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Research Article

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Dynamic Regulation of Leukocyte Beta Adrenergic Receptor-Agonist Interactions by Physiological Changes in Circulating Catecholamines

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ABSTRACT β-Adrenergic receptors on human mononuclear leukocytes were assessed using [^{125}I]iodohydroxybenzylpindolol binding. Subjects were studied supine and after being ambulatory, a maneuver that increases plasma catecholamines approximately twofold. β-Receptor affinity for agonists, measured by the competition of [^{125}I]iodohydroxybenzylpindolol binding by (−)isoproterenol was significantly reduced with ambulation and this reduction was associated with a reduction in the proportion of β-receptors binding agonist with a high affinity from a mean (±SEM) of 42±5 to 24±2% ($P < 0.01$). In a parallel series, β-adrenergic-stimulated adenylate cyclase activity was also reduced with postural change from 4.6±1.1 to 2.4±0.6 pmol [^{32}P]cAMP/min per mg protein ($P < 0.05$) after ambulation. Similar reductions in the proportion of receptors binding agonist with a high affinity were seen after infusion of norepinephrine. We conclude that the maneuver of ambulation reduces leukocyte β-receptor responsiveness and affinity for agonists, probably by the effect of increased plasma catecholamines mediating an uncoupling of the β-receptor-adenylate cyclase complex.

INTRODUCTION

β-Adrenergic receptor-mediated function is attenuated by persistent exposure to catecholamines (1–9). This process has been referred to as tachyphylaxis, or desensitization (3), and has been correlated with decreased β-receptor mediated cyclic AMP (cAMP)[1] production (1–9) and, in some circumstances, with β-receptor loss (6–9). This loss in β-receptor density has been demonstrated in vitro (6–9), as well as in man (10–15). In man, leukocytes have been used to monitor β-receptor properties ex vivo, since it has been suggested that changes in leukocyte β-receptors may reflect alterations in β-receptors on less accessible tissues (e.g., heart, lung) (10, 12, 13, 16).

Recently, it has been appreciated that important changes in β-receptor function may occur before the apparent loss of β-receptors from target cells. Harden et al. (17) demonstrated that short-term (15 min) incubation of astrocytoma cells with agonist resulted in a rapid reduction in β-adrenergic-stimulated adenylate cyclase activity that paralleled a reduction in receptor affinity for agonists, but was not correlated with a reduction in receptor density. Krall et al. (18) incubated human leukocytes with 0.01 nM isoproterenol, a concentration of agonist that would occupy only a very small fraction of the total β-receptor population, and found that the ability of isoproterenol to elevate cAMP levels was impaired without any change in receptor density. Similarly, these investigators observed that infusion of isoproterenol into humans promoted a loss in β-adrenergic-mediated cAMP accumulation

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[1] *Abbreviations used in this paper:* B_{max}, beta receptor density; cAMP, cyclic AMP; G, guanine nucleotide regulatory protein; Gpp(NH)p, 5′-guanylylimidodiphosphate; GTP, guanosine 5′-triphosphate; [^{125}I]HYP, [^{125}I]iodohydroxybenzylpindolol; K_H, dissociation constant of the high-affinity state; K_L, dissociation constant of the low-affinity state; R_H, proportion of receptors binding agonist with high affinity; R_L, proportion of receptors binding agonist with a low affinity.

in leukocytes, with no change in leukocyte β-receptor density. These data suggest that loss of β-adrenergic responsiveness without change in β-receptor density might occur in vivo at low levels of β-agonists comparable to the concentrations of circulating catecholamines occurring physiologically.

