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EFFECTS OF KETOTIFEN ON THE RESPONSIVENESS OF PERIPHERAL BLOOD LYMPHOCYTE β-ADRENERGIC RECEPTORS

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Abstract — The effects of ketotifen therapy on the responsiveness of lymphocyte β-adrenergic receptors was evaluated by measuring cyclic AMP elevations caused by isoproterenol in cells isolated from patients treated with ketotifen for more than 1 year. Binding of ^{3}H-dihydroalprenolol to β-receptors was also evaluated. The isoproterenol-induced rise in cyclic AMP relative to each individual's baseline level was greater in patients on current ketotifen therapy than in other asthmatic patients or non-asthmatic subjects. Ketotifen therapy increased the apparent equilibrium dissociation constant for specific ^{3}H-dihydroalprenolol binding to the receptors. Receptor numbers in symptomatic asthma patients on standard drug therapy were decreased.

The results indicate that long term ketotifen therapy is associated with increased responsiveness of β-receptors to stimulation by catecholamines and that this alteration may involve changes in the receptors themselves, their membrane environment, adenylate cyclase or components of the adenylate cyclase coupling system.

Ketotifen is an orally effective drug used in the therapy of patients with bronchial asthma. Although the mechanism of action of this drug has not been clearly elucidated, the possibility that ketotifen might prevent or reverse the β-adrenergic receptor subsensitivity that occurs in asthmatic patients (Szentivanyi, 1968) has been suggested (Craps & Ney, 1984).

The major objective of this investigation was to determine whether long-term ketotifen therapy is associated with alterations in β-adrenergic receptor function. This was assessed using isolated peripheral lymphocytes from patients to determine: (1) the effects of ketotifen on cyclic AMP (cAMP) accumulation in response to isoproterenol; and (2) the effects of ketotifen on the specific binding of ^{3}H-dihydroalprenolol (^{3}H-DHA) to β-receptors.

EXPERIMENTAL PROCEDURES

Protocol

Patients and non-asthmatic volunteers of both sexes ranging in age from 16 to 70 years were divided into five groups based on their histories and therapies as follows: (1) individuals with no history of asthma; (2) patients with a history of asthma, but who were asymptomatic at the time of the study and receiving no medication for asthma; (3) patients with asthma who were receiving conventional drug therapy for control of their asthma at the time of the study; (4) patients with asthma who were receiving conventional drug therapy as in group 3, above, but who were also being treated with ketotifen; and (5) patients who had received ketotifen for 1–2 years, but who had discontinued that agent for 30–60 days. All asthmatic patients studied were "mixed" asthmatics, that is, the asthma was perennial and there were evidences of an atopic factor by history and positive skin tests.

Conventional drug therapy included use of β-adrenergic agonists and methylxanthines in standard doses. Patients treated with ketotifen received 1 mg (approximately 6 μg/kg) twice daily. Blood samples were collected between 9:00 and 10:00 a.m. on the day of each experiment. Lymphocytes were isolated and incubated in the presence of varying concentrations of isoproterenol and their cyclic AMP content measured as described below. Separate aliquots of freshly isolated lymphocytes were

homogenized and the binding of ^{3}H-DHA to the membrane fraction was determined using the methods described for receptor binding.

Materials

Ketotifen (Zaditen®), a benzocycloheptathiophene with a mol. wt of 426, was supplied by Sandoz Research Institute, East Hanover, NJ, U.S.A.

The composition of the balanced salt solution (BSS) utilized was as follows (as mM): NaCl, 127; Tris HCl (pH 7.6), 146; glucose, 5.56; KCl, 5.54; and $MgCl_2$, 0.98. The composition of the Hank's balanced salt solution (HBSS) utilized was as follows (mM): NaCl, 138; Na_2HPO_4, 39.6; glucose, 5.56; KCl, 5.36; $NaHCO_3$, 2.98; $CaCl_2$, 1.26; $MgCl_2$, 0.49; KH_2PO_4, 0.44; $MgSO_4$, 0.41; phenolsulphonphthalein, 0.028.

