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Research Paper

The Influence of Hyperlipoproteinemia on *in Vitro* Distribution of Amiodarone and Desethylamiodarone in Human and Rat Plasma

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Purpose. To study the effect of hyperlipoproteinemia on *in vitro* distribution of amiodarone (AM) and its prevalent metabolite desethylamiodarone (DEA) in human and rat plasma.
Materials and Methods. Human and rat normolipidemic (NL) and hyperlipidemic (HL) plasma were spiked with AM and DEA. The fractions (high and low density lipoproteins, triglyceride rich lipoproteins and lipoprotein deficient plasma) were separated using ultracentrifugation.
Results. Human and rat displayed similar patterns in terms of association of AM and DEA in NL plasma, in which the highest and lowest associations were observed in lipoprotein deficient (LPDP) and triglyceride (TRL) rich plasma fractions, respectively. In HL a substantial shift was observed in partitioning of AM and DEA mostly to TRL. The shift of AM and DEA into TRL fraction of HL plasma was more drastic for rat than human. In HL, association of AM with rat LPDP and HDL fractions were 10 and 26-fold lower than in the corresponding human fractions, respectively. The DEA:AM ratio in rat, but not human, was significantly affected by HL.
Conclusion. HL caused a major shift of AM and DEA to TRL fraction in both species. The findings were consistent with the higher AM concentrations previously noted in HL rats given the drug.

KEY WORDS: amiodarone; desethylamiodarone; distribution; hyperlipidemia; pharmacokinetics.

INTRODUCTION

Amiodarone (AM) is an important drug in the management of life threatening ventricular and supraventricular arrhythmias and heart failure (1,2). Amiodarone possesses a very large volume of distribution and long terminal half-life (*t* 1/2) in both human and rat (3,4). Amiodarone also has erratic absorption characteristics resulting in low and unpredictable bioavailability after oral administration (5). The highly lipophilic structure of AM (6) might help to explain some of the pharmacokinetic behaviors of this molecule (5). In plasma, AM is extensively bound to circulating proteins (7). The lipoprotein binding of AM has been estimated to comprise 33.5% of its overall protein binding in plasma (7). Due to its extensive metabolism and its low to moderate hepatic extraction ratio of AM, its clearance is largely attributed to plasma protein binding and intrinsic metabolic clearance (3). Recently, it was shown that hyperlipoproteinemia (HL) caused by systemic HL or high fat intake in rat caused 11.5 and 1.5-fold decreases in the clearance of AM, respectively. Also, the volume of distribution (Vd) of AM was decreased 23-fold in HL rats (3). These findings, which paralleled those observed for the antimalarial drug halofantrine (8), suggest that lipoprotein binding of AM may affect the pharmacokinetics of the drug.

Amiodarone has both hepatic and extra hepatic metabolism (9). The major active metabolite of AM is desethylamiodarone (DEA) (10). This metabolite is known to possess significant electrophysiological effects that can add to the activity of the parent drug (10,11). To date little information is known of the effect of HL on the nature of the lipoprotein binding of AM and DEA. Based on a previous study in rats, in which a large increase in plasma AM concentrations was observed in HL rats compared to normolipoproteinemic (NL) rats, an increase in the localization to lipoprotein-rich fractions of plasma would be anticipated. In this manuscript

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ABBREVIATIONS: AM, amiodarone; DEA, desethylamiodarone; NL, normolipidemic; HL, hyperlipidemic; LPDP, lipoprotein deficient plasma; TRL, triglyceride rich plasma; LDL, low density lipoprotein; HDL, high density lipoprotein; P407, poloxamer-407; CHOL, cholesterol; TG, triglyceride; LP, lipoprotein; LC/MS, liquid chromatography-mass spectrometry; HPLC, high performance liquid chromatography; t 1/2, terminal half-life; AUC, area under the plasma concentration *versus* time curve; CL, clearance; Vd, volume of distribution; EDTA, ethylene diamino tetra acetic acid; FDA, Food and Drug Administration; SIR, selective ion recorder; CV, coefficient of variation.

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we describe the distribution of AM and DEA within NL and HL human and rat plasma.