Loss of responsiveness (desensitization) without reduction in receptor density suggests a functional uncoupling of the β-receptor-adenylate cyclase system. Characterization of the molecular components of this system and how they interact has allowed greater insights into the possible molecular mechanisms involved in receptor-cyclase coupling. The multicomponent β-receptor-adenylate cyclase system is composed of a catalytic subunit responsible for synthesis of cAMP, the receptor that binds agonists and a guanine nucleotide-binding regulatory protein (G) that couples receptor occupancy to the stimulation of catalytic activity (19, 20). Analysis of radioligand binding to the β-receptor has characterized the formation of a high-affinity complex between the β-receptor (R) and β-adrenergic agonists (H) (21). The high-affinity state has been identified as the ternary complex of agonist-receptor-G (H-R-G) (22). Guanine nucleotides, via binding to G, mediate the dissociation of the presumed agonist-β-receptor-G complex and the conversion to the low-affinity state of the receptor for agonists (agonist-receptor complex H-R) (23). Since formation of the high-affinity complex, agonist-receptor-G (H-R-G), appears to be required for agonist activation of adenylate cyclase, alterations in the ability to form high-affinity complexes could account for the observed changes that accompany desensitization. In fact, in vitro studies of desensitization of the β-receptor adenylate cyclase complex in animal model systems have demonstrated a reduction in the number of high-affinity receptor agonist complexes that can be detected subsequent to chronic catecholamine exposure (23).

To test the hypothesis that functional uncoupling of the adenylate cyclase system might occur in man, we investigated the effect of acute elevations in plasma catecholamines on the human leukocyte β-receptor and coupled adenylate cyclase system. To raise endogenous catecholamines within the levels that occur physiologically, we used the maneuver of upright posture, which has previously been shown to evoke an acute rise in plasma epinephrine and norepinephrine levels (24).

METHODS

Samples were drawn by venipuncture from 14 healthy male volunteers aged 26–39 yr, drug free for at least 10 d before the time of study. The subjects had no evidence of abnormality on routine history or physical examination and were normotensive.

Subjects remained in bed overnight in the Vanderbilt University Clinical Research Center. Before 7:00 a.m. and before the subject arose, a 100-ml blood sample was drawn while the subject remained supine in the darkened hospital room. A second 100-ml blood sample was drawn after the subject had been ambulatory for 3 h. Samples from 10 subjects were analyzed for β-receptor binding and plasma catecholamines. On different occasions, with the same protocol, lymphocyte adenylate cyclase activity was determined in eight subjects, four of whom had previously participated in the receptor binding studies. Additionally, four subjects were also studied during infusion of norepinephrine. After an overnight stay at the Clinical Research Center, norepinephrine was infused at 0.05 μg/kg per min for 3 h. Blood samples (50 ml) for β-receptor binding studies and plasma catecholamines was obtained through an indwelling needle in the contralateral antecubital vein at 0, 30, 90, and 180 min.

Lymphocytes were isolated from fresh citrated blood according to the method of Boyum (25). Whole blood was centrifuged at 300 *g* for 14 min. The platelet-rich plasma was aspirated and the buffy coat collected and diluted with phosphate-buffered saline (pH 7.4). A Ficoll/Hypaque (6%/10%) solution was carefully subfused and the samples centrifuged at 400 *g* for 40 min. After careful removal of the plasma/saline layer, the lymphocyte band (consisting of at least 85% small lymphocytes) was harvested by aspiration and a mononuclear broken cell lysate prepared according to a modification of the methods of Aarons et al. (26). The lymphocyte band was resuspended in 30 ml 0.9% NaCl with 12.5 mM $MgCl_2$, 1.5 mM EDTA buffered with 20 mM Tris-HCl (pH 7.4 at 37°C), and centrifuged for 10 min at 40,000 *g* at 4°C. The pellet was resuspended in ice-cold deionized water, homogenized for 5 s (Brinkmann Polytron, setting 5; Brinkmann Instruments, Inc., Westbury, NY), and centrifuged at 40,000 *g* for 10 min at 4°C. The pellet was resuspended in 20 ml of 0.9% NaCl with 20 mM Tris, 12.5 mM $MgCl_2$, and 1.5 mM EDTA and homogenized for 5 s (Brinkman Polytron, setting 5). Samples were frozen in an ethanol/dry ice bath and were rethawed before assay. Once thawed, samples were processed differently for [^{125}I]iodohydroxybenzylpindolol ([^{125}I]HYP) saturation binding than for competition binding studies and adenylate cyclase assays. For adenylate cyclase studies and isoproterenol competition binding studies, samples were thawed within 3 h of freezing. For saturation binding curves, samples were analyzed within 1 wk of processing. Freezing for up to 4 wk altered neither receptor density nor receptor affinity for antagonists. For determination of receptor density and antagonist affinity, samples were used directly after rethawing. To further reduce endogenous guanine nucleotides in competition binding studies, an additional wash was performed, followed by centrifugation at 40,000 *g* for 10 min. The pellet was resuspended in 10 ml of 0.9% NaCl with 20 mM Tris, 12.5 mM $MgCl_2$, and 1.5 mM EDTA and used immediately for binding studies. The additional wash did not alter receptor density or receptor affinity for the antagonist [^{125}I]HYP, as assessed using Scatchard transformation of saturation binding data.