Theophylline, L-isoproterenol-D-bitartrate and Histopaque 1077 were purchased from Sigma Chemical Co. (St Louis, MO). Radioimmunoassay reagents including ^{125}I labeled 2-*O*-succinyl cyclic AMP tyrosine methyl ester, cyclic AMP antiserum (rabbit), and anti-rabbit gamma globulin (goat) were obtained in kit form from Becton Dickinson (Orangeburg, New York).

Isolation of human blood lymphocytes

Venous blood (with citrate as anticoagulant) was diluted with an equal volume of BSS and 15 ml aliquots were carefully layered over 9 ml of Histopaque in siliconized 50 ml glass centrifuge tubes (Boyum, 1968). The diluted blood was then centrifuged at 400 **g** for 40 min at room temperature. The plasma layer was removed by aspiration without disturbing the leukocytes which settled in an opaque layer at the histopaque surface. The leukocytes were then suspended in BSS to which 15 μM $CaCl_2$ had been added and centrifuged at 60 **g** for 10 min. The supernatant fluid, which contained platelets, was decanted and the remaining pellet was resuspended and centrifuged again. The resulting leukocyte pellet contained about 25 – 40 million cells of which more than 90% were lymphocytes.

Cyclic AMP

Approximately 4.5 million cells were placed into 550 μl volumes of HBSS containing 2.5 mM theophylline and aerated with a 95% air, 5% CO_2 mixture. Isoproterenol was added and the cell suspensions were incubated in a 37°C water bath. After 15 min, the incubations were stopped by addition of 50 μl volumes of 5 M perchloric acid and the tubes were centrifuged at 1000 **g** for 10 min. The supernatant fluid was transferred to 1 ml tubes and adjusted to pH 7 using approximately 60 μl of K_2CO_3. After removal of the precipitate by centrifugation, the samples were stored at −80°C until assayed by the radioimmunoassay procedure of Steiner, Parker & Kipnis (1972) modified by acetylation of the antigen to increase sensitivity (Harper & Brooker, 1975). All assays were run in duplicate. On rare occasions assays failed to give closely matched values in duplicate assays or gave cAMP values that fell outside of two S.E. from the group mean. Those data points are not presented.

β-Adrenergic receptor binding

Freshly isolated lymphocytes were placed in 25 ml of ice-cold homogenization buffer and the cells broken using a motor-driven teflon/glass homogenizer. The homogenate was centrifuged at 26,500 **g** for 15 min. The resulting pellet was washed twice and resuspended in 3.2 ml of the same buffer, and 0.1 ml aliquots of this suspension were pipetted into 12 mm × 75 mm tubes for the binding reaction. After 20 min of incubation at 37°C, the binding reactions were started by additions of ^{3}H-DHA ranging in concentration from 0.2 to 10 nM. Non-labeled alprenolol (1 μM) was added to some of the tubes to determine non-specific binding. Steady-state binding was achieved by the end of 15 min of incubation and the reactions were terminated at that time by addition of 4.5 ml of cold buffer and filtered through GFC glass filters (Bishopric, Cohen & Lefkowitz, 1980; Williams, Snyderman & Lefkowitz, 1976). The radioactivity trapped on the filters was counted by liquid scintillation spectrometry.

Analysis of data

Binding data were analyzed for equilibrium dissociation constants (K_D) and maximum binding (B_{max}) using a Fortran IV G SCAFIT data processing program written by Faden and Rodbard (Hancock, DeLean & Lefkowitz, 1979), obtained through Biomedical Computing Technology Information Center (Nashville, TN) and run on an IBM 3033 mainframe computer. Statistical significance was tested using SAS computer programs for analysis of variance (ANOVA) coupled with Duncan's new multiple range test by SAS Institute Incorporated, Cary, NC. The Student's *t*-test for two independent means was employed. $P < 0.05$ was the criterion for statistical significance. Data are presented as means ± S.E.M.

