Neomycin inhibits secretion of apolipoprotein[a] by increasing retention on the hepatocyte cell surface

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Abstract Neomycin therapy reduces plasma levels of low density lipoprotein and lipoprotein[a] (Lp[a]). To determine whether neomycin directly alters the biogenesis of Lp[a], we have examined the effect of neomycin on apolipoprotein[a] (apo[a]) synthesis and secretion in primary cultures of baboon hepatocytes. Using this system, we have previously shown that apo[a] is synthesized as a lower molecular weight precursor that upon maturation becomes associated with the cell surface before release into the culture medium. Treatment of hepatocytes with 10 mM neomycin reduced levels of apo[a] in the culture medium by as much as 12-fold. Although a portion of the reduced secretion could be accounted for by a reduction in total protein synthesis, the greatest effect of neomycin on apo[a] secretion was to decrease the release of mature apo[a] from the hepatocyte cell surface into the culture medium. Treatment of hepatocyte cultures with trypsin confirmed that mature apo[a] in neomycin-treated cells was still transported to the cell surface. Examination of related antibiotics demonstrated that inhibition of apo[a] secretion is a general property shared by the deoxystreptamine antibiotics. ▪▪ The mechanism by which neomycin affects the apo[a]–cell surface interaction is not known, but neomycin is known to perturb cell surface membranes, inhibit the interaction of some ligands with their cell surface receptors, and inhibit the metabolism of phosphatidylinositol 4,5 biphosphate. These studies suggest that cell surface association of apo[a] may play a role in Lp[a] biogenesis in vivo.—**Lanford, R. E., L. Estlack, and A. L. White.** Neomycin inhibits secretion of apolipoprotein[a] by increasing retention on the hepatocyte cell surface. *J. Lipid Res.* 1996. **37:** 2055–2064.

Supplementary key words apolipoprotein B • lipoprotein[a] • primary hepatocytes • serum-free medium • phosphatidylinositol

Lipoprotein[a] (Lp[a]) is a modified form of low density lipoprotein (LDL) (1) in which apolipoprotein (apo) B is modified by covalent linkage to apo[a] (2) via a single disulfide bond. Elevated plasma levels of Lp[a] are associated with an increased incidence of cardiovascular diseases, including coronary heart disease, stroke, and peripheral vascular disease (3–5). However, the function of this lipoprotein, the mechanism of its involvement in cardiovascular disease, and the factors that regulate its biogenesis and clearance from the circulation are poorly understood.

Apo[a] is synthesized by the liver (6–8), and with the exception of the hedgehog (9, 10), Lp[a] is found only in Old World primates. The size of apo[a] is highly variable ranging from <300,000 to >800,000 in molecular weight (11–14). Apo[a] is homologous to plasminogen containing a single K5 kringle, an inactive protease domain, and a variable number of K4 kringle repeats (15). The number of K4 kringle repeats determines the size of the polypeptide (13, 16, 17). Studies with human apo[a] have demonstrated that the number of K4 repeats vary between 12 and 52 (13). The polypeptide is highly glycosylated with 28% of the mass composed of carbohydrate (18). Each kringle repeat has 1 N-linked glycosylation site and 6 potential O-linked glycosylation sites (15, 19).

The plasma levels of Lp[a] vary tremendously between individuals, ranging from <1 to >100 mg/dl (20). The levels are heritable and are very stable over an individual's lifetime with greater than 90% of the interindividual variation controlled by the APO[a] locus (21). An inverse correlation exists between the size of the apo[a] isoform and the plasma levels of Lp[a] (11, 12, 17). This correlation is not absolute, as large differences in Lp[a] concentrations are also noted within apo[a] isoforms of the same size (12, 17, 22). The plasma levels of Lp[a] are primarily controlled by the rates of synthesis rather than the rate of clearance (23, 24). In studies with cynomolgus monkeys, the level of apo[a] mRNA and the size of the apo[a] isoform accounted for 58% of the variation in Lp[a] levels. No correlations between the level of mRNA and the size of the isoform were observed (25). Thus,

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Abbreviations: apo, apolipoprotein; LDL, low density lipoprotein; Lp, lipoprotein; SDS, sodium dodecyl sulfate; SFM, serum-free medium; ER, endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; PDGF, platelet-derived growth factor.
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both transcriptional and posttranscriptional regulation appear to influence plasma Lp[a] levels.

