G-Protein Function Is Reduced in Hypertension

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Abstract A functional impairment in vasodilator tone may be important in the pathogenesis and/or maintenance of elevated peripheral vascular resistance in hypertension. Previous studies of hypertensive subjects have demonstrated impaired β-adrenergic-activated vasodilation paralleling a reduction in lymphocyte β-adrenergic-stimulated adenyl cyclase activity. We have suggested that this impairment is related to a defect in G-protein function. To determine whether this defect alters the coupling between the G-protein complex and adenyl cyclase, we performed [3H]forskolin binding studies in lymphocytes from hypertensive subjects, older normotensive subjects, and younger normotensive control subjects. Maximal specific [3H]forskolin binding was used as an index of adenyl cyclase binding sites Gpp(NH)p- and NaF/AlCl3-stimulated [3H]forskolin binding was significantly inversely correlated with blood pressure. No differences in NaF/AlCl3-stimulated [3H]forskolin binding were detected between groups. The studies indicate that G-protein/adenyl cyclase coupling is impaired in lymphocytes from younger hypertensive subjects and may contribute to the blood pressure-related defect in β-adrenergic-stimulated adenyl cyclase activity.
dependent of the determination of cAMP levels. It utilizes the affinity of the diterpene forskolin to bind selectively to adenyl cyclase. Notably, the effects of forskolin on adenyl cyclase are modulated by G-protein interactions. The extent of forskolin-stimulated activation of adenyl cyclase is enhanced by an interaction with Gs. Furthermore, forskolin binding to adenyl cyclase is enhanced by stabilization of the interaction between the Gs, and adenyl cyclase. With this technique, G-protein function has been assessed by the extent to which G-protein- and/or receptor-specific ligands enhance specific $[^3H]$forskolin binding. This approach has been used successfully to assess the regulation of G-protein function.

Therefore, to assess alterations in G-protein function in hypertension independent of the functional assessment of cAMP concentrations, we have determined the extent of G-protein-mediated enhancement of $[^3H]$forskolin binding. The data to be presented demonstrate that G-protein–enhanced $[^3H]$forskolin binding is reduced in lymphocytes from hypertensive but not older normotensive subjects. Further, G-protein–enhanced labeling is significantly inversely correlated with blood pressure independent of age, consistent with a blood pressure–related reduction in human lymphocyte G-protein function.

Subject Protocol
Younger and older normotensive subjects and younger borderline hypertensive subjects were studied. Younger normotensive subjects were between the ages of 20 to 36 years, healthy, and not taking any medications on a regular basis or any medications for at least 1 month before study. Older normotensive subjects were between the ages of 45 and 63 years, healthy, and not taking any medications on a regular basis or any medications for at least 1 month before study. Hypertensive subjects were all white, between the ages of 22 and 36 years, otherwise healthy, and had neither renal nor cardiovascular complications. Hypertensive subjects had not taken any antihypertensive medications (or any other mechanisms) for at least 1 month before study before the protocol was approved by the Human Subjects Review Committee, University of Western Ontario. Informed consent was obtained from each subject before study.

Assessment of $[^3H]$Forskolin Binding in Human Lymphocytes
$[^3H]$Forskolin binding was assessed in mononuclear leukocyte preparations. Mononuclear leukocytes were separated from whole blood by the method of Boyum and as described previously. Mononuclear cells were harvested via separation on Ficoll-Hypaque gradients and resuspended in Hank's balanced salt solution (HBSS, pH 7.4 at 4°C) with 30 mmol/L HEPES, 0.5 mmol/L EDTA, and 1 mmol/L magnesium sulfate (buffer A), followed by centrifugation for 10 minutes at 400g and resuspension in buffer A with digitonin (10 μg/mL) for 15 minutes at 4°C Cells were washed twice at 400g for 10 minutes at 4°C in buffer A without digitonin and resuspended in HBSS (pH 7.4 at 4°C) with 33 mmol/L HEPES, 1.25 mmol/L EDTA, and 5 mmol/L magnesium sulfate. The cells were incubated at a concentration of 8×10^6 cells/mL for 3 hours at 4°C. Initial studies showed $[^3H]$forskolin binding reached a steady state before 3 hours at 4°C at the concentrations of radioligand used. Incubations were terminated by vacuum filtration with rapid washing (less than 5 seconds) with a 1/10 dilution of buffer A at 4°C. Radioactivity on filters was measured by liquid scintillation spectroscopy (Beckman LS 6000). Nonspecific binding was determined as the extent of $[^3H]$forskolin binding in the presence of excess unlabeled forskolin (10 μmol/L) at the $[^3H]$forskolin concentrations used, nonspecific binding accounted for less than 25% of maximal G-protein–enhanced binding (σe, in the presence of NaF/AlCl3, see below). Binding site density and $K_d$ for specific $[^3H]$forskolin binding was determined by computerized nonlinear curve fitting (Inplot4. GraphPad Software).

