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EFFECTS OF ALPHA-1-ACID GLYCOPROTEIN ADMINISTRATION ON PROPRANOLOL BINDING AND BETA BLOCKADE IN RATS*

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Abstract—Alpha-1-acid glycoprotein (AAG), 750 mg/kg, was administered to rats to determine its effect on propranolol binding and beta blockade. Anesthetized rats received [^{3}H]propranolol i.v., followed in 15 min by human AAG or bovine serum albumin, 750 mg/kg. AAG treatment produced a human AAG concentration in serum of 7.76 ± 1.17 mg/ml, several times higher than the endogenous serum AAG concentration in stressed rats. AAG treatment significantly increased the heart rate response to isoproterenol, compared to albumin (95.4 ± 19.6 vs 28.3 ± 16.7% of baseline value, measured 45 min after propranolol, $P < 0.001$). AAG-treated rats had greater [^{3}H]propranolol binding in serum (93.0 ± 3.2 vs 76.7 ± 3.0%, $P < 0.01$) and a lower calculated unbound [^{3}H]propranolol concentration in serum (2.7 ± 1.3 vs 7.4 ± 3.1 × 10^6 dpm/ml, $P < 0.001$) than albumin-treated rats. These data demonstrate that AAG can alter propranolol pharmacokinetics and pharmacodynamics even when administered after the propranolol effect is evident. Because the reported affinity of propranolol for cardiac beta receptors is 10,000 times greater than its affinity for AAG, these data suggest that AAG acted by altering propranolol disposition rather than by directly competing with beta receptors for drug.

The ability of drug-binding proteins to alter drug action or toxicity has been demonstrated by the therapeutic use of drug-specific antibodies. Digoxin-specific antibody Fab fragments administered to patients with digoxin toxicity rapidly decrease the unbound digoxin concentration and reverse clinical toxicity [1]. Antibodies are an attractive means of increasing drug binding because of their ability to bind drug with a very high affinity (K_a = 10^8 to 10^{10} M^{-1} for digoxin-specific Fab) [2, 3]. It has been postulated that the efficacy of digoxin-specific antibodies resides in their ability to bind digoxin more avidly than the cardiac digoxin receptor [4], but this hypothesis has not been specifically tested. High-affinity antibodies are, in some cases, difficult to obtain and may be expensive to produce in large quantity [5]. Whether lower affinity antibodies or other drug-binding proteins with more modest affinities can alter drug pharmacokinetics and pharmacodynamics is therefore of interest.

Alpha-1-acid glycoprotein (AAG) binds a variety of basic, cationic drugs in serum, with K_a values in the range of 10^4 to 10^6 M^{-1}. Studies of drugs that bind to AAG suggest that changes in serum AAG concentration can alter substantially drug disposition and action. For example, Huang and Oie [6] pretreated rabbits with AAG and found the electrocardiographic effect of disopyramide to be reduced. AAG administration increased disopyramide binding in serum, but did not alter the concentration–response curve for unbound drug. Similar results were obtained when AAG was administered during *S*-disopyramide infusion, rather than as a pretreatment [7].

These data regarding disopyramide are generally supported by studies of propranolol in disease states such as inflammation, surgery or myocardial infarction that alter the serum AAG concentration [8–10]. Studies of propranolol are of particular interest because the binding of propranolol to cardiac beta receptors has been characterized and is of high affinity, with a K_a (7×10^{10} M^{-1}) that is 10,000 times greater than its affinity for AAG (2.4×10^5 to 1.6×10^6 M^{-1}) [10–12]. Belpaire *et al.* [8] pretreated rats with i.m. turpentine to produce inflammation, and administered propranolol as an i.v. bolus 48 hr later. In the animals with inflammation, the unbound fraction of propranolol in serum was reduced to 20% that of controls, and beta blockade was reduced by 50%. Yasuhara *et al.* [10] obtained similar results in rats pretreated with laparotomy. In laparotomized animals, the serum AAG and total propranolol concentrations increased, whereas beta blockade was reduced compared to controls. These data suggest that increases in the serum AAG concentration can increase propranolol binding in serum, reduce the unbound propranolol concentration, and decrease propranolol effect. A causal relationship is not established, however, because both surgery and inflammation produce effects in addition to increasing the serum AAG concentration.

Each of the studies of propranolol discussed above used pretreatment protocols, so that the AAG concentration was increased before drug administration. AAG could have exerted its effect by limiting initial propranolol distribution to receptors. The possibility

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of therapeutic efficacy would be more directly addressed by administering AAG after the onset of drug effect. In the present study, AAG was administered to rats after propranolol to assess the effect of AAG on propranolol-induced beta blockade.