In some individuals, no plasma Lp[a] is detectable leading to the hypothesis of null alleles (11). In some instances, especially in earlier studies, the lack of detection of Lp[a] can be ascribed to the sensitivity of Lp[a] assays (12, 26). However, null alleles are also required to explain the preponderance of single band apo[a] phenotypes compared to heterozygous genotypes (13). Studies in baboons have demonstrated the presence of two types of null alleles. Transcript-negative null alleles do not give rise to detectable levels of apo[a] mRNA, whereas transcript-positive null alleles have easily detectable levels of mRNA despite the lack of detectable plasma Lp[a] (16, 27, 28).

The properties of human and baboon Lp[a] are very similar (16, 22, 29, 30) which has permitted the use of primary cultures of baboon hepatocytes to examine the biogenesis of apo[a] and Lp[a] (6, 27, 28, 31–33). Our studies have demonstrated that apo[a] is first synthesized as a lower molecular weight precursor (27, 28, 32). The immature form of apo[a] has a prolonged residence time in the endoplasmic reticulum (ER), and the size of the apo[a] isoform influences the duration of ER residence (28). The ability of apo[a] to undergo posttranslational processing and exit from the ER appears to be responsible for the inverse correlation between apo[a] isoform size and plasma Lp[a] level (28). In addition, the inability of some apo[a] polypeptides to exit the ER accounts for the lack of detectable apo[a] associated with transcript-positive null alleles (28). Maturation of apo[a] involves processing of N-linked and addition of O-linked oligosaccharides (27, 28, 32). Cell-associated, mature apo[a] is bound to the cell surface. Interaction of LDL with cell surface-associated apo[a] results in the formation of Lp[a] and the release of newly formed Lp[a] into the culture medium (31).

In the current studies, we utilized the primary baboon hepatocyte culture system to examine the effects of the antibiotic neomycin on the secretion of apo[a]. Previous studies in humans demonstrated that neomycin lowers serum LDL cholesterol levels and that the mechanism is related to inhibition of cholesterol adsorption from the intestine (34). Gurakar and coworkers (35) demonstrated that neomycin treatment, especially when combined with niacin, lowers Lp[a] levels; however, the mechanism is not understood. Treatment of primary baboon hepatocyte cultures with neomycin resulted in an inhibition of apo[a] secretion. Further studies indicated that the inhibition of apo[a] secretion was primarily due to the lack of release of mature apo[a] from the cell surface. Examination of a series of deoxystreptamine antibiotics, of which neomycin is a member, suggested that inhibition of apo[a] secretion is a general property shared by these antibiotics. Potential mechanisms by which deoxystreptamine antibiotics may inhibit apo[a] release from the cell surface are discussed.

MATERIALS AND METHODS

Hepatocyte cultures

Hepatocytes were isolated by a two-step collagenase perfusion method as previously described (36). Liver wedge surgery was performed in accordance to procedures approved by the institutional animal research committee. Animals were immobilized with ketamine hydrochloride, and anesthesia was maintained using nonhepatotoxic pentobarbitol. Hepatocytes were cultivated on collagen-coated Primaria (Falcon) plates using serum-free medium III (SFM) as previously described (36) with the exception that thyrotropin releasing factor was omitted. All experiments were performed using confluent cultures that had been in culture for 5–7 days.