For determination of adenylate cyclase activity, the samples were rethawed and centrifuged at 40,000 *g* for 10 min. The supernatant was discarded and the pellet resuspended in 0.5 ml of 75 mM Tris with 12.5 mM $MgCl_2$ and 7.5 mM dithiothreitol (pH 7.4) and manually homogenized using glass/teflon homogenizer (20 strokes up and down) and used immediately.

β-Receptor binding assay. β-Receptor binding studies were performed according to the methods of Aarons et al.

(26). An aliquot (0.1 ml) of membranes containing 50–150 μg of protein was incubated in a final volume of 250 μl containing 0.5 mM ascorbic acid, 60 μg/ml of bovine serum albumin, 0.03 mM phentolamine mesylate, 12 mM Tris HCl (pH 7.4, 37°C), 0.054% NaCl, 7.5 mM $MgCl_2$, and 0.9 mM EDTA and [^{125}I]HYP (Amersham Corp., Arlington Heights, IL). For competition curves, one concentration of [^{125}I]HYP (45–65 pM) was used in each assay. For saturation binding curves, seven concentrations of [^{125}I]HYP (20–150 pM) were used in each assay. Saturation binding curves were performed with 0.1 mM guanosine 5'-triphosphate (GTP) (12).

Samples were incubated for 60 min at 37°C, at which time binding at all radioligand concentrations used had reached steady state. The reaction was stopped by addition of 10 ml of 0.9% NaCl, 10 mM Tris HCl, 12.5 mM $MgCl_2$ (at 37°C), followed by rapid filtration through Whatman GF/C filters (Whatman, Inc., Clifton, NJ). Each filter was then washed with an additional 10 ml of buffer. Radioactivity retained on the filter was determined in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was defined as that binding not competed for by 0.1 mM isoproterenol. Specific binding was calculated as total minus nonspecific binding. β-Receptor density (B_{max}) and K_d for [^{125}I]HYP was determined from saturation curves of specific [^{125}I]HYP binding analyzed by the method of Scatchard (27).

Protein concentration was determined by the method of Lowry et al. (28), using bovine serum albumin as a standard.

[^{125}I]HYP binding to lymphocyte broken cell preparations was characteristic of binding to a physiologically relevant β-receptor population. Binding was reversible, showed appropriate kinetics, was stereoselective (i.e., (−)isoproterenol was 1,000-fold more potent than (+)isoproterenol), and demonstrated an order of agonist potency appropriate for a β-receptor of the β_2-subtype; i.e., isoproterenol ≫ epinephrine > norepinephrine. Nonspecific binding at the K_d [^{125}I]HYP (40–60 pM) was 35±2%.

Receptor affinity for agonist was derived from the data from isoproterenol competition for [^{125}I]HYP binding at 14 concentrations of isoproterenol from 100 μM to 1 nM. The IC_{50} for isoproterenol and pseudo-Hill coefficients were determined from logit transformations of competition curves. For each experiment, binding was analyzed by a nonlinear curve-fitting procedure, using a generalized model for complex ligand-receptor systems (29). The quality of the fit of the data to one- vs. two-affinity-state models was compared by F test and the fit to the two-affinity-state model was deemed significantly better when $F < 0.05$. For the two-affinity-state model, estimates were provided by the computer of the dissociation constants for the high (K_H) and a low (K_L) affinity states and the proportions of the receptor population (percentage of R_H and R_L, respectively) in each state.

