H) cholesterol as internal standard (0.04 μCi/sample). Proteins were determined according to Lowry *et al.* [17]. The unsaponifiable material was extracted with low-boiling petrol ether and counted for radioactivity. To evaluate the incorporation of labelled acetate into cellular sterols, these were separated from the unsaponifiable fraction by thin-layer chromatography [18].

HMGCoA reductase assay. Cells were incubated for 24 hr in the medium containing 5% human LPDS, with or without drugs at the concentrations indicated. At the end of incubation the medium was removed, the cells were washed with PBS, scraped off the plates and centrifuged (4000 rpm, 4°, Beckman microfuge). The pellets were frozen and thawed twice, resuspended in 0.2 ml of buffer [19] and added with Kyro EOB. After 10 min of incubation at 37°, the suspensions were centrifuged (12,000 rpm, 1 min.) and the cell-free extracts from 2 or 3 petri dishes (1–1.5 μg protein/μl) were used for HMGCoA reductase assay for each experimental point. Incubations (final volume 0.3 ml) were carried out at 37° for 30 min, according to Cighetti *et al.* [20]; unlabelled HMGCoA (150 μM) was used as the substrate. The incubations were stopped by the addition

Table 1. Effect of selective and non-selective β-adrenoceptor antagonists on the incorporation of [2-^{14}C]-acetate into the unsaponifiable fraction of different lines of normal human skin fibroblasts

β-adrenoceptor antagonists	Concentration (μM)	Incorporation of [2-^{14}C]-acetate (pmoles hr^{-1} mg^{-1} cell protein) $\bar{X} \pm$ SEM	Increase (%)
Selective			
Control	—	112 ± 10	—
Atenolol	50	110 ± 10	−1
	100	114 ± 5	1
Control	—	504 ± 2	—
Metoprolol	50	539 ± 47	7
	100	632 ± 19*	25
Control	—	125 ± 10	—
Practolol	50	111 ± 6	−11
	100	110 ± 8	−11
Control	—	112 ± 10	—
ICI 188,551	50	339 ± 19†	203
	100	539 ± 49†	381
Non-selective			
Control	—	407 ± 2	—
Pindolol	50	495 ± 18‡	22
	100	516 ± 32*	27
Control	—	504 ± 2	—
Labetalol	50	892 ± 5†	77
	100	1243 ± 42†	146
Control	—	504 ± 2	—
Sotalol	50	558 ± 17	11
	100	529 ± 14	5
Control	—	112 ± 10	—
Propranolol	50	262 ± 5†	134
	100	450 ± 11†	302

Cells were incubated, in the absence of LDL, the 6th day after seeding. Values are mean of triplicate incubations $P < 0.01$ (*); <0.001 (†); <0.05 (‡); Student's *t*-test.

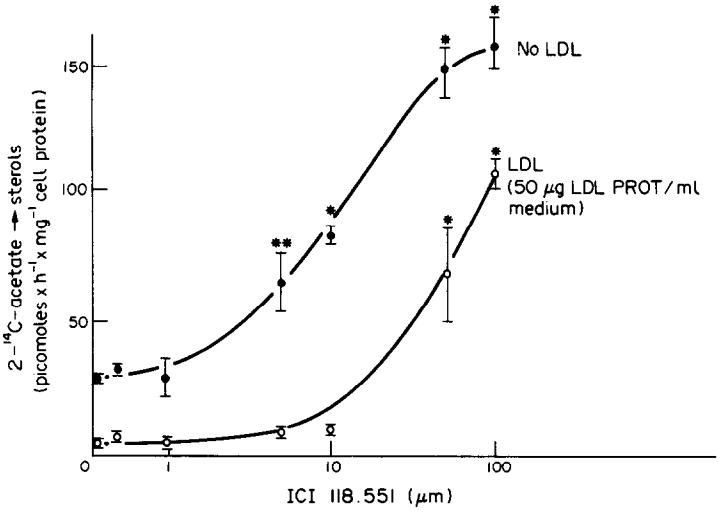

Fig. 1. Dose–response curves for the activation of sterol biosynthesis by the β_2-adrenoceptor antagonist ICI 118,551 in human skin fibroblasts. The curves refer to the absence or presence of LDL (50 μg LDL protein/ml) in the incubation medium. Cells were incubated the 6th day after seeding; values are mean of triplicate incubations ± SEM. $P < 0.05$ (**) and <0.01 (*) vs the respective controls: Student's *t*-test.

of 5 N NaOH (50 μl), and 0.5 μg [$^2H_4$] mevalonolactone was added as internal standard. The activity of HMGCoA-reductase (EC 1.1.1.34) was evaluated by selected ion monitoring technique [12] and expressed as pmoles of mevalonate formed min^{-1} mg^{-1} of detergent solubilized protein.