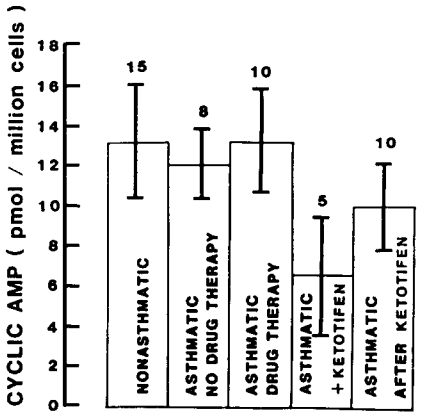

Fig. 1. Non-stimulated levels of cAMP in lymphocytes among the different patient groups. Means, S.E.s and number of patients are presented.

RESULTS

Cyclic AMP

Levels of cAMP in lymphocytes incubated in the absence of isoproterenol (non-stimulated levels) are illustrated in Fig. 1. The mean non-stimulated cAMP level for asthmatic patients who were treated with ketotifen was only about half of the level for non-asthmatic controls, but there was no statistically significant difference among these groups. However, it seems possible that these data, although not statistically significant, may indicate a trend.

The variation among patients was large, as is indicated by the large S.E.s. Because of this interindividual variation, levels of cAMP stimulated by isoproterenol were expressed as the percentage increase above control, each patient's own non-stimulated level serving as his or her control value. Stimulated levels of cAMP are illustrated in Fig. 2.

Isoproterenol produced concentration-dependent rises in cAMP that reached a maximum of 187% above control levels in non-asthmatic individuals. The mean elevation in asthmatics not treated with ketotifen tended to be lower than the rest, particularly at the lowest isoproterenol levels. This general trend did not show statistical significance in the present study, but may be indicative of a biologically significant depression in β-receptor responsiveness in these patients. By contrast, the cAMP responses to isoproterenol in ketotifen-treated patients were significantly greater than in other asthmatic patients and reached a peak elevation of 519% over control at 10 μM isoproterenol. The β-receptor blocking agent alprenolol (1 μM) attenuated the elevations produced by isoproterenol (10 μM) in all groups.

^{3}H-DHA binding

Specific binding of ^{3}H-DHA to lymphocyte membranes was saturable and yielded linear Scatchard plots indicating consistency with a homogenous population of binding sites. Representative binding data are shown in Fig. 3.

Dissociation constants (K_D) and maximum ligand binding (B_{max}) in the five groups of patients are summarized in Table 1. The mean K_D of all asthmatic patients except those on ketotifen therapy tended to be lower than nonasthmatics, but this tendency was not statistically significant because of variations. By contrast, the K_D of asthmatic patients on ketotifen therapy had returned to at least normal values and was significantly higher than other asthmatic groups. This indicated a lowering of the apparent affinity of β-receptors for the ligand in ketotifen-treated patients.

The mean B_{max} for asthmatic patients receiving drug therapy without ketotifen was only 56% of the B_{max} in normal subjects ($P < 0.05$) indicating a decrease in the number of receptors. This decrease was also statistically significant with respect to patients with history but no current symptoms of asthma, and with asthmatic patients after discontinuation of ketotifen (Table 1).

DISCUSSION

Ketotifen is an orally effective pharmacologic agent that is of prophylactic benefit to patients with bronchial asthma (Tinkelman, Moss, Bukantz, Sheffer, Dobken, Chodosh, Cohen, Rosenthal, Rappaport, Buckley, Chusid, Deutsch, Settipane & Burns, 1985). Because the *in vivo* effects of ketotifen are most apparent after long-term therapy (Craps & Ney, 1984; Tinkelman *et al.*, 1985) the present study was designed to examine β-receptor function in lymphocytes of patients who had been on therapeutic dosages of ketotifen for at least a year.