MATERIALS AND METHODS

Chemicals

Amiodarone HCL was obtained from Sigma (St. Louis, MO, USA). Desethylamiodarone was a kind gift from Wyeth-Ayerst Research (Princeton, NJ). Methanol, acetonitrile, hexane and water (all HPLC grades) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Pluronic-F127 (poloxamer-407; P407), sodium chloride, sodium bromide and sodium hydroxide were purchased from Sigma Aldrich (St. Louis, MO). Enzymatic assay kits for determination of total cholesterol (CHOL) and triglyceride (TG) in human and rat plasma samples were purchased from Diagnostic Chemicals Limited (Charlottetown, Prince Edward Island, Canada).

Prescreening and Characterization of Human Plasma Pools

Hyperlipidemic human plasma was purchased from Bioreclamation (Hicksville, NY). Human plasma was derived from whole blood donations obtained in the United States from donations at FDA-registered blood centers. All blood was treated with sodium EDTA as an anticoagulant to obtain the plasma. The HL plasma was obtained from donors with chronically elevated lipid levels as determined at the site of collection.

Collection of Rat Plasma

The study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. A total number of 12 Sprague-Dawley rats (weight=300–400 g) were used in this study. Rats were allocated in two groups of NL and HL. About 36 h before the plasma collection, one group of rats (n=6) was rendered HL by intraperitoneal injection of 1 g/kg of P407 (0.13 g/ml in normal saline) as previously described (3). In NL rats (n=6), the same amount of normal saline was injected intraperitoneally. Normolipoproteinemic and HL plasma was obtained by exsanguination of rats by cardiac puncture under anesthesia with halothane. Blood was collected in heparinized tubes, centrifuged for 10 min at 2,500g and plasma separated.

Treatment of Plasma Samples

Pooled human and rat plasma samples were added to Beckman Ultraclear™ ultracentrifuge tubes in 3 ml aliquots. The mixture of AM and DEA was added such that the final concentration in the plasma was 1,000 ng/ml AM and 500 ng/ml DEA. Both AM and DEA were dissolved in methanol and the final volume of methanol added to each plasma sample was not more than 0.01%. At this volume, the composition of plasma lipoproteins is not affected by methanol (12). The plasma samples were incubated at 37°C for 60 min. The incubation was stopped by the addition of 1.020 g of sodium bromide to adjust the density of the plasma and then the samples were rapidly cooled on ice to 4°C for a minimum of 2 h.

Separation of Lipoprotein Components

Sodium bromide density solutions (1.006δ, 1.063δ and 1.21δ) were prepared by dissolving appropriate amounts of sodium chloride, sodium bromide and sodium hydroxide in water. The solutions were cooled to 4°C prior to preparation of the gradient. Three ml of the cooled, treated plasma samples, adjusted to a density of approximately 1.25δ with sodium bromide, was added to an ultracentrifuge tube (Beckman Coulter: Fullerton, CA). Density solutions were carefully layered on top of the plasma in the order 1.21δ, 1.063δ then 1.006δ. The tubes were balanced and placed into individual titanium buckets and capped. The buckets were loaded on a SW 41 Ti swinging bucket rotor (Beckman Coulter) and centrifuged at 40,000 rpm at 15°C for 18 h in a Beckman L8-80M Ultracentrifuge (Beckman Coulter) or a Beckman LE-80 Ultracentrifuge (Beckman Coulter). Upon completion, the tubes were removed and four distinct regions were observed and labeled. The layers correspond to the triglyceride-rich lipoproteins (TRL) comprised of very low density lipoproteins and any chylomicrons present, low density lipoproteins (LDL), high density lipoproteins (HDL) and the lipoprotein deficient plasma fraction (LPDP). These layers were removed using a Pasteur pipette and added to disposable glass test tubes and the volumes measured and recorded (13).