RESULTS

Among β_1-selective antagonists tested, only metoprolol stimulated the incorporation of [2-^{14}C]-acetate into the unsaponifiable fraction; ICI 118,551, a novel erythro aryloxybutanolamine antagonist of β_2-adrenoceptors [21], was active as well. Among non-selective β-antagonists, pindolol, labetalol and propranolol were increasingly effective (Table 1). Figure 1 depicts the log dose-effect of the compound ICI 118,551 on the incorporation of labelled acetate into cellular sterols: the drug stimulates the conversion of labeled acetate into sterols regardless of the presence of LDL, which down regulate cholesterol biosynthesis.

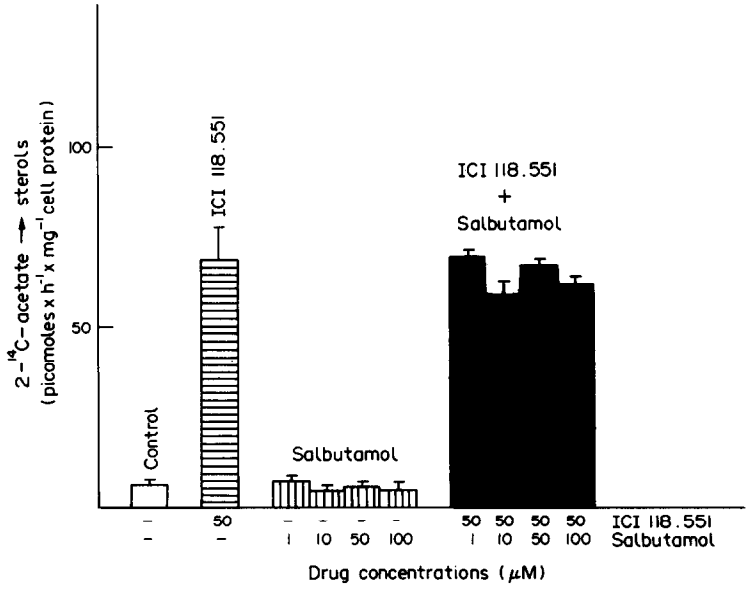

Fig. 2. Competition experiment between salbutamol and ICI 118-551 for sterol biosynthesis in human skin fibroblasts. Cells were incubated the 6th day after seeding in the absence of LDL; values are mean of triplicate incubations ± SEM. The increased sterol biosynthesis in the presence of ICI 118,551 alone or plus salbutamol is significantly different from the respective control, but not from each other (Duncan's test, $P < 0.01$).

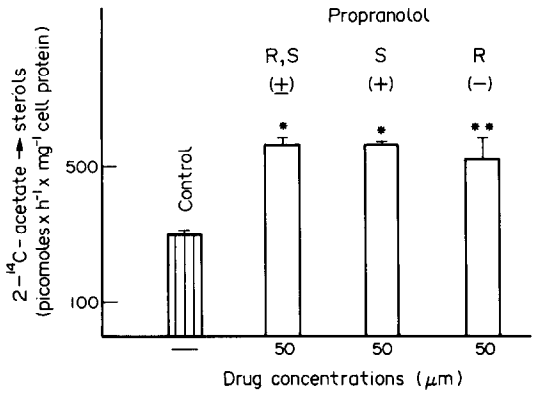

Fig. 3. Effect of racemic propranolol and its enantiomers on sterol biosynthesis in human skin fibroblasts. Cells were incubated the 6th day after seeding, in the absence of LDL; values are mean of triplicate incubations, ± SEM. $P < 0.01$ (**) and <0.001 (*) vs control; Student's *t*-test.

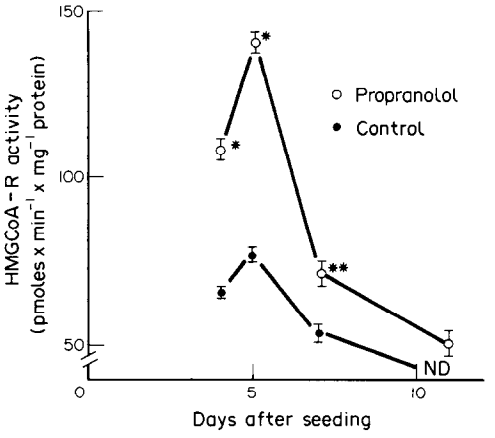

Fig. 5. Effect of propranolol (50 μM) on mevalonate formation in human skin fibroblasts at various days after cell plating. The incubation medium did not contain LDL. Values are the mean of triplicate incubations, ± SEM. $P < 0.01$ (**) and <0.001 (*); Student's *t*-test.