In the postural studies, K_L was obtained experimentally as the K_i for isoproterenol competition for [^{125}I]HYP binding in the presence of 0.1 mM 5'-guanylylimidodiphosphate (Gpp(NH)p). This hydrolysis-resistant analog of GTP has been shown to mediate transition of the entire receptor population to the low-affinity state for agonist (21, 23). Thus the K_i of isoproterenol obtained in the presence of Gpp(NH)p corresponds to the K_d for the low-affinity state (K_L).

Adenylte cyclase activity determination. Adenylate cyclase activity was measured as described previously (30), with minor modifications as detailed below. The conditions of the assay differed from those of the binding studies as follows: membranes (30–70 μg protein) were added to a final incubation volume of 50 μl with 1 μCi of α-[^{32}P]ATP, 0.3 mM ATP, 6 mM $MgCl_2$, 3 mM dithiothreitol, 0.1 mM AMP, 5 mM phosphenolpyruvate, 40 μg/ml of pyruvate kinase, and 20 μg/ml of myokinase. Incubations were carried out at 25°C for 30 min and the reaction terminated by addition of 1 ml of an ice-cold solution containing 100 μg of ATP, 50 μg of cAMP, and ~15,000 cpm of [^{3}H]cAMP. cAMP was isolated by sequential Dowex and alumina chromatography as described by Salomon et al. (31), and corrected for recovery using [^{3}H]cAMP as the internal standard. Adenylate cyclase activity was linear with time over the 30-min incubation interval.

Catecholamine assay. Plasma epinephrine and norepinephrine were determined by radioenzymatic assay (32).

Statistical analysis. The statistical significance of differences was determined using analysis of variance and Student's t test for paired data.

RESULTS

The binding of [^{125}I]HYP in the presence of the β-receptor agonist isoproterenol resulted in shallow competition binding curves (pseudo-Hill coefficients, −0.65±0.03; range, −0.52 to −0.78), consistent with the model of receptor binding agonist with more than one affinity (Fig. 1). Computer curve fitting of isoproterenol competition binding data was improved in all subjects by assuming a two-affinity-state model for agonist receptor interactions and the improvement from the one-affinity state was significant at the $P < 0.05$ level in 8 out of 10 subjects. With the addition of 0.1 mM Gpp(NH)p, the agonist competition curve

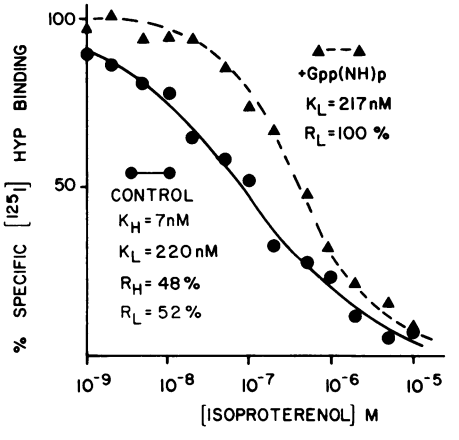

FIGURE 1 Alteration in isoproterenol competition curves with guanine nucleotides. The competition curve with Gpp(NH)p (– – –) is shifted to the right, is steeper, and is adequately described by a one-affinity-state model, whereas the competition curve in the absence of Gpp(NH)p (——) is better described by a two-affinity-state model ($P < 0.05$). K_H, K_L, dissociation constants of high- and low-affinity states, respectively. R_H, proportion of receptors in high-affinity state. R_L, proportion of receptor in low-affinity state. Nonspecific binding is defined as [^{125}I]HYP binding in the presence of 0.1 mM isoproterenol. Each point is the mean of triplicate determinations. The figure is representative of 10 replicate studies.

was shifted rightward and steepened (pseudo-Hill coefficient, −0.89±0.05; range, −0.80 to −1.08) (Fig. 1) and was consistent with receptor binding agonist according to the laws of simple mass action; i.e., a model with one homogeneous receptor population binding agonist with a single affinity. The rightward shift and increase in IC_{50} in the presence of Gpp(NH)p (from 199±9 to 532±17 nM, $P < 0.01$) indicates that guanine nucleotides induce a transition to the lower affinity state of the receptor. This effect of Gpp(NH)p was guanine nucleotide specific and was not observed with the addition of 0.1 mM App(NH)p, a hydrolysis-resistant analog of ATP ($n = 3$).