Lipid Measurement

Total cholesterol and TG concentrations were determined using enzymatic cholesterol and triglyceride assay kits. Two ml of CHOL or TG reagents was added to 10 µl of human or rat NL or HL plasma samples or 10–100 µl of each NL or HL fractions. Normolipidemic plasma was defined as having CHOL and TG concentrations of <200 mg/dl. Tubes were incubated at 37°C for 5 or 10 min and scanned at 505 or 515 nm, respectively, using an ultraviolet spectrophotometer (14).

LC/MS Analysis of Amiodarone and Desethylamiodarone

A LC/MS method was used to measure the concentrations of both AM and DEA. The assay had a validated lower limit of quantification of 2.5 ng/ml for both AM and DEA in 100 µl of rat plasma (15). Measurements of AM and DEA were made in 100 µl of each lipoprotein fractions. All calibration curves were made in the same AM or DEA-spiked matrix (e.g. blank rat or human HDL, LDL, TRL or LPDP fraction) as the sample being quantified for analyte. Briefly, to each 0.1 ml sample was added 0.03 ml of internal standard solution (ethopropazine). To extract the analytes from each fraction, 0.3 ml of acetonitrile were added in a 2 ml polypropylene microcentrifuge tube. The tubes were briefly vortex mixed (5 s) to precipitate plasma proteins. The tubes were subsequently centrifuged for 2 min and the supernatants were carefully transferred to new glass tubes using Pasteur pipettes. To each tube, 0.3 ml of HPLC water and 3 ml of hexane were added. The tubes were then vortex mixed for 30 s and centrifuged at 3,000 g

for 3 min. The organic layer was transferred to new glass tubes and evaporated to dryness in vacuo. The residues were reconstituted using 1 ml of methanol and aliquots of 5–10 µl were injected into the LC/MS system (15).

The LC/MS analyses were performed using a Waters Micromass ZQ™ 4000 spectrometer coupled to a Waters 2795 HPLC separations module (Milford, MA, USA). The mass spectrometer was operating in positive ion and selective ion recorder (SIR) acquisition modes. The nebulizing gas was obtained from an in house high purity nitrogen source. The temperature of the source was set at 150°C and the voltage of the capillary was 3.2 kV. The cone voltage was set at 25, 40 and 50 V for IS, DEA and AM, respectively. The gas flow of desolvation and cone were set at 550 and 80 l/h, respectively. Chromatographic separation was achieved using a C18 3.5 µm (2.1×50 mm) column as the stationary phase (XTerra® MS, Ireland). The mobile phase was pumped as a linear gradient from methanol: formic acid 0.2%, 40:60 to 90:10 v/v over 12 min, then back to the original 40:60 v/v composition over 3 min. A constant flow rate of 0.2 ml/min was used throughout. The column was heated to 45°C during the chromatographic run. The intraday and interday CV and mean error were each <20%. Total analytical run time was 15 min.

The mixture of standard compounds and internal standard were analyzed on the mass spectrometer using flow injection in scan mode to determine optimal fragmentation for each compound and establishment of the *m*/*z* values of the molecular ions (15).

Data and Statistical Analysis

The partitioning of analyte was calculated using the measured concentrations in the fraction aliquots multiplied by the volumes obtained at the time of separation. The percentage recovered was determined by comparing the total mass recovered from all fractions to the total mass added to the plasma at the onset of the ultracentrifugation.

Compiled data were expressed as mean±SD unless otherwise indicated. Statistical analysis was performed using SigmaStat version 3.5 (Systat Software). Each data set was analyzed for normality and homogeneity of variances in order to determine the choice of statistical method. For ranking analyte association in the various fractions, Kruskal–Wallis One Way Analysis of Variance on Ranks with Dunn's Method or Student-Newman–Keuls method of post-hoc tests were used. Student unpaired *t* test were also used for the interpretation of differences between species and between NL and HL plasma and fractions in TG and CHOL content. The alpha value was set a priori at 0.05.

RESULTS

Triglyceride and Cholesterol Measurement

Hyperlipidemia caused 4.21 and 2.24-fold increases in the TG and total CHOL levels in human plasma, respectively (Table I). In HL rat however, the increase in TG and CHOL levels were more drastic than HL human. Poloxamer 407 caused 32.9 and 42.3-fold increases in TG and CHOL in rat plasma, respectively (Table I).