METHODS

Animal preparation. Small male Holtzman rats (120–140 g) were used because of the high cost of AAG. Rats were anesthetized with pentobarbital (Abbott Laboratories, Chicago, IL), 30 mg/kg i.m., initially followed by 20 mg/kg every 45 min. Polyethylene (PE50) cannulas were placed in the right femoral vein for drug administration and the left femoral vein for blood sampling. Body temperature was maintained using a heating lamp. Heart rate was monitored using subcutaneous needle electrodes and a polygraph. Blood pressure was not measured because preliminary experiments showed that the administered dose of propranolol produced only a small decrease in systolic blood pressure (< 20 mm Hg).

Drugs. Pentobarbital, 30 mg/ml, was prepared in distilled water. *d*,l-Propranolol (Sigma Chemical Co., St. Louis, MO), 0.50 mg/ml, was prepared daily in 0.9% saline. Human AAG (Sigma), 250 mg/ml, was prepared by gentle agitation at 4° in 0.45% saline. This preparation is homogeneous as assessed by sodium dodecyl sulfate (SDS)polyacrylamide gel electrophoresis and immunoelectrophoresis [13]. Bovine serum albumin (Sigma) was prepared in a similar manner. [^{3}H]Propranolol was obtained from the Amersham Corp. (Arlington Heights, IL), sp. act. 20 Ci/mmol, with a purity of >97% as assessed by high pressure liquid chromatography (see below).

Protein binding. Propranolol binding in serum was measured by equilibrium dialysis using 1 ml semi-micro teflon cells (Spectrum Medical Industries Inc., Los Angeles, CA). Spectrapor 4 membranes with a molecular weight cutoff of 12,000–14,000 daltons were prepared by soaking in distilled water for 15 min, then in 30% ethanol for 15 min, and rinsing with water. Membranes were placed in Sorenson's buffer (0.13 M phosphate, pH 7.4) and used within 1 week. Dialysis cells were filled on one side with buffer and on the other side with serum adjusted to pH 7.45. An additional 7.4×10^6 dpm (55 ng) of [^{3}H]propranolol was added to each serum sample prior to dialysis. Cells were placed in a circulating water bath at 37° and rotated at 10 rpm. Preliminary studies demonstrated that equilibrium was reached at 2 hr, and all subsequent dialyses were peformed for 4 hr. Volume shifts were negligible. Data were collected only if serum pH at the end of dialysis was between 7.35 and 7.45. Reported values for binding are the mean of two serum samples. Protein binding studies were performed on only six of eight animals because insufficient serum was available from two animals to run duplicate assays.

Assays. Serum AAG concentration was measured by a radial immunodiffusion assay (Behring Diagnostics, La Jolla, CA) that does not cross-react with rat serum [13]. [^{3}H]Propranolol was measured by placing 0.1 ml of serum or dialysate directly into scintillation fluid. The purity of [^{3}H]propranolol was measured using a high pressure liquid chromatographic assay initially described for quantitation of desipramine [14] modified as follows: mobile phase acetonitrile:methanol:methoxyethylamine 94.6:5:0.4, fluorescence excitation 220 nm, emission cutoff 370 nm.

Table 1. Protocol for drug administration

0	Isoproterenol*
15 min	Isoproterenol
30 min	Propranolol, 0.5 mg/kg i.v., over 2 min
40 min	Blood sample
45 min	AAG or albumin, 750 mg/kg i.v., over 15 min
60 min	Isoproterenol
75 min	Isoproterenol
90 min	Isoproterenol
105 min	Blood sample

* Isoproterenol, 0.12 μg/kg, was administered i.v. over 6 min.

Protocol (Table 1). Propranolol, 0.5 mg/kg, was administered to anesthetized rats i.v. over 2 min. [^{3}H]Propranolol, 2×10^6 dpm, was added to the unlabeled propranolol because preliminary experiments showed the propranolol concentrations in some serum samples to be below the sensitivity of the HPLC assay. The response to isoproterenol was then tested repeatedly by administering isoproterenol, 0.12 μg/kg, over 6 min and measuring heart rate at the end of isoproterenol infusion. An interval of 15 min was chosen for repeated testing to allow heart rate to return to the pre-isoproterenol value.