In contrast to the properties of the β-receptor-agonist interactions, competition for [^{125}I]HYP binding by the β-adrenergic antagonist propranolol demonstrated competition profiles of normal steepness (pseudo-Hill coefficient not significantly different from 1). Furthermore, the addition of 0.1 mM Gpp(NH)p did not alter the competition curves for propranolol ($n = 3$). Thus, these data are consistent with the interpretation that the leukocyte β-receptor binds antagonist with one homogeneous affinity unaltered by guanine nucleotides.

In normal subjects, the assumption of upright posture resulted in an acute elevation of plasma norepinephrine and plasma epinephrine (Fig. 2). Associated with the change in plasma catecholamines was an increase in the IC_{50} for isoproterenol competition for [^{125}I]HYP binding to leukocyte membranes from 199±29 to 313±43 nM, $P < 0.05$ (Figs. 2 and 3). This decrease in β-receptor affinity for agonist (increase in IC_{50}) was also associated with ~50% reduction in the proportion of receptors binding agonist with a high affinity ($\%R_H$) upon the assumption of upright posture (Table I). Furthermore, $\%R_H$ was inversely correlated with the log plasma norepinephrine concentration ($R = -0.72$, $P < 0.001$), and with log plasma epinephrine ($R = -0.50$, $P < 0.025$). Despite the change in the proportion of β-receptors possessing a high affinity for agonist (R_H), the estimated K_d values for both the high- and low-affinity states were unchanged with upright posture (Table I).

These significant changes in receptor-agonist interactions occurring with postural change were not paralleled by alterations in receptor-antagonist interactions. Thus the K_d and B_{max} for [^{125}I]HYP binding were unchanged between samples from subjects taken when supine and upright (K_d 46±7 supine, 39±5 pM upright; B_{max} 15±2 supine, 13±1 fmol/mg protein upright).

To monitor whether the washing techniques used to generate our final membrane preparation adequately removed catecholamines present in the plasma, we added norepinephrine to a final concentration of 1 μM to samples (whole blood) and prepared control and norepinephrine-containing samples according to our typical protocol. Isoproterenol competition curves, from control and norepinephrine-added samples were indistinguishable (IC_{50} control, 202±60 nM; IC_{50} norepinephrine added, 146±39 nM), indicating that our membrane washing procedure was adequate for removal of residual catecholamines.

To assess the effect of alterations in receptor-agonist binding on β-receptor responsiveness, we measured adenylate cyclase activity as another parameter of β-receptor-mediated effects. There was a significant reduction in isoproterenol-stimulated adenylate cyclase activity seen with upright posture (2.4±0.6 pmol [^{32}P]cAMP/min per mg protein compared with supine; 4.6±1.1 pmol [^{32}P]cAMP/min per mg protein, $P < 0.05$). However, no alteration in basal activity was detected (supine, 12±2 pmol [^{32}P]cAMP/min per mg