Table I. The Triglyceride and Cholesterol Present in Human and Rat Plasma Before Separation of the Lipoprotein Fractions

Species	Triglyceride (mg/dl)	Cholesterol (mg/dl)
NL-Human	92.0±5.9	144±15
HL-Human	388±49	322±170
NL-Rat	109±22*	57.1±0.86
HL-Rat	3570±430	2420±520

Asterisks represent significant differences between normolipidemic (*NL*) and hyperlipidemic (*HL*) groups of the same species (Student's unpaired *t* test, $p<0.05$)

In HL human plasma, the concentrations of TG were higher in all of the isolated fractions compared to NL plasma (Table II). The largest increase was observed in the TRL fraction. Although the HDL fraction was less than the lower limit of quantitation of the assay, in each of the other fractions tested the HL rat plasma had, similar to human, increases in the TG levels compared to NL plasma. For CHOL, a somewhat different pattern emerged. For example, compared to NL plasma, in HL human plasma a large increase in CHOL was observed only in the TRL fraction. In contrast, in the HL rat plasma the CHOL concentrations were greatly increased in the TRL and the LDL fractions, compared to NL plasma.

Recovery

Overall recovery of AM from the spiked plasma samples ranged from 59 to over 100%. For DEA the overall recovery was less variable than AM, ranging from 62 to 79% (Table III). The presence of HL caused a significant decrease in the recovery of AM. The total recoveries of AM from NL human and rat plasma were higher than that from HL plasma (Table III). Similarly, in rat the recovery of DEA was higher from NL plasma. In contrast, the recovery of DEA in human NL fractions was lower than that of HL (Table III).

Table II. Mean ± SD Triglyceride and Total Cholesterol Concentrations (mg/dl) in Plasma Lipoprotein Fractions from Normolipidemic (NL) and Hyperlipidemic (HL) Plasma Specimens

Species	TRL	LDL	HDL	LPDP
Triglyceride				
NL-Human	17.1±3.3*	12.9±5.4*	9.90±0.30*	6.64±0.21*
HL-Human	278±67	94.6±5.5	52.4±3.6	48.2±3.0
NL-Rat	15.4±1.7*	7.29±2.1*	5.99±0.93	4.38±1.0*
HL-Rat	1861±150	93.3±8.1	ND	33.3±8.6
Total cholesterol				
NL-Human	10.0±3.1*	51.8±30	19.4±0.68*	5.41±1.1
HL-Human	135±25	60.7±1.7	9.93±4.6	ND
NL-Rat	8.14±1.9*	10.6±0.23*	19.8±4.8	1.89±0.48
HL-Rat	2698±186	237±42	18.9±6.0	2.23±1.1

ND Denotes not detectable
Asterisks represent significant differences between NL and HL of the same species (Student's unpaired *t* test, $p<0.05$).

Table III. Association of Amiodarone and Desethylamiodarone (Mean ± SD) in each Fraction of Normolipidemic (NL) and Hyperlipidemic (HL) Plasma of Human and Rat (n=6 each for NL-Human, NL-Rat and HL-Rat; n=5 for HL-Human)

Sample	Recovery in Each Fraction (ng)				Total Recovery (% Incubated)
	TRL	LDL	HDL	LPDP	
Amiodarone					
NL-Human	432±52*	292±170	592±100*	1213±130*	84.3±2.1*
HL-Human	911±170	408±150	247±38	350±26	63.8±6.5
NL-Rat	261±55*	533±350*	821±310*	1780±250*	103±8.0*
HL-Rat	1370±230	167±82.4	15.2±6.7	32.0±41	59.0±7.3
Desethylamiodarone					
NL-Human	93.6±17*	119±49*	285±51*	438±60*	62.4±3.9
HL-Human	347±120	210±66	190±28	296±27	69.6±13
NL-Rat	30.6±7.3*	72.3±52*	204±75*	875±110*	78.8±14
HL-Rat	705±130	196±53	40.4±12	79.3±29	69.6±8.3

Asterisks represent significant differences between NL and HL of the same species (Student's unpaired t test, p<0.05)

Comparison of Normolipidemic and Hyperlipidemic Plasma

Upon normalizing the fractional recoveries to overall recovery from plasma, it was observed that in all of the NL plasma from both species, AM and DEA were most associated with LPDP (p<0.05). The LPDP fraction represents the most polar of the fractions (Fig. 1). The next most polar lipoprotein fraction, HDL, was second in abundance for both drug and metabolite in all species. The lowest degree of association of both drug and metabolite occurred in the most apolar lipoprotein fractions, LDL and TRL, respectively (Fig. 1).

