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EFFECT OF A CHOLESTEROL SYNTHESIS INHIBITOR (BM 15.766) IN THE PRESENCE AND ABSENCE OF HDL ON CORTICOSTEROIDOGENESIS OF ISOLATED ZONA GLOMERULOSA AND FASCICULATA CELLS

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Summary—The effect of the cholesterol synthesis inhibitor BM 15.766, 4-[2-[1-(4-chlorocinnamyl)piperazin-4-yl] ethyl]-benzoic acid on the corticosteroid production was studied in order to reveal the importance of endogenous cholesterol synthesis in the function of zona glomerulosa and zona fasciculata cells of rats. Attempts were made to compensate the effect of BM 15.766 through the application of high-density lipoproteins (HDL). Electron microscopy was used to trace the binding and intracellular accumulation of colloidal gold-labelled HDL (HDL-Au, a cholesterol carrier), in the presence of the cholesterol biosynthesis inhibitor. The stimulation of both types of cells with ACTH was less effective in the presence of 2×10^{-5} M BM 15.766. The inhibitory effect of BM 15.766 was most marked on the aldosterone production of the zona glomerulosa cells, and could not be reversed by addition of a small amount of HDL-Au. Corticosterone–aldosterone conversion was inhibited by 2×10^{-5} M BM 15.766. ACTH-stimulated, short-term HDL uptake and internalization was not affected by the cholesterol synthesis inhibitor. The results suggest that certain metabolites of *de novo* cholesterol biosynthesis may participate in the control of aldosterone production.

INTRODUCTION

We have previously reported that a reduced serum cholesterol level in rats does not affect the aldosterone production of the zona glomerulosa cells, whereas the hormone production of the zona fasciculata cells is seriously affected [1]. In the present investigation, we set out to establish the role of *de novo* synthesized cholesterol in the functional activity of the zona glomerulosa and the zona fasciculata cells, by using BM 15.766, 4-[2-[1-(4-chlorocinnamyl)piperazin-4-yl] ethyl]-benzoic acid a cholesterol synthesis inhibitor recently developed by Boehringer Mannheim GmbH [2, 3].

An additional aim was to obtain information on the relationship between the inhibition of *de novo* cholesterol synthesis and the uptake and internalization of the extracellular cholesterol source, the high-density lipoproteins (HDL).

MATERIALS AND METHODS

Experimental: cell incubation and steroid determinations

Male CFY rats weighing 200–250 g were used. The rat adrenal cell preparation applied in our laboratory was described previously [4, 5]. Briefly: cell suspensions were prepared by collagenase digestion of adrenal capsular strippings to yield zona glomerulosa cells, and of decapsulated adrenal glands to yield zona fasciculata cells. The zona fasciculata cell contamination in the zona glomerulosa cell suspension was $< 5\%$. In general, adrenal glands from 40 rats were digested for each adrenal cell preparation. The cells were prepared in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2 g/l glucose and 40 g/l human serum albumin (HSA). Forty zona glomerulosa and 40 zona fasciculata (0.9 ml) cell suspensions aliquots (approx. 3×10^5 cells/ml) were incubated in one session, in a shaking water-bath at 37°C under an atmosphere of 95% O_2 and 5% CO_2 for 2 h. BM 15.766 was dissolved in dimethylsulphoxide (2 g/l final concentration); ACTH was dissolved in physiological saline containing 5 g/l HSA and adjusted to pH 3.5. The concentration of BM 15.766 added to the zona fasciculata and zona glomerulosa cell suspensions was from 2×10^{-7} to 2×10^{-5} M if it was added alone, or 2×10^{-6} and 2×10^{-5} M if it was added together with 10^{-9} M ACTH. In the experiments where the effect of BM 15.766 on HDL uptake was stud-

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ied, the cells were incubated with HDL-Au (0.1 ml, protein content $< 70\ \mu g$) either alone or together with 2×10^{-5} M BM 15.766 for 5 h. Experiments were performed in a randomized block format to eliminate bias due to systematic error. The experiments were carried out with 3 or 4 incubations at each dose in duplicate or triplicate.

The corticosterone contents of the incubation media (both zona glomerulosa and zona fasciculata) were determined by fluorimetry [6] after chloroform extraction. Aliquots of the chloroform extract of the zona glomerulosa incubate were assayed for aldosterone content by radioimmunoassay without chromatographic separation [7].

