Nicanartine Improves in Vitro Resistance of Lipoproteins to Oxidation

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Purpose. The aim of this study is to investigate the plasma protein binding of nicanartine and to measure its antioxidant effect on lipoproteins.

Methods. The blood binding was studied with an erythrocyte partitioning method and the lipoprotein oxidation with the conjugated dienes method.

Results. Nicanartine was 24.7% LDL (low density lipoprotein)-bound and 29.2% AAG (alphal-acid glycoprotein)-bound. Nicanartine delayed but did not stop the oxidation of the three density classes of lipoprotein HDL (high density lipoprotein), LDL, VLDL (very low density lipoprotein). The addition of AAG to LDL in the conjugated dienes method decreased the nicanartine fraction bound to LDL and decreased its antioxidant effect. The decrease of nicanartine LDL-bound fraction and the decrease in antioxidant effect were not parallel.

Conclusions. This suggested that (a) AAG-bound nicanartine dissociated to equilibrate the decrease in LDL-bound nicanartine consummed by oxidation, or (b) the oxidation conditions could involve chemical modifications of nicanartine and therefore modify its affinity for AAG.

KEY WORDS: nicanartine; lipoprotein; oxidation; plasma binding.

INTRODUCTION

It is currently believed that oxidative modifications of LDL is an important event in atherogenesis. In vitro, oxidized LDL shows increased affinity to the macrophage scavenger receptor; this uptake of oxidized LDL by macrophages is not regulated by internalized LDL-cholesterol and leads to lipid loading of these cells (1–2). Another mechanism which may lead to these foam cells is the phagocytosis of LDL immune complexes by macrophages. These immune complexes are likely to be a consequence of the autoimmune response to oxidized LDL (3). In early atherosclerotic lesions foam cells are found to have accumulated in the subendothelial space. As the disease progresses most of these cells die, generating fatty streaks that may ultimately develop into plaques. Moreover, oxidized LDL is cytotoxic toward vascular cells (4) and exerts chemotactic activity toward monocytes, but inhibits migration of resident macrophages (5). All these properties suggest that LDL oxidation is involved in atherogenesis. Thus, drugs with antioxidant properties are developed in order to protect plasma lipoproteins from oxidation. Nicanartine is a recent antioxidant drug whose chemical structure results from the combination of a substituted phenol radical and a pyridyl radical by an ether bond (figure 1). This chemical structure indicates that nicanartine is mainly lipophilic with poor water solubility. This drug appears to be able to target the atherosclerotic process on different levels. Animal studies show that nicanartine has lipid lowering properties (6). Besides these properties, nicanartine is a drug with antioxidant effect.

The aim of this study is (a) to investigate the plasma protein binding of nicanartine with special attention to the distribution in lipoproteins, and (b) to measure its antioxidant effect on lipoproteins.

MATERIALS AND METHODS

1) Lipoprotein Preparation

Fresh whole blood was obtained by venipuncture from healthy human volunteers (20–25 years old) and was supplemented with EDTA (1 mg/ml) and BHT (4.4 μg/ml). VLDL, LDL, HDL were prepared by sequential ultracentrifugation within density gradients according to Havel (7). The Lipoprotein (HDL or LDL or VLDL) stock solutions were stored at 4°C in the dark in nitrogen atmosphere for 1 week. A sample of stock solution was dialysed in oxygen free phosphate buffer (Na_2HPO4 0.066 M, NaH_2PO4 0.66 M, NaCl 0.16 M, pH = 7.4).

2) Lipoprotein Oxidation (Conjugated Dienes Method (8))

The EDTA- and BHT-free lipoprotein sample was diluted with oxygen saturated phosphate buffer (Na_2HPO4 $8.4 \cdot 10^{-3}$ M, NaH_2PO4 $1.6 \cdot 10^{-3}$ M, NaCl 0.16 M, pH = 7.4) to obtain a 0.15 mg/ml (0.01 μM) final VLDL concentration or a 0.25 mg/ml (0.1 μM) final LDL concentration or a 0.25 mg/ml (1 μM) final HDL concentration. A microvolume (10 μl) of nicanartine in ethanol was incorporated before adding $CuCl_2$ (1.66 μM). The kinetics of lipoprotein oxidation was monitored by the change in absorbance at 234 nm.