In HL plasma, the pattern of association of both drug and metabolite were markedly changed (Fig. 1). For both species, there was a shift of both AM and DEA in HL plasma from the more polar LP fractions to the less polar fractions.

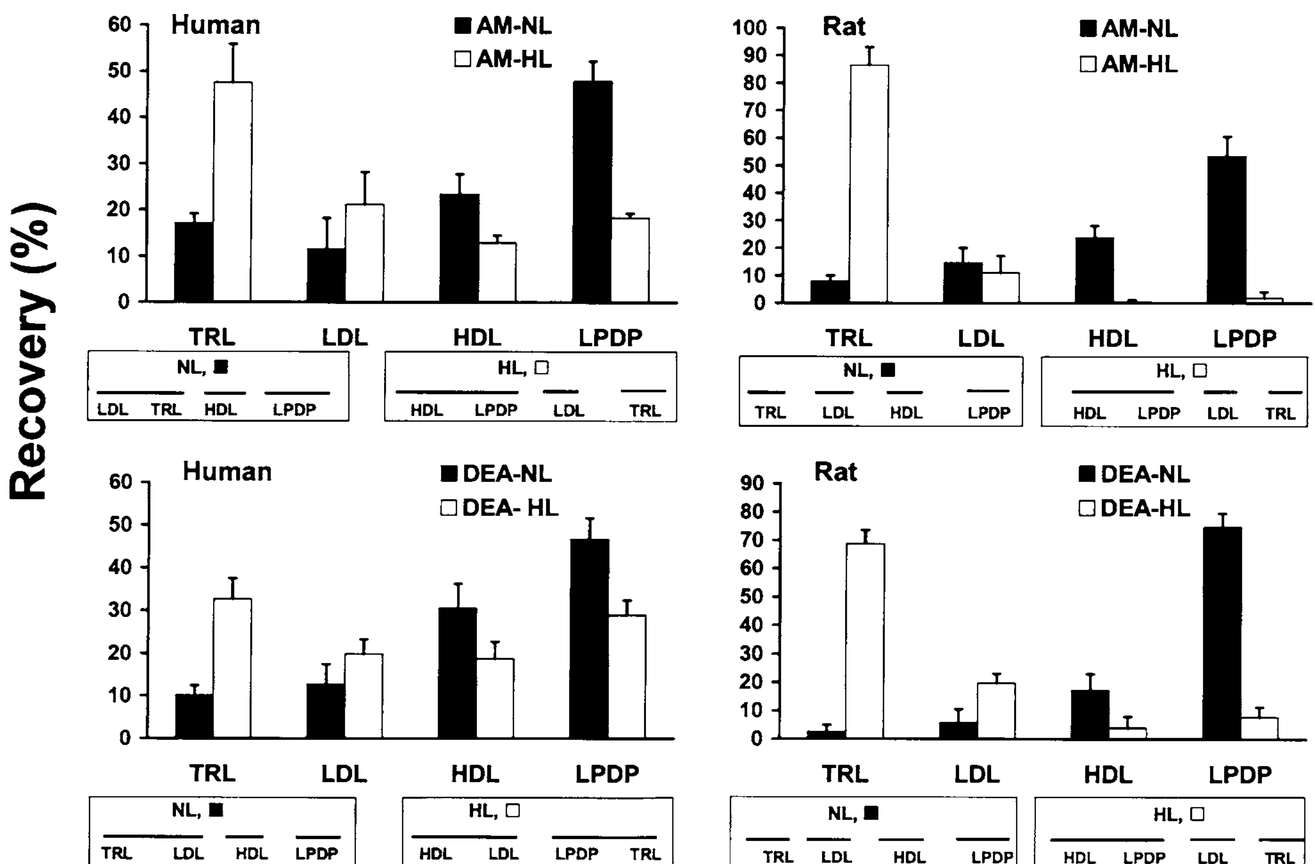

Fig. 1. Association of amiodarone (*AM*) and desethylamiodarone (*DEA*) with lipoprotein fractions isolated from hyperlipidemic (*HL*) or normolipidemic (*NL*) plasma, expressed as percent of total recovery of drug and metabolite. *Continuous lines over fractions* indicates lack of significance between fractions encompassed by the lines; *fractions not encompassed within lines* are significantly different from those fractions encompassed by the lines (Kruskal–Wallis One Way Analysis of Variance on Ranks, p<0.05). Each *bar* represents the mean ± SD of five to six samples.