G-protein–stimulated forskolin binding was assessed by the concomitant with either the hydrolysates-resistant GTP analogue guanylylimidodiphosphate (Gpp[NH]p, 100 μmol/L), isoproterenol (100 μmol/L with Gpp[NH]p), or NaF/AlCl3 (20 mmol/L and 20 μmol/L, respectively) and $[^3H]$forskolin (50 μmol/L, a concentration approximating the $K_d$ for basal, Gpp[NH]p-, and isoproterenol-stimulated $[^3H]$forskolin binding and a saturating concentration for NaF/AlCl3-stimulated binding; see below) The enhancement of $[^3H]$forskolin binding by the stimulants was expressed as a percentage of basal specific binding (σe). $[^3H]$forskolin binding in the absence of stimulants subtracted from nonspecific binding. This approach was chosen prospectively based on two major considerations. First, although absolute levels of adenyl cyclase activity may differ between lymphocyte subpopulations, relative stimulation is comparable. Second, in preliminary studies we determined that the coefficient of variation is lower when stimulated $[^3H]$forskolin binding is expressed relative to basal specific binding.

Data Analysis
Because measures of potency and relative proportions are normally distributed when logarithmically transformed, these parameters are expressed as a geometric mean ± average SEM. Differences in binding were assessed by ANOVA followed by group comparisons as appropriate. A value of $P<0.05$ on a two-tailed test was considered significant.

Results
Assessment of Specific $[^3H]$Forskolin Binding
Forskolin mediated a concentration-dependent and saturable increase in binding. In initial saturation binding studies ($n=3$), a $B_{max}$ of 5.0±1.4 fmol/million cells (3012

![Graph](image-url)
Alterations in \([H]Forskolin Binding in Older and Borderline Hypertensive Subjects

No significant alterations in overnight sodium excretion were detected between older and hypertensive subjects versus younger normotensive control subjects. No significant difference in blood pressure was apparent between older and younger control subjects. Normotensive and hypertensive subjects were comparably aged. Body mass did not differ among the three groups (Table).

Basal specific \([H]Forskolin binding (ie, binding in the absence of G-protein and/or \(\beta\)-adrenergic agonist) was not significantly altered in older and hypertensive subjects compared with younger normotensive controls (younger normotensives, 4.5±0.3 fmol/million cells [2710 sites/cell]; older normotensives, 5.2±0.3 fmol/million cells [3132 sites/cell]; younger hypertensives, 4.9±0.5 fmol/10^6 cells [2951 sites/cell]). Isoproterenol- and Gpp(NH)p-enhanced \([H]Forskolin binding were significantly reduced in lymphocytes from hypertensive subjects (Fig 2). In contrast, no alterations in isoproterenol- or Gpp(NH)p-stimulated \([H]Forskolin binding were apparent in lymphocytes from older subjects (Fig 2). Further, no alterations in NaF/AlCl₃-mediated enhancement of \([H]Forskolin binding were evident between hypertensive subjects and either older or younger normotensive groups (Fig 2). Overall, isoproterenol- and Gpp(NH)p-mediated enhancement of \([H]Forskolin binding were highly correlated (r=0.76, P<0.001, Fig 3). However, neither were correlated with basal \([H]Forskolin

Alterations in \([H]Forskolin Binding in Hypertensive and Older Subjects

The ability of isoproterenol, Gpp(NH)p, and NaF/AlCl₃ to increase \([H]Forskolin binding in lymphocytes from younger normotensive subjects is compared with those from older normotensive and younger hypertensive subjects. Data represent the mean±SE. *P<.05 vs younger normotensive subjects.
Binding (data not shown). Both isoproterenol-stimulated and Gpp(NH)p-stimulated [3H]forskolin binding were significantly inversely correlated with blood pressure (isoproterenol-stimulated [3H]forskolin binding versus 24-hour mean arterial pressure: \( r = -0.47, P = 0.02 \); Gpp(NH)p-stimulated [3H]forskolin binding versus 24-hour mean arterial pressure: \( r = -0.47, P = 0.04 \), Fig 4). In contrast, there was no correlation between age and either isoproterenol- or Gpp(NH)p-stimulated [3H]forskolin binding (data not shown).

Discussion

Alterations in receptor-stimulated and G-protein-stimulated adenylyl cyclase activity have previously been demonstrated in lymphocytes from hypertensive subjects (as discussed above). Furthermore, alterations in G-protein function have been suggested based on reduced cholera toxin-mediated substrate labeling of the \( \alpha \)-subunit of the stimulatory G-protein. The present studies demonstrate that these alterations in lymphocyte G-protein properties parallel a reduction in G-protein-stimulated [3H]forskolin binding. In contrast, no alterations in G-protein-stimulated [3H]forskolin binding were apparent in lymphocytes from older normotensive subjects.