Treatments (albumin or AAG) were administered starting 15 min after propranolol because preliminary experiments showed that beta blockade was maximal by that time. Groups of eight rats received treatments of AAG or albumin, 750 mg/kg i.v., over 15 min. This AAG dose was chosen to produce a serum concentration of approximately 5–10 mg/ml [13]. Blood, 0.3 ml, was obtained at 40 min, and 1–3 ml was obtained at the end of each experiment.

Analysis of data. The baseline unstimulated heart rate and the baseline response to isoproterenol (percent increase in heart rate) were calculated as the mean of two measurements (0 and 15 min). For statistical comparisons, data were normalized by expressing them as a percent of the baseline value for each animal. The percent of the baseline value for the unstimulated heart rate and the percent increase in heart rate after isoproterenol stimulation were then compared between AAG and the albumin treatments using ANOVA with repeated measures. When this test indicated group differences, individual comparisons of values at 60, 75 and 90 min were made using the two-tailed, unpaired Student's *t*-test and the Bonferroni correction. The unbound radiolabel concentration was calculated as the product of the serum radiolabel concentration and the fraction of unbound [^{3}H]propranolol determined by equilibrium dialysis. Comparisons of the change in serum radiolabel concentration, the unbound radiolabel concentration and the percent unbound [^{3}H]propranolol were made using the two-tailed, unpaired Student's *t*-test.

Table 2. Effects of treatments on heart rate

	Unstimulated heart rate (beats/min)		Stimulated heart rate (% increase)	
	Albumin	AAG	Albumin	AAG
Baseline	468 ± 19	455 ± 29	12.8 ± 3.4	16.3 ± 3.9
After prorpanolol (45 min)	404 ± 23	393 ± 29		
After AAG or albumin (60 min)	413 ± 25	408 ± 22	3.2 ± 1.7	11.2 ± 1.7*
(75 min)	420 ± 26	423 ± 27	3.2 ± 2.6	12.3 ± 3.2*
(90 min)	425 ± 27	429 ± 29	3.9 ± 3.2	15.1 ± 2.7*

Mean ± SD, N = 8.
* $P < 0.001$ compared to albumin.

RESULTS

AAG infusion produced a human AAG concentration in serum of 7.76 ± 1.17 mg/ml. Only six of eight animals given AAG were used for equilibrium dialysis experiments because insufficient serum was available from two animals for duplicate determinations; the serum human AAG concentration of these 6 animals was 8.16 ± 0.71 mg/ml.

Albumin- and AAG-treated rats had comparable unstimulated heart rates prior to administration of propranolol (Table 2, baseline values). The unstimulated heart rate decreased in both groups after propranolol administration to comparable values. The percent of baseline unstimulated heart rate after AAG treatment was not significantly different from the percent of baseline unstimulated heart rate after albumin treatment, as assessed by ANOVA with repeated measures ($P < 0.35$).

Albumin- and AAG-treated rats had comparable increases in the stimulated heart rate prior to administration of propranolol (Table 2). The percent of the baseline response after AAG treatment was significantly greater than after albumin treatment, as assessed by ANOVA with repeated measures ($P < 0.001$). Individual comparisons at 60, 75 and 90 min showed the response of the AAG group at each of these times to be significantly greater than that of the albumin group (Fig. 1).

Serum radiolabel concentrations prior to administration of albumin or AAG did not differ between groups (Table 3). The decrease in serum radiolabel concentration between 40 and 105 min was greater after albumin than after AAG. The percent of unbound [^{3}H]propranolol and the unbound radiolabel concentration were lower after AAG treatment than after albumin (Table 3). Serum pH after equilibrium dialysis was 7.44 ± 0.02 (albumin) and 7.43 ± 0.04 (AAG, $P = 0.64$).

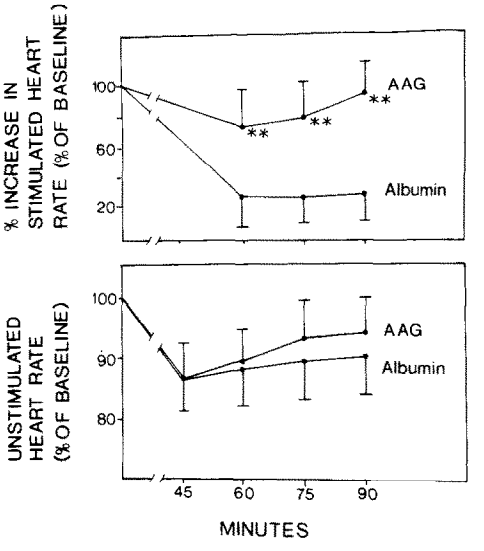

Fig. 1. Top: Heart rate response to isoproterenol. Propranolol was administered at 30 min, and AAG or albumin was infused from 45 to 60 min. The percent increase in heart rate (see Table 2) is expressed as a percent of the baseline response. The response after AAG, determined by ANOVA with repeated measures, was significantly greater than the response after albumin ($P < 0.001$). Individual comparisons at 60, 75 and 90 min also showed significant differences at each time (** $P < 0.01$). Bottom; Unstimulated heart rate (see Table 2) expressed as a percent of the baseline value. Unstimulated heart rates did not differ between groups ($P = 0.35$).