To determine whether the observed effect of β-adrenoceptor antagonists occurred through interaction with their specific receptors, a constant concentration of compound ICI 118,551 was incubated with increasing concentrations of salbutamol, a β_2-adrenoceptor agonist with no effect on acetate conversion to sterols. Salbutamol did not interfere with the stimulating action of ICI 118,551 (Fig. 2). The equipotency of the propranolol enantiomers, differing in their β-adrenolytic activities, in increasing the synthesis of sterols in the cells (Fig. 3) further suggests that this effect is independent from β-adrenoceptors.

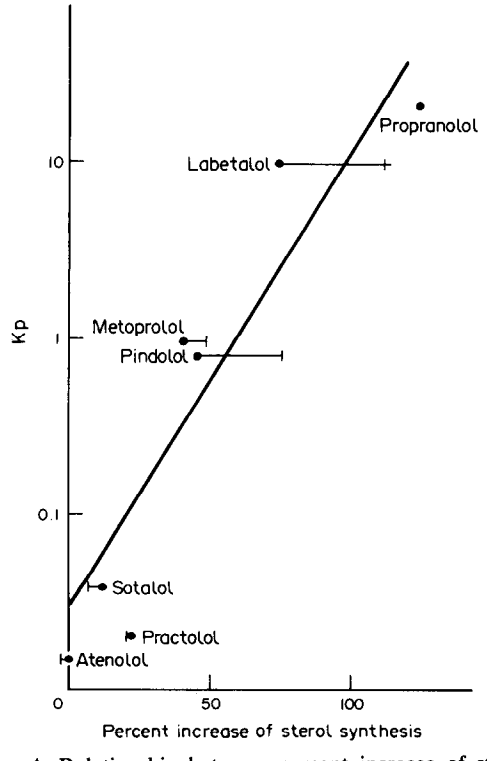

Fig. 4. Relationship between percent increase of sterol biosynthesis in human skin fibroblasts and n-octanol/pH 7.4 buffer partition coefficient (Kp, log scale) for a series of β-adrenoceptor antagonists. Cells were incubated the 6th day after seeding, in the absence of LDL. Final concentration of drugs: 50 μM. Values are mean of triplicate incubations, ± SEM. $r = 0.89$, $P < 0.01$.

In the experiment reported in Fig. 4 a series of β-adrenoceptor antagonists varying in lipophilicity was evaluated on cholesterol biosynthesis in fibroblasts, as expressed by [2-^{14}C]-acetate incorporation into cellular sterols. Lipophilic properties were derived from literature in terms of partition coefficients in *n*-octanol/pH 7.4 buffer at 37° for atenolol, labetalol, metoprolol, pindolol, propranolol and sotalol [22], and *n*-octanol/pH 7.5 buffer at 37° for practolol [23]. A linear plot correlated significantly the percent increase of sterol biosynthesis and log partition coefficient of β-adrenoceptor antagonists. HMGCoA reductase, the feed-back regulated key enzyme of cholesterol biosynthesis, is stimulated by propranolol and its enantiomers. The effect of the racemic drug on the activity of the enzyme in fibroblasts was evaluated at various days after cell plating: at any time explored, the transformation of HMGCoA into mevalonic acid was higher in the presence of the drug (Fig. 5). Enantiomers of propranolol, which share lipophilicity [23], were studied in two lines of human skin fibroblasts with different

Table 2. HMGCoA-reductase activity (pmoles mevalonate min^{-1} mg^{-1} protein) in two cell lines of normal human skin fibroblasts: effect of racemic propranolol and its enantiomers

	Experiment I $\bar{X} \pm$ SEM	Experiment II $\bar{X} \pm$ SEM
Control	33 + 3	187 ± 13
(±) Propranolol (50 μM)	202 ± 17*	473 ± 11*
(−) Propranolol (50 μM)	166 ± 14*	—
(+) Propranolol (50 μM)	—	490 ± 15*

Cells were incubated in the absence of LDL, the 5th day after seeding. Values are mean of triplicate incubations ± SEM.

$P < 0.001$ (*) vs the respective control; Student's *t*-test.

basal rates of HMGCoA conversion into mevalonic acid. Both agents were equally effective in this respect (Table 2), as they were on the incorporation of labeled acetate into cellular sterols.