ELISA

ELISA assays for apo[a], apoB and apoA-I were performed with culture medium from primary cultures of baboon hepatocytes grown in 6-well dishes. For each assay, microtiter wells were coated with 100 µl per well of a polyclonal antibody at 1.5 µg/ml in 0.1 M sodium bicarbonate buffer, pH 9.0. The apo[a] and apoB antibodies were from Biodesign (Kennebunkport, ME), and the apoA-I antibody was from Fitzgerald Industries, Inc. (Concord, MA). Wells were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 3% bovine serum albumin (BSA). Wells were washed one time with PBS/Tween and were incubated for 3 h at 37°C with 100 µl of culture medium. Wells were washed three times with PBS/Tween and incubated for 2 h at 37°C with alkaline phosphatase-conjugated antibody diluted in PBS/Tween containing 1% BSA. Apo[a]-conjugated antibody was obtained from American Diagnostica, Inc. (Greenwich, CT), and apoB- and apoA-I-conjugated antibodies were purchased from The Binding Site (San Diego, CA). Wells were washed three times with PBS/Tween and incubated for 15 min with citrate buffer, pH 5.0, containing 0.3% H_2O_2 and 5 µg/ml OPD (O-phenylenediamine dihydrochloride). The reaction was stopped with 4 N H_2SO_4 and adsorbance at 490 nm was determined. Optical density values were captured into a Microsoft Excel™ spreadsheet and were analyzed using simple least squares linear regression techniques. The estimates of sample concentrations (ng/ml) were calculated using a regression equation fitted to a standard curve based on log–log transformed optical density data of five calibrators having known concentration. All ELISA data are the mean

of four values, duplicate assays on duplicate cultures. The protocol, reagents and standardization for these ELISA assays were a generous gift from Drs. Anil Dundale and Margaret Prack of Oncogene Sciences, Inc. (Uniondale, NY).

Radiolabeling and immunoprecipitation

Radiolabeling and immunoprecipitation experiments were conducted with primary baboon hepatocytes grown in 60-mm dishes as previously described (31, 32). For steady-state labeling, cultures were incubated in SFM containing 0.1 times the normal concentration of methionine and cysteine and 125 μCi/ml each of [^{35}S]cysteine and Expre^{35}S^{35}S label (New England Nuclear). For pulse-chase analysis, cells were preincubated for 1 h in methionine/cysteine-free SFM and then labeled for 10 min in the methionine/cysteine-free SFM containing the same concentrations of labeled isotopes as for steady-state labeling. Isotopic label was chased for 30 min to 8 h in SFM containing the normal concentration of methionine and cysteine. Cultures were washed two times with PBS and extracted with EB (50 mM Tris/HCl, pH 9.0, 100 mM NaCl, 1% NP40) as previously described (31, 32). Cell extracts and culture medium were clarified at 12,000 g for 5 min, the medium was adjusted to contain 1% NP40, and proteins were immunoprecipitated with antibodies bound to protein A agarose. Immunoprecipitated proteins were eluted from the beads in SDS gel sample buffer and analyzed by 3–10% gradient polyacrylamide SDS gels (SDS-PAGE) followed by fluorography.

Protease digestion and immunoblot analysis

Cultures of baboon hepatocytes grown in 60-mm dishes were treated with or without 10 mM neomycin for 24 h. The cultures were washed with PBS and then exposed to trypsin (1 μg/ml in PBS) for 20 min at 0°C. The trypsin solution was removed and digestion was stopped by washing two times for 5 min at 0°C with PBS containing 1 μg/ml of soybean trypsin inhibitor. Cells were then lysed in EB containing the same concentration of soybean trypsin inhibitor. Aliquots of each cell lysate were subjected to 3–10% SDS-PAGE followed by electrophoretic transfer to a PVDF blotting membrane. Membranes were probed with anti-apo[a] antibodies (Biodesign) followed by ^{125}I-labeled protein A as previously described (36).