The conversion of corticosterone to aldosterone was studied according to Campbell *et al.* [8]. Zona glomerulosa cells were incubated with [^{3}H]corticosterone (50,000 cpm, 3.4 TBq/mmol, Amersham). After incubation for 2 h the cells were sedimented by centrifugation. The incubation medium was decanted, $10\ \mu g$ each of unlabelled aldosterone and corticosterone were added and the medium was extracted with 3 vol of chloroform. The extracts were dried under vacuum and after paper-chromatographic separation, (with paper development in benzene–methanol–water (2:2:1, v/v), the migrations of aldosterone and corticosterone standards were visualized with an ultraviolet lamp and the radioactivities of the corresponding zones were determined by liquid scintillation spectroscopy (LKB). The results were expressed as % [^{3}H]-corticosterone converted to [^{3}H]aldosterone/3×10^5 cells/120 min.

Materials

Synthetic human α^{1-39} ACTH and aldosterone antiserum (Sheep 088) were supplied by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Md, U.S.A. The following materials were purchased: collagenase (type I), Worthington Chemical Corp., U.S.A.; TC Medium 199, DIFCO Laboratories, U.S.A.; 1,2-[^{3}H]-aldosterone, Radiochemical Centre, Amersham, England. BM 15.766 was kindly donated by Boehringer–Mannheim GmbH.

Preparation of HDL and conjugation to colloidal gold

HDL (density: 1.121–1.125 g/ml) were isolated from fresh human plasma by the modified method of Chung *et al.* [9], and HDL were conjugated to colloidal gold granules as described earlier [10]. The lipoproteins and their conjugates were stained with uranyl acetate, then characterized by electron microscopy as previously described [10].

Electron microscopy

Cell suspensions were processed for electron microscopy as described earlier [10, 11]. Eleven to 23 electron micrographs were taken at a magnification of 6600× on each cell type, for each treatment.

Statistical analysis

Analysis of electron micrographs: gold granules representing the HDL particles were counted on the cell surface and intracellularly. The comparisons of the number of gold particles were made by Kruskal–Wallis non-parametric analysis [12]. The median values obtained in this way were used to calculate the percentages of the intracellular gold granules.

Hormone values were expressed as percentages of the related controls. These percentages were compared by analysis of variance and Dunnet contrasts [13].

RESULTS

Effects of BM 15.766 on hormone production

BM 15.766 at 2×10^{-6} and 2×10^{-5} M inhibited the basal corticosterone and aldosterone productions of isolated zona glomerulosa cells, but did not affect markedly the basal corticosterone production of isolated zona fasciculata cells (Fig. 1 and Table 1). The ACTH-stimulated corticosteroid production was inhibited by 2×10^{-5} M BM 15.766 in both cell types (Fig. 1 and Table 1). The inhibitory effect of BM 15.766 was most definitive on aldosterone production. At the highest dose used (2×10^{-5} M), BM 15.766 inhibited corticosterone–aldosterone conversion both in the presence and in the absence of ACTH (Fig. 2).

Internalization of HDL-Au by ACTH-stimulated cells, in the presence or absence of BM 15.766. Fine structural observation

Zona glomerulosa cells. In both the BM 15.766-treated and non-treated cells most of the labelled HDL was attached to the cell surface at the sites of microvilli. Some of them were observed in coated pits, coated vesicles and also in non-coated vesicles. The major part of the intracellular HDL-Au accumulated in the lysosomes.

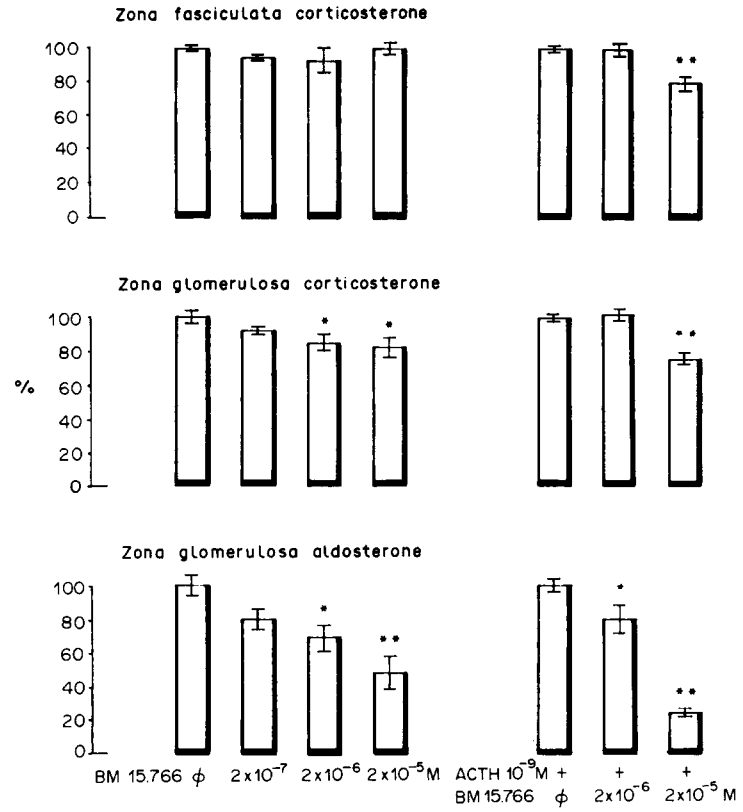

Fig. 1. Effect of BM 15.766 on basal and ACTH stimulated adrenocortical steroid production (mean ± SEM, $n = 10$, *: $P < 0.05$; **: $P < 0.01$).