DISCUSSION

The data of this study indicate that β-adrenoceptor antagonists interfere with cholesterol biosynthesis in human skin fibroblasts. It was recently reported that stimulation of β_2-adrenergic receptors suppresses sterol synthesis in human mononuclear leukocytes, an effect opposed by selective blockade of this receptor [15]. Our results show a direct stimulating effect of β-antagonists on cholesterol biosynthesis which is independent on β-adrenoceptors, as suggested by competition experiments, by the equipotent effects of propranolol enantiomers, which differ in β-adrenolytic activities [24] and have different affinities for β-adrenoceptors in human fibroblasts [25], and by the drug concentrations needed. The β-adrenoceptor antagonists studied differ in lipophilicity. Linear regression analysis indicated a positive correlation between stimulation of sterol biosynthesis and log partition coefficient in *n*-octanol/pH 7.4 buffer. Compound ICI 118,511 was not included owing to its unreported partition coefficient. This drug has a membrane-stabilizing action similar to that of propranolol [21]: since this property is linearly correlated with lipid/H_2O partition coefficient [5] and since the compound clearly increased cellular synthesis of cholesterol, it would have probably fitted in the correlation considered. Thus, lipid solubility appears to be a determinant of the stimulating effect of β-adrenergic blockers on cholesterol biosynthesis in human fibroblasts *in vitro*. HMGCoA reductase is a microsomal enzyme that determines the rate of cholesterol synthesis in a variety of mammalian cells, including fibroblasts [1]. The capacity of propranolol and its enantiomers to stimulate HMGCoA reductase suggests that β-adrenoceptor antagonists interfere with an enzymatic activity which is central to the homeostasis of cellular cholesterol, and that this action in the intact cell is linked to the lipid solubility of these drugs. Thus a parallelism exists in human skin fibroblasts between the stimulation by β-adrenoceptor antagonists of cholesterol biosynthesis from acetate and the stimulation of HMGCoA reductase activity, and the latter effect may mediate the former.

The need for cholesterol in fibroblasts is met by LDL-carried esterified cholesterol, which is hydrolyzed in lysosomes, and by cholesterol synthesis, which is regulated by HMGCoA reductase. The resultant free cholesterol is utilized by the cell for a number of regulatory purposes, including down regulation of LDL receptors and suppression of HMGCoA reductase activity [26]. Any interpretation of the effect of lipophilic β-adrenoceptor antagonists on cholesterol biosynthesis in human skin fibroblasts is at present only speculative. These drugs could enter the cells and act directly on the enzyme(s) involved in cholesterol biosynthesis such as HMGCoA reductase. Alternatively, it is tempting to speculate, at the light of non-stereoselective lysosomal accumulation of lipophilic β-adrenoceptor antagonists in cultured cell systems [27] and of their capacity to inhibit lysosomal enzymes involved in lipid metabolism [28], that the increased cholesterol biosynthesis could be secondary to inhibition of lysosomal cholesterol ester hydrolase. Preliminary studies in our laboratory indicate an increase of esterified cholesterol in cells incubated with lipophilic β-adrenoceptor antagonists and their effect on lysosomal cholesterol ester hydrolase is now under investigation.

This interpretation would explain the capacity of propranolol to stimulate the HMGCoA reductase activity in the presence of LDL. However, since lipophilic β-adrenoceptor antagonists stimulate cholesterol synthesis also in the absence of LDL, other mechanisms have to be involved. According to the model proposed by Goldstein and Brown [29], the increased internalization of LDL elicited by these drugs [3, 30] should lead to a decreased cholesterol synthesis by feed-back inhibition of HMGCoA reductase. It should be noted that the effects evoked by lipophilic β-adrenoceptor antagonists: increased cholesterol formation, enhanced expression of LDL receptors [3, 30] and decreased activity of acyl cholesterol acyltransferase (ACAT) [4, 31], are all events that would be expected, according to Goldstein and Brown [29], whenever a decrease of intracellular free cholesterol occurs. It follows that such drugs could act either by reducing the intracellular pool of free cholesterol, or by interfering with the mechanism(s) by which intracellular free cholesterol mediates its own homeostasis, hypothesis already considered [4].

Various antihypertensive drugs, including β-adrenoceptor antagonists, interfere with lipid metabolism and alter plasma lipoprotein levels [32]; it is currently discussed whether these agents may by this way offset, in terms of risk factors for coronary heart disease, the beneficial effects of lowering blood pressure [2]. Plasma levels of β-adrenoceptor antagonists following clinical doses are lower than concentrations used in this study. The apparent volume of distribution of lipophilic β-adrenoceptor antagonists largely exceeds, however, the physiological body fluids, indicating concentration in some tissues, where they could interfere with local cholesterol homeostasis.

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