RESULTS

Time course of neomycin inhibition of apo[a] secretion

To determine the effect of neomycin on the secretion of apo[a], cultures of primary baboon hepatocytes were treated for various lengths of time with concentrations of neomycin ranging from 1 to 100 mM. The culture medium was analyzed in an ELISA that detects both free apo[a] and Lp[a], as much of the apo[a] secreted from baboon hepatocyte cultures is not bound to apoB (32). Inhibition of apo[a] secretion by neomycin was apparent as early as 6 h after the initiation of the treatment (data not shown). The inhibition was more pronounced at 24 and 48 h of drug treatment. The level of apo[a] secretion was reduced 5.5-fold after 48 h of treatment with 10 mM neomycin (**Fig. 1**). The level of inhibition was even greater at 100 mM neomycin, but this level of the antibiotic was toxic to the hepatocyte cultures.
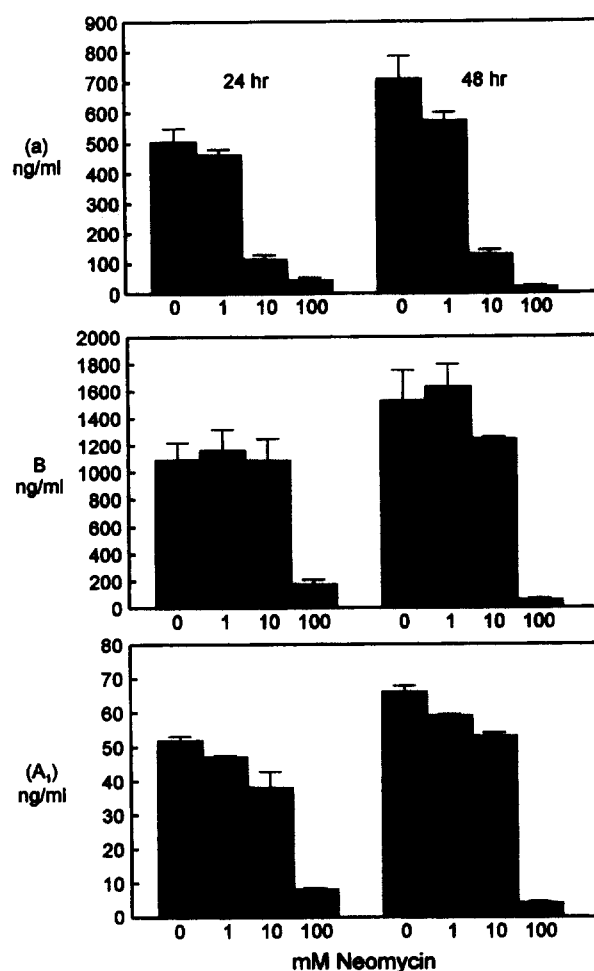

Fig. 1. Time course and specificity of neomycin for inhibition of apo[a] secretion. Hepatocyte cultures were treated with 0, 1, 10, or 100 mM neomycin. The media were harvested after 24 and 48 h of treatment and were analyzed by ELISA for apo[a], apoB, and apoA-I as described in Materials and Methods. The standard error of the mean is indicated by the thin line at the top of each bar. Treatment with 100 mM neomycin resulted in toxicity to the cultures and inhibition of all protein secretion, whereas 10 mM neomycin primarily inhibited the secretion of apo[a].
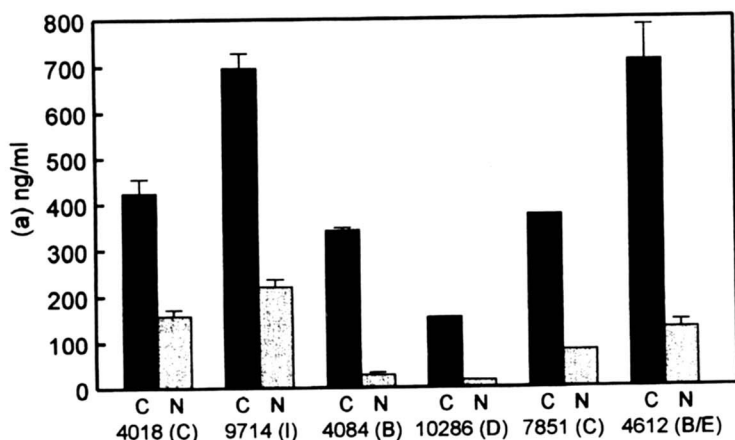
Fig. 2. Neomycin inhibits the secretion of different apo[a] isoforms. Hepatocyte cultures derived from baboon donors expressing different isoforms of apo[a] were treated with 10 mM neomycin. The media were harvested after 48 h of treatment and were analyzed by ELISA for apo[a]. Values from control and treated cultures are indicated by C and N, respectively. The standard error of the mean is indicated by the thin line at the top of each bar with the exception of cultures from animals 10286 and 7851 for whom replicate measurements were not available. The animal identification numbers (4018, 9714, 4084, 10286, 7851, 4612) are given followed by the isoform designations in parentheses. Apo[a] isoforms in the baboon have been grouped into 12 size classes designated A for the largest to L for the smallest (22).