The AAG (Behring, 99% pure) stock solution (97.6 μM) was made in phosphate buffer (Na_2HPO4 0.066 M, KH_2PO4 0.66 M, pH = 7.4). A sample of AAG stock solution, mixed with a EDTA- and BHT-free LDL sample, added with nicanartine, was diluted with oxygen saturated phosphate buffer

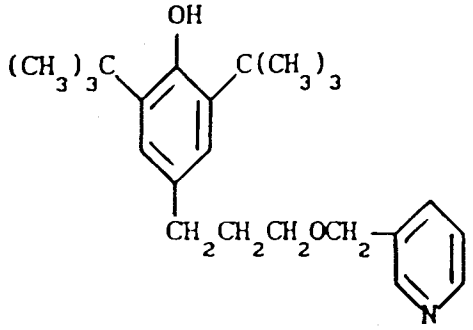

Fig. 1. Chemical structure of nicanartine.

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ABBREVIATIONS: AAG = alphal-acid glycoprotein; BHT = butylated hydroxytoluen; EDTA = ethylenediaminetetraacetic; fu = unbound fraction; HDL = high density lipoprotein; HSA = human serum albumin; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein.

Table I. Nicanartine Binding Parameters to Isolated Plasma Proteins

Plasma proteins	HSA	AAG	HDL	LDL	VLDL
nK_A l/μmol	0.03 ± 0.014	4.5 ± 1.9	3.1 ± 1.5	31.8 ± 11.5	123 ± 49

Note: nKa is the total binding constant for each protein and is determined by the erythrocyte partitioning method. The values are presented in the form of an estimate ± standard deviation.

(Na_2HPO4 $8.4 \cdot 10^{-3}$ M, NaH_2PO4 $1.6 \cdot 10^{-3}$ M, NaCl 0.16 M, pH = 7.4) to obtain a 8 μM final AAG concentration and a 0.1 μM final LDL concentration before adding $CuCl_2$ (1.66 μM).

3) Erythrocyte Partitioning Method

Blood binding of nicanartine could not be investigated by classical methods such as equilibrium dialysis or ultracentrifugation because the drug did not diffuse across dialysis membrane and there was a high degree of non specific adsorption on cells and membrane. A rapid method which measures the partitioning of the drug between protein and erythrocytes was used (9). The erythrocyte partitioning method assumes that the free concentration of drug in plasma is in equilibrium with that in erythrocytes. An aliquot of radiolabelled nicanartine (^{14}C/^{3}H nicanartine: 1μCi/ml (^{14}C); 2 μCi/ml (^{3}H)) dissolved in ethanol was added to 1.6 ml of buffered protein solution (115 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, pH = 7.4). For each protein, different protein concentrations, Pt, were studied (HSA 2 to 30 μM, AAG 0.02 to 0.1 μM, HDL 0.005 to 0.15 mg/ml, LDL 0.05 to 0.4 mg/ml, VLDL 0.03 to 0.21 mg/ml). Then, 0.4 ml of erythrocyte pellet was added to the 1.6 ml samples containing the protein and the radiolabelled nicanartine to obtain a 20% hematocrit. These samples were incubated in capped glass vials (37°C, 50 min) with gentle orbital shaking in a Brunswick water bath. Duplicate aliquots (50 μl) of the whole suspension (WS) were taken for counting. After centrifugation (2000 g, 15 min at 37°C in a preheated Z2320K Centrifuge, BHG, Gosheim, Germany) of an aliquot of WS at 37°C, duplicate aliquots (50 μl) of supernatant (P) were counted. The ratio of drug concentration in erythrocytes and protein solution (E/P) was calculated from the following equation:

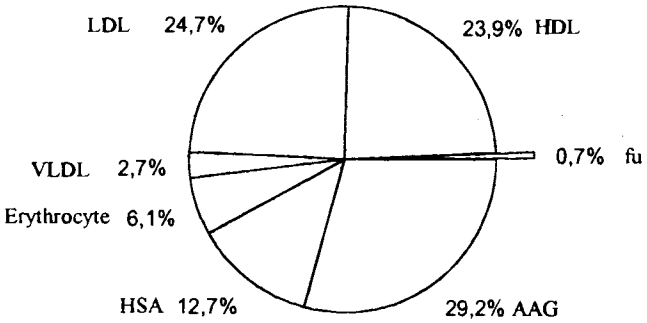

Fig. 2. Simulation of blood distribution of nicanartine. Calculations are done according to table I and human physiological values which are HSA (650 μmol/l), AAG (10 μmol/l), HDL (3 g/l), LDL (3 g/l), VLDL (0.5 g/l), hematocrit (40%).

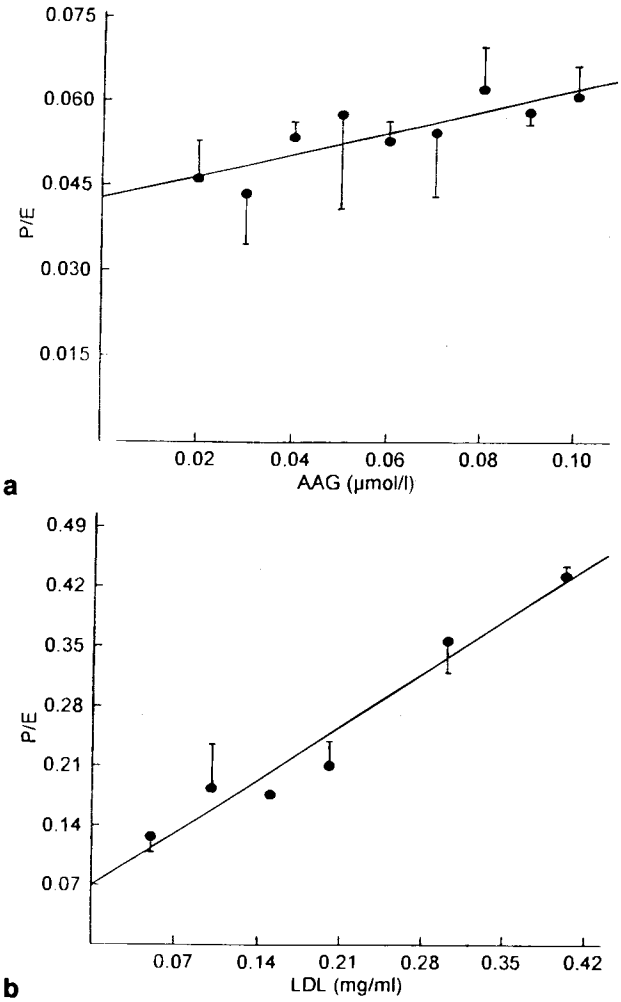

Fig. 3. Partitioning of nicanartine between AAG (figure 3-a) LDL (figure 3-b) and erythrocytes at 37°C. The protein solution in glucose buffer saline containing a trace amount of (^{14}C–^{3}H)nicanartine is incubated with erythrocytes (Hematocrit 20%). At equilibrium, the protein solution or plasma-to-erythrocyte concentration ratio (P/E) of nicanartine is determined and plotted versus protein concentration. Line is fitted according to equation (2). Experimentally observed values are means +/− standard deviation of duplicate measurements in the same experiment.

$$\frac{E}{P} = \frac{(WS/P) - (1 - H)}{H} \qquad (1)$$

where H denotes the hematocrit.