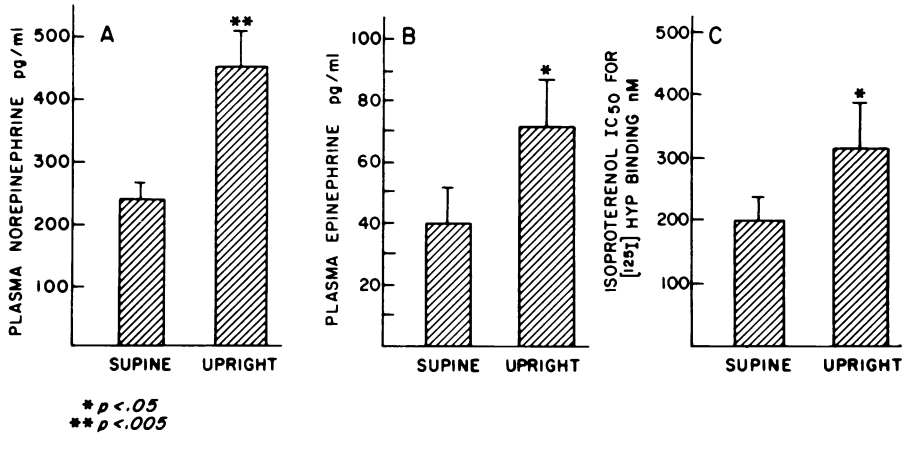

FIGURE 2 Effect of upright posture on plasma norepinephrine (*A*), plasma epinephrine (*B*), and receptor affinity for agonist (*C*). Values shown are mean±SEM from 10 subjects.

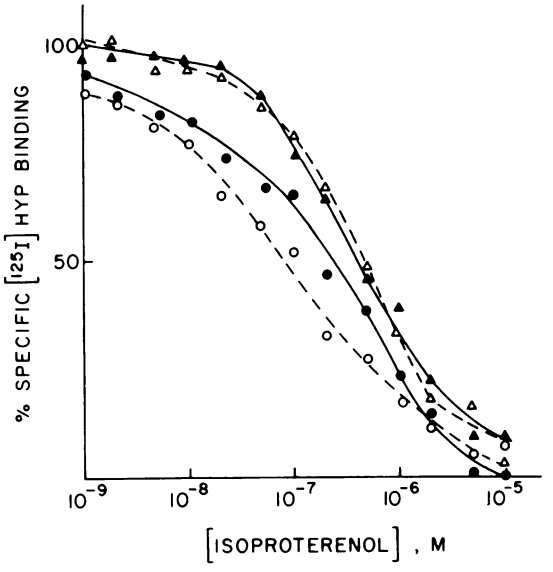

FIGURE 3 Effect of posture on receptor affinity for agonist. Isoproterenol competition curve after 3 h of upright posture (●) is shifted to the right compared with supine sample (○). Addition of Gpp(NH)p to competition curves from both supine (△) and upright (▲) samples resulted in identical curves and indicates that no alteration in K_L has occurred. The figure is representative of 10 replicate studies.

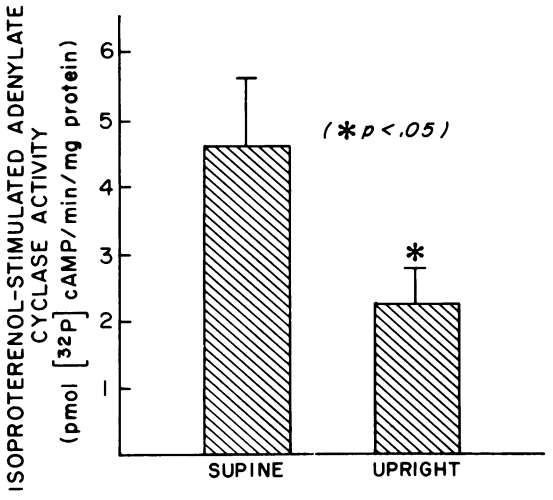

FIGURE 4 Effect of upright posture on β-adrenergic-stimulated adenylate cyclase activity. Values represent the difference between basal activity and 0.1 mM isoproterenol-stimulated activity. Values shown are mean±SEM from eight replicate studies.

protein; upright, 13±43 pmol [^{32}P]cAMP/min per mg protein) (Fig. 4).

To directly assess the effect of elevations in plasma catecholamines on β-receptor affinity for agonist without concomitant postural change, we studied lymphocyte β-receptors during infusion of the adrenergic agonist norepinephrine. After infusion of norepinephrine, there was a decrease in $\%R_H$ from 63±7% before norepinephrine infusion to 32±5% ($P < 0.02$) after 30 min of infusion, 36±5% at 90 min and 41±3% ($P < 0.05$) after 180 min of infusion.