DISCUSSION

AAG treatment in this study substantially reduced propranolol-induced beta blockade. AAG administration was associated with a reduction in both the unbound fraction of [^{3}H]propranolol and the unbound radiolabel concentration in plasma. These data demonstrate that AAG administration can alter both the pharmacokinetics and pharmacodynamics of propranolol even when administered after drug effect is evident.

The AAG dose used in this study was chosen to produce a serum AAG concentration several times higher than stresses such as surgery or inflammation. Concentrations of rat AAG were not measured because the assay used is specific to human AAG, but reported values for the rat range from 0.2 mg/ml for non-stressed animals to 2.8 mg/ml after surgery [15]. The human AAG concentration in serum achieved in this study of 7.76 ± 1.17 mg/ml was in the desired range. We have shown previously that i.v. administration of a larger dose, 2.2 g/kg, of human AAG to rats is tolerated without evidence of

Table 3. Effects of treatment on [^{3}H]propranolol binding in serum

	Serum total radiolabel (dpm/ml × 10^3)				
	Before treatment (40 min)	After treatment (105 min)	Change (105 min − 40 min)	[^{3}H]Propranolol (% unbound)	Unbound radiolabel (dpm/ml × 10^6)
Albumin	51.0 ± 9.3	31.2 ± 12.1	19.8 ± 15.1	23.3 ± 3.0	7.43 ± 3.1
AAG	44.1 ± 5.3	41.5 ± 11.9	2.6 ± 10.1*	7.0 ± 3.2†	2.69 ± 1.3†

Values are means ± SD; N = 8 for serum total [^{3}H]propranolol measurement, and N = 6 for measurement of unbound [^{3}H]propranolol and percent unbound radiolabel.

* $P < 0.05$ compared to albumin.

† $P < 0.001$ compared to albumin.

toxicity and does not alter heart rate or blood pressure [13]. Thus, AAG infusion would not be expected in itself to produce any effects that might be confused with beta adrenergic responsiveness or beta blockade.

Albumin was chosen as a control treatment because it has approximately the same molecular weight and volume of distribution as AAG and should provide comparable intravascular volume expansion. This is important because intravascular volume status could influence heart rate or the heart rate response to beta blockade or stimulation. Albumin does bind propranolol, although to a lesser extent than AAG [16, 17]. Albumin infusion could have potentially increased propranolol binding in serum, but that increase would be expected to be small because of the high concentration of albumin normally present in serum (10–20 times that of AAG). It is therefore unlikely that albumin administration substantially altered propranolol binding in serum.

The use of propranolol to assess beta blockade is potentially complicated by recent reports that isoproterenol binds weakly to AAG ($K_a = 10^4\,M^{-1}$) and that isoproterenol can displace propranolol from AAG [18]. In either case, however, isoproterenol binding to AAG would serve to minimize the observed effect of AAG in the current study. Isoproterenol binding to AAG would not explain the observed inhibition of beta blockade in AAG-treated animals.

Beta blockade, as measured by isoproterenol-induced tachycardia, was reduced significantly by AAG immediately after AAG infusion. This effect persisted for the duration of the study, consistent with the long half-life of human AAG in the rat [13]. In contrast with this measure of beta blockade, AAG had little effect on the unstimulated heart rate. This mimimal effect of AAG on the unstimulated heart rate may reflect a difference in the dose–response relationships for unstimulated and isoproterenol-stimulated beta blockade due to propranolol, so that the current study represented a flat portion of the dose–response curve for the unstimulated heart rate.

The observation that AAG reduced beta blockade when administered after propranolol suggests that AAG was not simply reducing the initial distribution of propranolol to tissues. Because the interval between administration of propranolol and of treatments was only 15 min, however, it is pertinent to examine the time course of propranolol distribution to myocardium and the development of beta blockade. In dogs, peak myocardial propranolol concentration occurs within 1–2 min of an i.v. dose [19]. In rats, Schneck *et al.* [20] found myocardial propranolol concentrations to be higher 20 min after i.v. propranolol (the time of first measurement) than at any later times. In the current study, unstimulated heart rate was decreased maximally within 15 min of propranolol administration. In addition, pilot experiments demonstrated that cardiac beta blockade, measured by isoproterenol challenge, was maximal within 6 min of propranolol administration. These observations suggest that propranolol distribution to myocardium, and the onset of cardiac beta blockade, occur rapidly after propranolol administration in the rat. It is therefore unlikely that reduction of beta blockade by AAG in the current study was due to decreased initial distribution of propranolol to tissues.