Because we used low concentrations of nicanartine (8–10 μM), we may assume that the C_P/C_E ratio is linearly related to the protein concentration (Pt) in the extracellular buffer solution (see references 10 and 11 for details)

$$\frac{C_P}{C_E} = \frac{1 + nK_A \cdot Pt}{1 + K_E} \qquad (2)$$

where nK_A is the total binding constant for ligand interaction with a protein (product of n, number of binding sites, by K_A, corresponding association constant), and K_E is the binding coefficient for ligand interaction with erythrocytes (dimension-less,

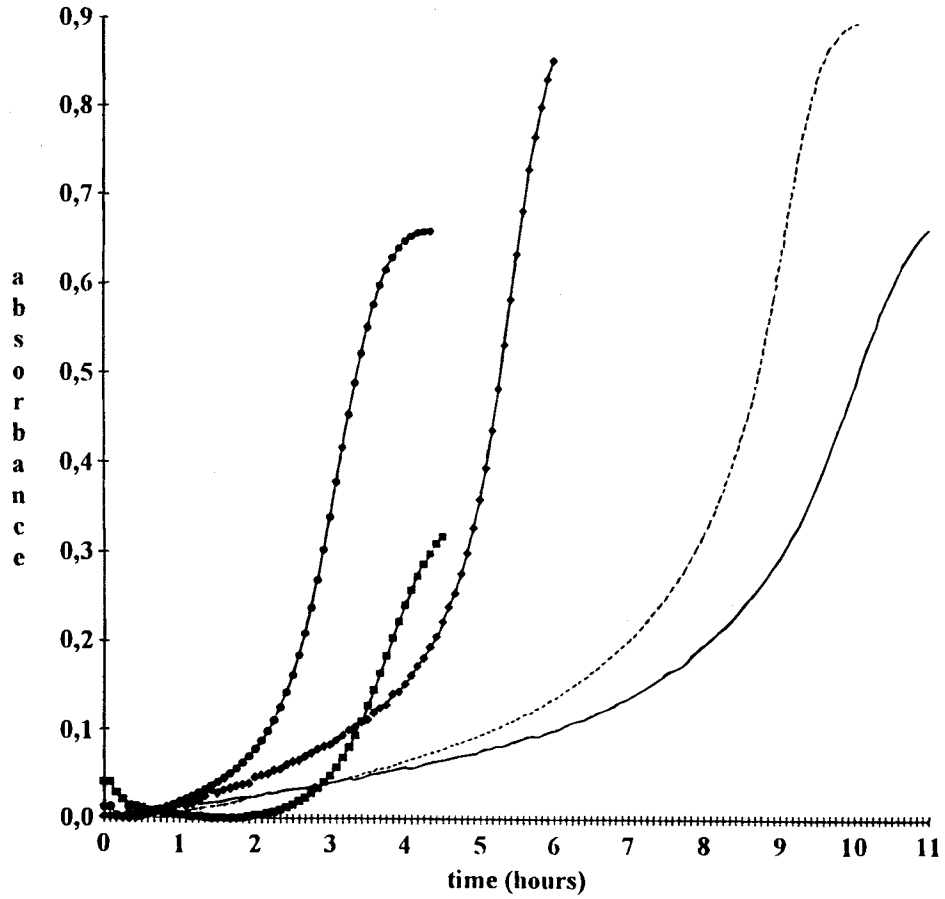

Fig. 4. Effect of nicanartine on the lag phase of LDL (0.1 μM) oxidation as measured by the increase of the diene absorption at 234 nm without AAG (figure 4-a) and with AAG = 8 μM (figure 4-b). The end of the lag-phase is defined by the intercept of the tangent to the slope of the propagation phase and the abciss axis. Nicanartine concentrations are 0 mol/mol LDL (●), 5 mols/mol LDL (■), 10 mols/mol LDL (◆), 15 mols/mol LDL (- -), 20 mols/mol LDL (—).

ratio of erythrocyte-bound to free ligand concentrations). Accordingly, the Cp/C_E ratios (CP/CE = P/E) were measured within a range of different protein concentration (Pt). These data were ascribed to equation 2 and analyzed by an iterative non-linear regression program (Microparm, INSERM, 1990)

Concentration of radiolabeled nicanartine in protein solution and in erythrocyte suspension were determined in duplicate using a Packard liquid scintillation counter (Tri-Carb 460 CD). Prior to counting, the erythrocyte suspension was bleached by the following procedure; an aliquot (50 μl) of erythrocyte suspension was mixed with sodium hypochloride (200 μl) by Vortex agitation for 10 s and after standing for 10 min, the mixture was revortexed, then 3 ml of liquid scintillation solution (Pico-Fluor™) were added to the resulting sample for counting. Supernatant were added to non-radioactive erythrocytes and processed as above to get comparable counting conditions.