Forskolin has been previously shown to interact with adenylyl cyclase with a high affinity, and the use of this reagent was instrumental in the original purification of the enzyme. It is notable that forskolin has cAMP-independent effects, including inhibition of glucose transport, of nicotinic receptor function, and of voltage-dependent potassium channels. However, these non-adenylyl cyclase-mediated effects of forskolin are common to 1,9 dihydroforskolin, a naturally occurring analogue of forskolin that neither inhibits high-affinity binding of [3H]forskolin nor activates adenylyl cyclase. The high affinity of [3H]forskolin binding demonstrated in these studies, and lack of effect of 1,9 dihydroforskolin in inhibiting [3H]forskolin binding, is consistent with forskolin binding to adenylyl cyclase. In the lymphocyte, consistent with other models, both receptor and G-protein specific stimulators significantly enhance [3H]forskolin binding in respect to maximal binding as well as to binding affinity. The estimate of basal binding site concentration of 3012 sites/cell is quantitatively similar to \( \beta \)-adrenergic receptor density (of approximately 10^5 sites/cell-12).

The reduction in Gpp(NH)p- and isoproterenol-stimulated [3H]forskolin binding in lymphocytes from hypertensive subjects is consistent with an impairment in G-protein-mediated function. Several lines of evidence would suggest that the locus of this defect is at the level of the G-protein. First, specific basal [3H]forskolin binding was not altered between groups. Second, isoproterenol-mediated enhancement of [3H]forskolin binding was highly correlated with Gpp(NH)p-stimulated binding and comparably reduced in hypertensive subjects, suggesting a common mechanism of regulation. Last, in vitro studies demonstrated that pretreatment of lymphocytes with isoproterenol did not alter isoproterenol-stimulated [3H]forskolin binding under conditions that impair \( \beta \)-adrenergic-mediated adenylyl cyclase activity. This would suggest that isoproterenol-stimulated [3H]forskolin binding is insensitive to the changes in receptor/G-protein coupling that occur with acute agonist exposure. These results parallel the finding in S49 lymphoma cells where agonist pretreatment of intact cells did not alter G-protein-stimulated [3H]forskolin binding assessed in permeabilized cells under conditions mediating homologous desensitization of the \( \beta \)-adrenergic receptor pathway.

NaF/AlCl3-mediated enhancement of [3H]forskolin binding was not altered in lymphocytes from hypertensive subjects. This is particularly notable because our recent studies have reported impaired NaF-stimulated adenylyl cyclase activity in lymphocytes from a similar group of hypertensive subjects. The explanation linking these findings is unclear. However, a previous report suggested that NaF-stimulated enhancement of [3H]forskolin binding is not a sensitive index of heterologous regulation of G-protein-stimulated adenylyl cyclase activation. In that study, Gpp(NH)p-stimulated [3H]forskolin binding was a more sensitive index, perhaps related to the lesser potency of Gpp(NH)p in enhancing the [3H]forskolin binding. Our finding that NaF/AlCl3-enhanced binding was unaltered in hypertensive subjects (assessed at a [3H]forskolin concentration associated with its "Bmax" effect), although Gpp(NH)p- and isoproterenol-enhanced binding were reduced (assessed at a [3H]forskolin concentration approximating their \( K_s \)) is consistent with that result. Overall, these data would suggest that G-protein regulation as assessed by [3H]forskolin parallels...
a shift in the $K_d$ but not the $B_{\text{max}}$ for binding. Additionally, NaF is a tyrosine phosphatase inhibitor. Recent studies have suggested that tyrosine kinases may have additional G-protein–independent effects on adenylyl cyclase.

As discussed above, impaired G-protein–stimulated adenylyl cyclase activity in lymphocytes from hypertensive subjects has been previously reported. Additionally, we have recently demonstrated a decrease in cholera toxin–mediated ADP ribosylation of Go, in lymphocytes from a comparable group of younger hypertensive subjects studied under identical conditions.

In those studies, there were no alterations in Go, expression as assessed by immunodetection. Together with the present studies, a consistent impairment in G-protein function in lymphocytes from younger white hypertensive subjects has now been demonstrated using three distinct approaches.

Finally, these studies provide further evidence that alterations in lymphocyte β-adrenergic responses in hypertension are distinct from the pattern seen with aging. As discussed above, an impairment in lymphocyte β-adrenergic responsiveness has variably been seen with aging. However, these studies have been generally confounded by the age-dependent increase in blood pressure. In contrast, in the present study, blood pressures in older and younger normotensive groups were virtually identical. Our finding that overall G-protein–mediated enhancement of $[H]_{\text{Forskolin}}$ binding was inversely correlated with mean arterial pressure over all age groups studied is consistent with the hypothesis that age-related alterations in this transmembrane signaling process may be confounded with the age-related increase in blood pressure.

In summary, these studies have demonstrated the utility of $[H]_{\text{Forskolin}}$ binding in lymphocytes as a measure of G-protein function. Furthermore, they demonstrate an impairment in G-protein–mediated function specific to lymphocytes from hypertensive subjects. Overall, the present data, and those from previous studies, have suggested that in these younger white hypertensive subjects, an impairment in G-protein function may underlie the impairment in transmembrane signaling characteristic of the defect in the β-adrenergic receptor/G-protein/adenylyl cyclase complex in the hypertensive state.

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References

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