Differences were not observed regarding the accumulation of HDL-Au in the treated and non-treated cells (Table 2).

Zona fasciculata cells. The HDL pathway was similar to that in the zona glomerulosa cells. No qualitative or quantitative differences were observed in the accumulation of HDL-Au by treated and non-treated cells (Table 2).

No noteworthy qualitative changes were observed in the fine structure of the zona glomerulosa and zona fasciculata cells.

Effect of BM 15.766 on stimulated steroid production of adrenocortical cells in the presence of HDL-Au

BM 15.766 markedly decreased the ACTH-stimulated aldosterone production of zona glomerulosa cells even in the presence of a small amount of HDL-Au (Table 3). The corticosterone production of the zona glomerulosa cells seemed not to be affected. The corticosterone production of the ACTH-stimulated zona fasciculata cells decreased slightly in the presence of the cholesterol synthesis inhibitor supplemented by HDL-Au (Table 3).

DISCUSSION

Previously we described that the decrease in the plasma lipoproteins, i.e. the decrease in the exogenous cholesterol source, after 4-APP (4-aminopyrazolo-(3,4-d)pyrimidine) treatment decreases the lipid content and ACTH responsiveness of the zona fasciculata cells, but leaves intact the lipid content and ACTH responsiveness of the zona glomerulosa cells [1]. We con-

Table 1. Basal and ACTH stimulated adrenocortical steroid production

	Zona glomerulosa		Zona fasciculata
	Aldosterone (pmol/ml)	Corticosterone (pmol/ml)	Corticosterone (pmol/ml)
Saline	4.1 ± 0.04	106.0 ± 41.0	110 ± 54.4
ACTH 10^{-12} M	148.0 ± 12.0	639.0 ± 282.0	1807 ± 628

The hormone values used as 100%, for calculation of the effect of BM 15.766; see Fig. 1. (Mean ± SEM, $n = 10$.)

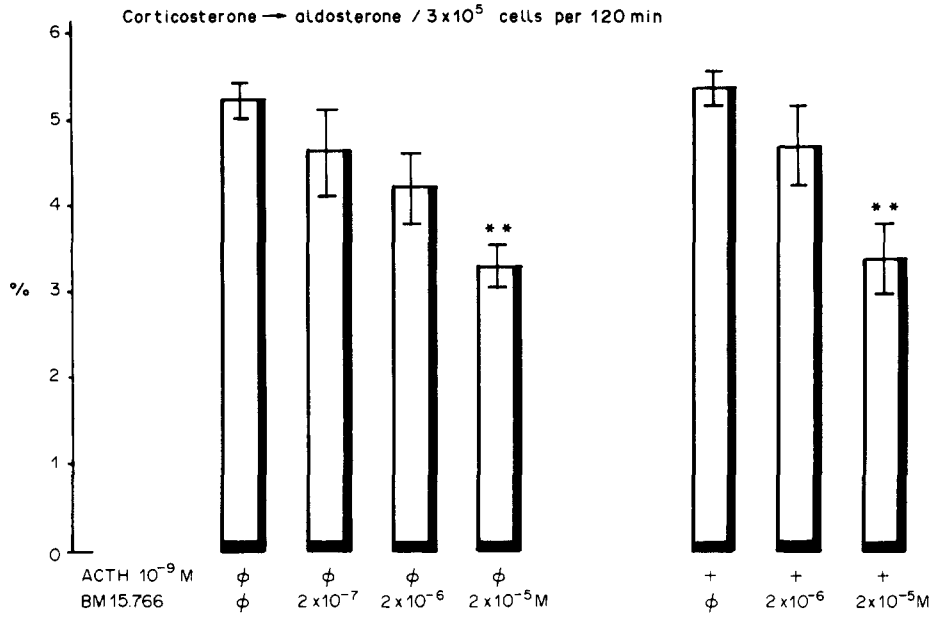

Fig. 2. Effect of BM 15.766 on the conversion of [^{3}H]corticosterone to [^{3}H]aldosterone in zona glomerulosa cells. Each values represents the mean ±SEM ($n = 6$, $^{**}P < 0.01$).

cluded that an endogenous cholesterol source is more important than exogenous lipoproteins for the corticosterone and aldosterone production of zona glomerulosa cells, while as concerns the corticosterone production of the zona fasciculata cells the exogenous cholesterol uptake seems to play a dominant role.