A significant result of this study is the finding that patients being treated with ketotifen responded to the β-adrenergic agonist isoproterenol with increased accumulations of cAMP relative to non-stimulated levels. When the data were examined without reference to each patient's control level of cAMP, the ketotifen-treated patients as a group did not show higher levels (expressed as pmoles/million cells) than other groups. However, when each

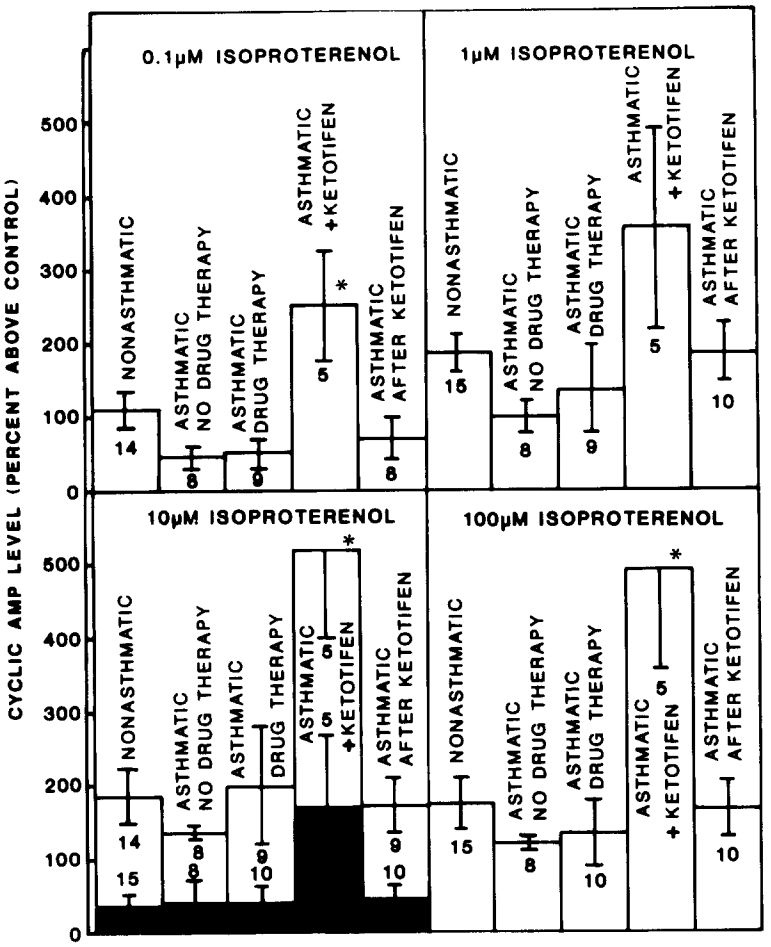

Fig. 2. Isoproterenol-induced rises in cAMP among the different patient groups. The non-stimulated level in each patient's cells served as his or her control level. Data are presented in terms of percentage increases above control levels and indicate means, S.E.s and number of patients. Asterisks indicate statistical significance with respect to all other groups ($P < 0.05$). Shaded areas show the rises produced by 10 μM isoproterenol when 1 μM alprenolol was added as a blocking agent.

Table 1. ^{3}H-DHA binding to human lymphocyte membranes

Subject groups	n	K_D(nM)*	B_{max} (fmol/mg protein)*
1. Non-asthmatic volunteer	24	0.9 ± 0.1	121 ± 15
2. History of asthma without drug therapy	9	0.5 ± 0.1	108 ± 17
3. Asthmatic with standard drug therapy	12	0.7 ± 0.1	68 ± 8‡
4. Asthmatic, standard drug therapy plus ketotifen	13	1.3 ± 0.3†	114 ± 29
5. Asthmatic, standard drug therapy after discontinuation of ketotifen	10	0.5 ± 0.1	112 ± 14

* Mean ± S.E.M.

† Statistically different ($P < 0.03$) from other asthmatic patients (groups 2, 3 and 5).

‡ Statistically different ($P < 0.05$) from groups 1, 2 and 5.