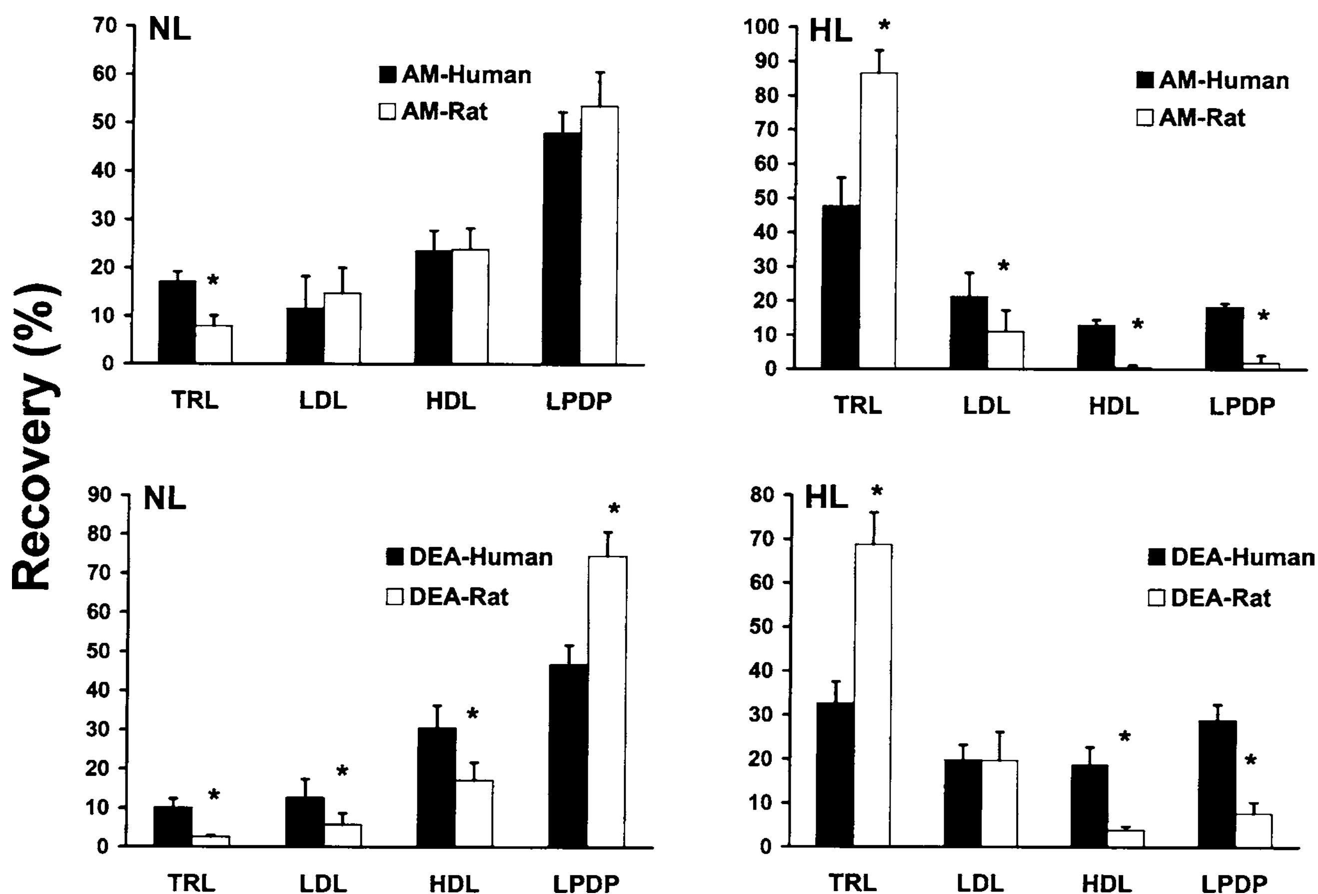

Fig. 2. Mean ± SD differences between human and rat for disposition of amiodarone (*AM*) and desethylamiodarone (*DEA*) within different lipoprotein fractions from normolipidemic (*NL*) and hyperlipidemic (*HL*) plasma. *Asterisks* represent significant differences (Student's unpaired *t* test, $p<0.05$).

In both species, the highest association of AM and DEA in HL plasma was in the TRL fraction. The lowest association of both analytes was in the HDL fraction. With the exception of DEA in human HL plasma, in the other incubations involving AM and DEA the LDL fraction possessed the second highest association of analyte, next to TRL.

Interspecies Differences

With respect to interspecies comparisons, the patterns of partitioning of both AM and DEA in the fractions were quite similar in both species (Figs. 1 and 2). However, there were some significant interspecies differences noted in the percent association within fractions. The shift of drug into the TRL fraction of HL plasma was more dramatic for rat than human plasma for drug and metabolite, with significantly higher levels (~2-fold) of association of AM and DEA in rat TRL fractions at the expense of the LPDP and HDL fraction uptake (Fig. 2). Indeed, the association of AM with rat LPDP and HDL fractions were 10 and 26-fold higher than in the corresponding human fractions, respectively (Fig. 2).

Comparison of the Ratio of DEA to AM in Different Fractions

The ratio of DEA to AM in human plasma lipoprotein fractions was affected only modestly by HL (Table IV). The ratios stayed fairly constant with the exception of LPDP, where the DEA:AM ratio significantly increased by approximately 1.6-fold. In contrast, in rat HL caused the metabolite to drug ratios to be markedly changed in each of the LP fractions The ratios of DEA to AM in TRL, LDL, HDL and

Table IV. Ratio (Mean ± SD) of DEA:AM Within each Fraction Derived from Normolipidemic (NL) and Hyperlipidemic (HL) Plasma

Plasma	TRL	LDL	HDL	LPDP
NL-Human	0.587±0.068	1.19±0.29	1.31±0.14	0.975±0.028*