DISCUSSION

Using the physiological stimulus of upright posture as a maneuver to raise catecholamines, we detected a significant reduction in β-receptor affinity for isoproterenol, which is correlated with a reduction in the proportion of receptors binding this agonist with a high affinity. In parallel studies, a significant reduction in β-adrenergic-stimulated adenylate cyclase activity was also observed. This reduction in β-adrenergic responsiveness, without an associated change in B_{max} suggests an uncoupling of the β-receptor complex. Our data would suggest this uncoupling is linked to an impairment in the formation of the high-affinity complex H-R-G, which, as previously noted, is a prerequisite for β-receptor-stimulated adenylate cyclase activation. Our findings are consistent with previous in vitro studies, which have demonstrated that decreases in receptor affinity for agonist parallel alterations in receptor-mediated adenylate cyclase activity (33, 34).

The R_H was significantly reduced with upright posture and the associated acute elevation in catecholamines, and was inversely correlated with plasma catecholamines, which suggests that elevations in catecholamines may mediate the reduction in R_H.

We feel confident that we have excluded the possibility of artifactual increases in IC_{50} for isoproterenol caused by the presence of persistent endogenous catecholamines in the membrane preparation. The presence of sufficient persistent catecholamines to alter the IC_{50} for agonists in competition studies would also alter the apparent K_d obtained for [^{125}I]HYP in saturation binding studies. K_d values for [^{125}I]HYP were not different in supine and upright samples. Furthermore, 1 μM norepinephrine added exogenously to samples

TABLE I
Alterations in β-Receptor-Agonist Interactions with Upright Posture

	Supine	Upright	P
$\%\ R_H$	42±5	24±2	<0.01
K_H (nM)	15±4	10±3	NS
K_L (nM)	257±25	261±24	NS

did not increase the IC_{50} for isoproterenol in subsequently prepared membranes, which implies that the membrane preparation techniques was adequate to remove catecholamines in solution. Alternatively, residual catecholamines binding persistently to β-receptors would result in an apparent reduction in β-receptor density. However, there was no change in B_{max} determined in leukocytes from supine and upright samples. Thus, there is no evidence that residual catecholamines interfered with our interpretation of the radioligand binding data. Preliminary studies (35) have reported a reduction in receptor density after upright posture using the radioligand [^{3}H]dihydroalprenolol. However, in subsequent studies with both [^{125}I]HYP (36) and [^{3}H]dihydroalprenolol,[2] we have not found any alterations in receptor density with postural change.

Our data thus suggest that in vivo acute increases in catecholamines over a small, physiological range result in detectable changes in agonist-receptor interactions. Although other hormones (e.g., renin, angiotensin) may change with catecholamines upon assumption of upright posture, the reduction in receptor affinity for agonist that we found during the infusion of norepinephrine suggests that alterations in catecholamines are either directly or indirectly related to the receptor changes. Methodologically, our study would suggest that consideration of posture (and of other factors that alter plasma catecholamines) may be important in the interpretation of studies of β-receptor responsiveness in man.

The present studies suggest that in response to acute changes in catecholamines leukocyte β-receptor function is primarily regulated by alterations in coupling between the receptor and the catalytic component. We have previously demonstrated (13) and recently confirmed using different techniques (36) that chronic physiological elevations in catecholamines are associated with down-regulation of lymphocyte β-receptor density. This mechanism must follow a longer time course and probably represents a more chronic adaptation to alterations in sympathetic activity.

Attenuation of β-receptor function in response to small increases in circulating catecholamines would act to dampen the effect of sudden changes in sympathetic activity. Alterations in receptor affinity for agonist with acute, physiological fluctuations in hormone levels might represent one mechanism of feedback control in the sympatho-adrenal system.

[2] G. A. FitzGerald, R. Feldman, and A. J. J. Wood. Unpublished observations.

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