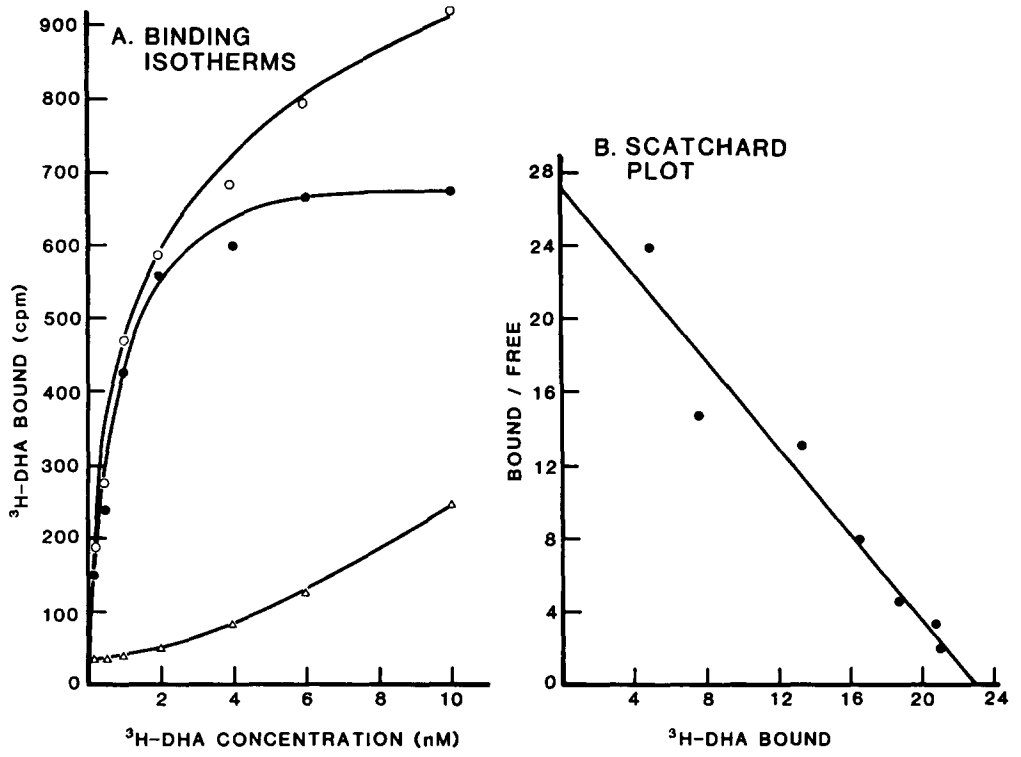

Fig. 3. Representative experiment demonstrating the binding of ^{3}H-DHA to human lymphocyte membranes. Experimental conditions are described under Experimental Procedures. A. Total binding ○; non-specific binding △; specific binding ●. B. Scatchard plot of specific binding.

patient's response to isoproterenol was examined relative to his or her non-stimulated levels, the results showed that ketotifen therapy increased the responsiveness of lymphocytes to beta adrenergic stimulation. Since elevations in cAMP are known to inhibit lymphocyte function (Coffey & Hadden, 1985), it appears likely that the lymphocytes of ketotifen-treated patients are therefore more sensitive to inhibition by circulating catecholamines and β-agonist drugs than patients not receiving ketotifen. Furthermore, assuming that lymphocyte responses are indicative of alterations in other cells, and in particular cells of the respiratory tree, it seems clear that increased β-receptor responsiveness produced by ketotifen would be protective against asthma. This concept is further supported by the findings of Bretz, Martin, Mazzoni, Engel & Reinert (1982) and Bretz, Martin, Mazzoni & Ney (1983), that ketotifen can prevent or reverse tachyphylaxis to the inhibitory effects of isoprenaline on rat passive cutaneous anaphylaxis.