HL-Human	0.703±0.17	0.993±0.22	1.47±0.39	1.58±0.18
NL-Rat	0.338±0.069*	0.390±0.15*	0.712±0.085*	1.40±0.11*
HL-Rat	0.793±0.035	1.94±0.36	5.02±2.8	6.62±3.8

Asterisks represent significant differences between NL and HL of the same species (Student's unpaired *t* test, $p<0.05$)

LPDP fractions of rat HL plasma were 2.34, 4.97, 7.0 and 4.7-fold higher ($p<0.05$), respectively, than in the corresponding fractions in rat NL plasma (Table IV).

DISCUSSION

It is commonly believed that it is the unbound fraction of drug in blood that is pharmacologically relevant, based on the premise that only the unbound drug can transverse cell membranes and reach its intended target for pharmacological activity. The plasma proteins most commonly involved in binding of drugs are albumin and alpha-acid glycoprotein (16). In comparison, only a small number of drugs are known to significantly incorporate into lipoproteins (17). However, the association of drugs with lipoproteins may not involve simple reversible binding phenomenon, as is the case with the other major classes of plasma proteins. This is due to the complex nature of lipoproteins, which are heterogenous structures comprised of many different types of lipids and proteins (18). Indeed, one of the unique aspects of lipoproteins is in their inclusion of specific apoproteins that are involved in tissue mediated uptake of drug, which potentially can increase measures of drug action or toxicity beyond that anticipated by the change in unbound fraction. Given that each of the various classes of lipoproteins differs in the nature of the apoproteins involved in cellular recognition of the lipoprotein particle, it is important to understand into which lipoprotein fraction a drug preferentially associates. Although prior to this study there was some information in the literature indicating that AM could bind to lipoproteins in plasma (3,7), this is the first study to evaluate the intraplasma distribution of AM and its major metabolite (DEA) in lipoprotein fractions, in both NL and HL plasma.