[^{3}H]Propranolol was used to estimate the serum propranolol concentration because some serum propranolol concentrations were below the sensitivity of the HPLC assay for unlabeled drug. Because radiolabel from serum samples could also be associated with propranolol metabolites, additional [^{3}H]propranolol was added to serum prior to equilibrium dialysis to assure that binding data measured only parent drug. To assure this, the amount of radiolabel added *in vitro* was 100-fold greater than that present in serum from *in vivo* administration. The addition of this [^{3}H]propranolol can be calculated to increase the serum propranolol concentration by approximately 55 ng/ml. Propranolol binding in the rat is not markedly concentration dependent in the physiologic concentration range [61], and this additional propranolol would not be expected to alter binding. The percent unbound in control animals (23.3 ± 3.0%) is similar to values of 22–25% reported for propranolol in human serum [21]. Differences in temperature, pH, or duration of dialysis may account for the lower values reported by others [16, 22]. In the current study, binding of [^{3}H]propranolol was increased and the calculated unbound [^{3}H]propranolol concentration was lower in animals treated with the AAG. The measurement of radiolabel, rather than unlabeled propranolol concentration in serum, could have led to overestimation

of the total serum propranolol concentration, and thereby to overestimation of the unbound propranolol concentration. Although this possibly cannot be excluded, the current results are similar to those obtained by pretreatment of rats with surgery or inflammation prior to administration of unlabeled propranolol [8, 10]. The current study, then, supports the hypothesis that propranolol effect is mediated by unbound drug.

The total serum radiolabel concentration in albumin-treated animals decreased between 40 and 105 min. This decrease is consistent with the reported 40–110 min half-life of propranolol in rats [20, 23]. AAG treatment prevented the decrease in serum radiolabel concentration, presumably by increasing [^{3}H]propranolol binding in serum and reducing [^{3}H]propranolol distribution to tissues. An alternative possibility is that AAG decreased [^{3}H]propranolol clearance. This possibility is suggested by the finding that unbound propranolol clearance is impaired in rats with increased serum AAG concentration due to laparotomy [10]. While this observation could be due to other effects of surgery, impaired prazosin clearance has also been reported in rats pretreated with AAG [24]. Decreased [^{3}H]propranolol clearance in the current study could have contributed to the higher serum radiolabel concentration in AAG-treated animals, but would not account for the observed decrease in beta blockade. AAG may also alter the tissue partitioning or binding of drugs [24]; this possibility is not addressed by the current study.

It has been suggested that digoxin-specific Fab is effective in reversing digoxin toxicity because its affinity for digoxin exceeds that of the cardiac digoxin receptor [4]. The affinity of propranolol for cardiac beta receptors in the rat ($K_a = 7 \times 10^{10}\,M^{-1}$) is more than 10,000 times greater than the affinity of propranolol for human AAG [10]. The ability of AAG to alter propranolol effect demonstrates that a binding protein need not approach the affinity of a specific drug receptor in order to modify drug effect. Presumably the reduction in unbound drug concentration produced by AAG is rapidly reflected by a reduction in unbound drug concentration at the drug receptor. If this is generally true, then the ability of a drug-binding protein to modify drug effect may depend upon its ability to reduce unbound drug concentration rather than its ability to bind drug more avidly than a specific receptor. The implication of this possibility regarding drug-binding proteins is that relatively modest affinities for drug may be sufficient to produce pharmacokinetic and pharmacodynamic effects. Very high-affinity antibodies, which are often difficult to obtain, may not be necessary in all cases.

In summary, AAG administration to rats after [^{3}H]propranolol increased [^{3}H]propranolol binding in serum, reduced the unbound [^{3}H]propranolol concentration in serum, and reduced propranolol-induced beta blockade. These data support previous demonstrations that the effect of propranolol is dependent upon the unbound, rather than the total, drug concentration in serum. Because the affinity of propranolol for AAG is much lower than its affinity for cardiac beta receptors, these data suggest that drug-binding proteins with modest affinities for drug may be capable of modifying drug effect or toxicity.

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