RESULTS

The erythrocyte partitioning data obtained with all proteins studied fit satisfactorily equation 2. The binding parameters and the simulated blood distribution of nicanartine in the blood of a healthy individual are presented in table I and figure 2; experimental data for LDL and AAG are depicted in figure 3. Binding to gammaglobulins was negligible.

The lipoprotein oxidation could be divided into 3 phases: a lag phase during which the lipid hydroperoxides or conjugated dienes did not or slowly increase, a propagation phase during which the conjugated dienes reached their maximum value, and a decomposition phase during which the conjugated dienes decreased. Nicanartine delays the propagation phase for VLDL, for LDL with or without AAG (figure 4) and for HDL. The experimental relationship between the lag phase (y:hours) and the nicanartine concentration (x:mol/mol lipoprotein) is linear for all lipoproteins. The straight line equations defined by linear regression are $y = 0.39\ x + 6.52$ ($r^2 = 0.97$) for VLDL (0.01 μM), $y = 0.32\ x + 1.8$ ($r^2 = 0.96$) for LDL (0.1 μM) without AAG, $y = 0.168\ x + 1.4$ ($r^2 = 0.97$) for LDL (0.1 μM) and AAG (8 μM) and $y = 1.59\ x + 0.014$ ($r^2 = 0.98$) for HDL (1 μM). The slope of the LDL experimental straight line without AAG was significantly higher (nearly two-fold superior) than the slope of the LDL experimental straight line with AAG ($p < 0.01$). Given the nicanartine binding constants for plasma proteins, the binding percentage of nicanartine was calculated. With LDL (0.1 μM), 76% of nicanartine was bound to LDL.