High concentrations of ketotifen can elevate cAMP levels in cells *in vitro*. Work by Castillo, Sanz & Oehling (1986) suggests that the high concentrations are needed for the drug to penetrate and accumulate inside cells during the 1 or 2 days of incubation time ordinarily used in *in vitro* studies. Castillo *et al.* also suggested that the lower concentrations of ketotifen achieved *in vivo* could produce similar effects after weeks of accumulation of the drug within target cells.

Our investigation did not look at whether ketotifen alone raised cAMP levels *in vivo*, since cAMP was measured only after isolated lymphocytes were incubated in the presence of 2.5 mM theophylline with and without isoproterenol. However, it seems possible that the mechanism by which high concentrations of ketotifen elevated cAMP *in vitro* may also contribute to the increased responsiveness of β-receptors that was found in our study.

We also examined the binding of a specific β-receptor antagonist ^{3}H-DHA to lymphocyte membranes to determine whether any changes could be found in the receptors themselves that might explain the altered cAMP responsiveness. Previous reports have shown reduced numbers of receptors in asthmatics (Brooks, McGowan, Bernstein, Alterman

& Peagler, 1979; Kariman & Lefkowitz, 1977; Szentivanyi, 1979). Although some investigators have shown data that suggest the reductions are induced by β-agonist therapy (Davis, Simpson, Paget & Turi, 1986; Reinhardt, Becker, Nagel-Heimke, Schiffer & Zehmisch, 1983; Tashkin, Conolly, Deutsch, Hui, Littner, Scarpace & Abrass, 1982) others have reported reductions in β-receptor numbers in the absence of β-agonists (Motojima, Fukuda & Makino, 1983; Sano, Watt & Townley, 1983). The data reported herein did not reveal a decrease in receptors (indicated by maximal binding) in patients with a history of asthma who did not have active asthma or need for drug therapy. This may be a reflection of the lack of severity of the disease in these patients. Those patients with active asthma and receiving standard drug therapy (β-agonists and methylxanthines) possessed only 56% of the binding capacity of non-asthmatics, which indicates a decrease in receptor numbers. This decrease was apparently reversed by ketotifen since there was no similar decrease in patients who had ketotifen added to their therapy nor was there any after discontinuation of this agent.

Ketotifen therapy was associated with a significant increase in the K_D in our study. Increases in the apparent K_D for β-agonists are well known when GTP or a GTP substitute together with a guanine nucleotide-binding protein (required for activation of adenylate cyclase by agonist – receptor complexes) is added *in vitro* (Stiles, Caron & Lefkowitz, 1984). However, no such phenomenon has been reported for antagonists such as ^{3}H-DHA. Therefore, the meaning of the alteration in K_D associated with ketotifen and how it may relate to the increased cAMP responsiveness of β-receptors to agonist stimulation can only be the subject of speculation at the present time. The altered K_D may be an indication of some change in the receptor itself, a reflection of a change in the membrane environment of the receptor or of components of the adenylate cyclase coupling system which might influence catecholamine binding. Further study will be required to answer this question.

In summary, we have examined β-adrenergic function in the lymphocytes of asthmatic patients undergoing long-term ketotifen therapy. We found that those patients receiving ketotifen had a greater responsiveness to isoproterenol-stimulated elevation of cyclic AMP relative to non-stimulated levels than non-asthmatics or asthmatics not on ketotifen therapy. We also found a significant decrease in the apparent affinity of receptors for the antagonist ligand ^{3}H-DHA in ketotifen-treated patients. An apparent decrease in the number of β-receptors was found in patients with active asthma who were receiving conventional drug therapy. By contrast, the same decrease was not seen in non-symptomatic patients or patients on current or former ketotifen therapy. How the apparent alterations in receptors relate to the increased cAMP response to adrenergic stimulation is not clear and will require further study. However, in view of the role of intracellular cAMP levels in mediating the counterregulatory responses of many cells to allergic reactions, the increased cAMP responsiveness associated with ketotifen therapy offers a plausible, even if still tentative, explanation of ketotifen's action *in vivo*.

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