The role of endogenous cholesterol synthesis in adrenocortical steroidogenesis is not completely understood yet. In our present experiments the cholesterol synthesis inhibitor BM 15.766 inhibited both the basal and the ACTH-stimulated aldosterone and corticosterone secretions of the zona glomerulosa cells, but it inhibited only the ACTH-stimulated corticosterone secretion of the zona fasciculata cells and only at the highest concentration (2×10^{-5} M) used. The aldosterone production by the zona glomerulosa cells seems to be more dependent on *de novo* cholesterol synthesis than corticosterone production by the zona fasciculata cells. BM 15.766 was found to inhibit corticosterone–aldosterone conversion. This effect could not be reversed by supplementation with a small amount of HDL. Two explanations are possible: (i) BM 15.766 selectively inhibited the 49 K form of cytochrome P-$450_{11\beta}$, the key enzyme converting corticosterone to aldosterone [14], or (ii) certain metabolites of *de novo* cholesterol biosynthesis might control the aldosterone secretion. The latter possibility is plausible on the basis of recently acquired knowledge about *de novo* cholesterol biosynthesis, reviewed by Goldstein and Brown [15]: there is a finely tuned mechanism controlling the relationship between mevalonate synthesis, cholesterol homeostasis and several vital cell functions.

Table 2. Percentages of HDL-Au at the ACTH (10^{-9} M) stimulated cell surface and inside the cells in the presence or absence of BM 15.766 (2×10^{-5} M)

Treatment	No. of cellular profiles examined	Gold particles/ cellular profiles	Percentage inside the cells (%)
Z.g. cells			
HDL-Au + ACTH	20	35	24.6
HDL-Au + ACTH + BM 15.766	23	50	28.0
		NS	NS
Z.f. cells			
HDL-Au + ACTH	11	135	15.5
HDL-Au + ACTH + BM 15.766	10	108	9.7
		NS	NS

Z.g.: zona glomerulosa, Z.f.: zona fasciculata. NS: No significant differences were observed in the number of gold particles/cells profiles or inside the cells as revealed by the Kruskal–Wallis test.

Table 3. Effects of BM 15.766 (2×10^{-5} M) on ACTH-stimulated adrenocortical steroid production in the presence of HDL-Au

	Zona glomerulosa		Zona fasciculata
	Aldosterone (pmol/ml)	Corticosterone (pmol/ml)	Corticosterone (pmol/ml)
HDL-Au + ACTH 10^{-12} M	85.9 ± 13.6	644.4 ± 72.1	975.6 ± 137.5
HDL-Au + ACTH 10^{-12} M + BM 15.766	41.3 ± 7.3	668.8 ± 42.9	815.1 ± 169.8
HDL-Au + ACTH 10^{-9} M	336.2 ± 20.9	1287.2 ± 49.5	2754.8 ± 76.1
HDL-Au + ACTH 10^{-9} M + BM 15.766	62.5 ± 4.3	1402.7 ± 100.4	2044.5 ± 183

In steroidogenic cells there is a pool of free cholesterol which can be restored from (i) the endogenous *de novo* cholesterol synthesis, (ii) the exogenous, imported cholesterol, or (iii) the intracellular cholesterol store: the lipid droplets. The stimulation of the cells was less effective in the presence of BM 15.766, indicating that the intracellular lipid droplets, although still present in a great number in the cells, can not supply enough cholesterol for maximal hormone production. *In vitro*, only a proportion of the lipid droplets seem to be steroidogenic, or alternatively the metabolites of endogenous cholesterol synthesis also control the steroidogenesis. *De novo* cholesterol synthesis is possibly more intensive in zona glomerulosa cells than in the zona fasciculata cells. A small amount of HDL, which is below the HDL serum level in rats, but comparable to the extracellular HDL level, although significantly internalized, did not restore the decreased hormone production capacity. This amount of HDL was not sufficient to compensate the effect of the BM 15.766 inhibition of *de novo* cholesterol synthesis. Rebuffat *et al.* [16] reported that long-term *in vivo* administration of mevinolin, a drug similar in effect to BM 15.766, led to lower plasma and intra-adrenal cholesterol concentrations, and also to a slight decrease in the blood level of corticosterone. Heikkilä reported that in a primary tissue culture of undifferentiated zona glomerulosa-like cells mevinolin had no effect on corticosterone secretion [17].

The observations suggest that certain metabolites of *de novo* cholesterol synthesis may play a role in controlling aldosterone production of zona glomerulosa cells, and possibly to a much lesser extent corticosterone production of the zona fasciculata cells.

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