The data indicated that in NL plasma, both AM and its major metabolite are taken up by the lipoprotein rich fractions of both rat and human species (Table III, Figs. 1 and 2). For AM, approximately half of the association in NL plasma is with the lipoprotein rich fractions (Fig. 1). Of these, HDL has the highest affinity for the drug. The pattern of distribution of DEA was qualitatively similar with approximately 30 and 50% of metabolite residing, respectively, within the lipoprotein rich fractions of rat and human. Similar to AM, the association of DEA in the lipoprotein-rich fractions was highest in the HDL fraction in NL plasma.

In the presence of HL plasma, there was a substantial shift of drug and metabolite, apparently mostly from the LPDP fraction to the most apolar of the lipoprotein fractions, TRL, which is particularly rich in chylomicron and very low density lipoproteins (Fig. 1). The shift was more dramatic for the rat, but the pattern of change was similar for both species. The reason for the more dramatic change in the HL rat plasma compared to human is possibly due to the larger increases in plasma TG and CHOL in the rat P407-treated plasma opposed to that seen in the HL human plasma. Although recoveries of the drug and metabolite were not complete from the plasma, given the large differences between NL and HL in AM and DEA association in the fractions (Table III) it seems unlikely that the incomplete recovery would significantly influence the conclusions arising from the data when normalized for recovery, even if the recovery of drug or metabolite was not homogenous between fractions.

This change in the LP association in HL is in line with the changes observed in the pharmacokinetics of the drug in the HL state (3). In P407 treated rats, induction of HL caused a major change in the pharmacokinetics of the drug, with significant and substantial decreases being observed in the unbound fraction, clearance and volume of distribution. It was also found that there was a significance increase in AUC of the drug after oral dosing of AM to NL rats concomitantly administered oral lipid, and that part of the increase in AUC was due to a decrease in the clearance of the drug as an apparent consequence of increased lipoprotein binding. In the previous study in rats the chromatographic assay used was not able to characterize the pharmacokinetics of the metabolite in the NL vs. HL state (3). It was of note that in human, but not rat, the metabolite to drug ratio stayed relatively constant.

Amiodarone is a drug for which a narrow therapeutic range has been established, and for which therapeutic drug monitoring is warranted (19). Our findings in conjunction with the previous study in rats might explain part of the variability that might be associated with AM therapy, in that HL patients might also be expected to have higher plasma concentrations of the drug than NL patients given the same dose level. Another aspect of use with AM is the fact that it is associated with changes in lipoprotein levels with chronic use. In particular, it has been shown that HDL plasma concentrations are increased with long term administration of AM (20). This may be a secondary effect of hypothyroidism, which is a known side effect of treatment with the drug, and which can precipitate changes in lipid metabolism and plasma lipoprotein profile. Interestingly, in NL plasma the lipoprotein fraction with the highest level of affinity for AM and DEA was HDL, which raises the possibility of a change in plasma concentrations of the drug with repeated doses.

CONCLUSION

In the NL condition, most of the parent drug and its primary metabolite are distributed in the LPDP fraction of plasma. A similar trend of drug and metabolite localization in NL plasma was noted for rat and human. The presence of HL causes a major change of AM and DEA from the more polar lipoprotein fractions to the less polar fractions represented by LDL and TRL. The effect of HL was as anticipated based on the results of a recently published study examining the pharmacokinetics of AM in HL rats. Given the similarities in LP association of AM in the rat plasma, and the influence of HL on AM pharmacokinetics in rats, it seems likely that the influence of HL on the plasma AM concentrations in patients given the drug will be similar.

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