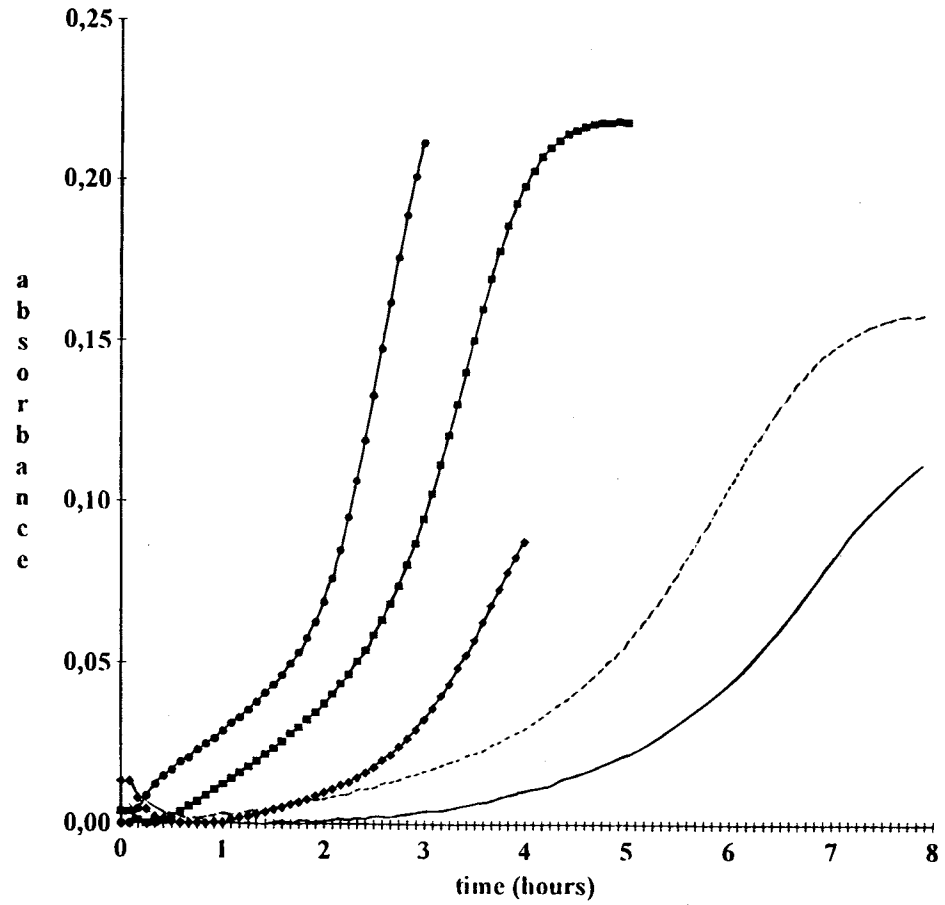

Fig. 4. Continued.

With AAG (8 μM) and LDL (0.1 μM), 89.6% of nicanartine was bound to AAG and 7.9% of nicanartine was bound to LDL. The addition of AAG in the LDL solution produced a nearly ten-fold decrease of the LDL-bound fraction of nicanartine.

DISCUSSION

Our results indicate that nicanartine was highly protein bound with an unbound fraction smaller than 1% and that interestingly 51.3% of nicanartine was lipoprotein bound. Nicanartine affinity for lipoprotein is related to the lipoprotein lipid content. This suggests that this large lipoprotein binding is due to its lipophilicity. Other lipophilic lipoprotein-bound drugs such as probucol (12) or vitamin E are very effective inhibitors of LDL oxidation. Moreover, a study on the antioxydant activity of calcium antagonists against LDL oxidation (13) indicated that the more lipophilic the drug, the greater the antioxidant effect. These results suggest that there could be a relationship between the potency of LDL oxidation inhibition and the drug affinity for LDL.

To study this relationship, we first investigated the affinity of nicanartine for the lipoproteins and the corresponding antioxidant effect. Nicanartine delayed but did not stop the oxidation of all lipoproteins but we had to study lipoprotein oxidation with different lipoprotein concentrations. The absence of relationship between affinity and antioxidant effect could be explained by the difference of lipoprotein concentrations which could have modified oxidative conditions and by different sensibilities of lipoprotein to oxidation (14).

The inhibition of lipid peroxidation is explained by a scavenging effect. The antioxidants scavenge lipid peroxyl radicals formed in LDL during the lag phase. When LDL is depleted from its antioxidant compounds, the rate of lipid peroxidation rapidly increases. Therefore, the inhibition potency of LDL oxidation should be related to the amount of antioxidant incorporated in LDL which is likely to relate to the drug affinity for LDL. Consequently, we modulated the amount of nicanartine in LDL by adding AAG in the conjugated dienes experiment. With a small amount of AAG, the interference with the rise of absorbance at 234 nm was weak and the decrease of nicanartine incorporated in LDL is important because nicanartine has a high affinity for AAG. The addition of AAG in the LDL solution involved a nearly ten-fold decrease of the LDL-bound fraction of nicanartine. This dramatic decrease in LDL-bound nicanartine resulted in a significant decrease in the antioxidant effect (two-fold) of nicanartine. This first suggests that the AAG-bound fraction of nicanartine was not available for antioxidant effect towards LDL. Secondly, since the observed decrease in antioxidant activity did not parallel the AAG-induced decrease in LDL-bound nicanartine, one may suggest that (a) the AAG-nicanartine complex is continously dissociated to compensate the disappearance in nicanartine consummed by oxidation or (b) the nicanartine affinity for plasma protein is modified in

highly oxidative conditions. These conditions would be able to involve chemical modifications of nicanartine which would decrease its affinity for AAG. To support this hypothesis, it would be interesting to study the chemical modification of nicanartine after oxidation.

These in vitro results suggest that it could be possible to delay and even to limit in vivo LDL oxidation with antioxidant drugs such as nicanartine. A dosage regimen that produces an effective concentration of nicanartine in LDL corresponding to the induced delay of LDL oxidation could limit the development of atherosclerosis process.

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