Specificity of neomycin for inhibition of apo[a] secretion

The toxicity at 100 mM neomycin was apparent by visual inspection of the cultures using phase-contrast microscopy and by an analysis of total protein synthesis performed during pulse-chase analyses (see below). The nonspecific effect of 100 mM neomycin and the specificity of 10 mM neomycin for the inhibition of apo[a] secretion were best illustrated by comparison of the levels of secretion for apo[a] with that of apoB and apoA-I (Fig. 1). At 100 mM neomycin, the level of secretion of all three apolipoproteins was markedly reduced. The level of apoB in the culture medium was reduced by 96.3% after 48 h of drug treatment, and the level of apoA-I decreased by 94.1%. However, at 10 mM neomycin, the secretion of apo[a] was reduced by a much greater level than that of apoB or apoA-I. Apo[a] secretion was reduced by 81.9%, while apoB and apoA-I were reduced by 18.7% and 21.5%, respectively. At 10 mM neomycin, the level of inhibition for apoB and apoA-I secretion was approximately equivalent to the level of inhibition observed for total protein synthesis during pulse-chase analyses (see below).

The effect of neomycin on the secretion of apo[a] was examined using primary baboon hepatocyte cultures derived from six different animals. The cultures expressed apo[a] isoforms of different sizes, and the untreated cultures secreted apo[a] at markedly different levels. Hepatocytes were cultured in medium containing 10 mM neomycin; the medium was changed after 6 h as a wash-out to remove previously synthesized apo[a]; and the cultures were maintained in 10 mM neomycin for 48 h. Apo[a] levels in the medium were determined by ELISA. The inhibition of apo[a] secretion by neomycin was apparent in all cultures (**Fig. 2**). The level of inhibition ranged from 62.7% to 91.8%.

Neomycin inhibits apo[a] release from the cell surface

To define the step in apo[a] synthesis and secretion being affected by neomycin, immunoprecipitation studies were performed. Cultures were treated with 10 mM neomycin for 24 h and then labeled for 24 h with ^{35}S methionine and cysteine in the presence of the antibiotic. Apo[a] and apoB were immunoprecipitated from the cell lysate and the culture medium and were analyzed by SDS-PAGE. In the untreated control cell lysates, the precursor and the cell surface-associated mature form of apo[a] were detected (**Fig. 3**). ApoB coprecipitated with apo[a] in the culture medium but not in the cell lysate due to the formation of Lp[a] after secretion of apo[a]. In the cell lysate from the neomycin-treated cultures, the level of precursor and mature apo[a] were very similar to that detected in the untreated cultures. However, in the medium, the level of apo[a] and coprecipitating apoB were dramatically reduced. Quantitation of the apo[a] bands in Fig. 3 by laser densitometry demonstrated that the level of apo[a] in the medium was
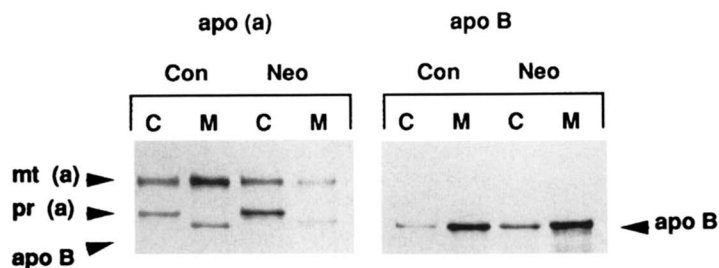

Fig. 3. Immunoprecipitation of apo[a] and apoB after steady-state labeling. Control cultures (Con) or hepatocytes treated with 10 mM neomycin for 24 h (Neo) were labeled with ^{35}S methionine and cysteine (125 µCi/ml each) for 24 h in the presence or absence of neomycin. Apo[a] and apoB were immunoprecipitated from the cell lysates (C) and the culture media (M) as described in Materials and Methods. Precipitated proteins were analyzed by 3–10% gradient SDS-PAGE and fluorography.
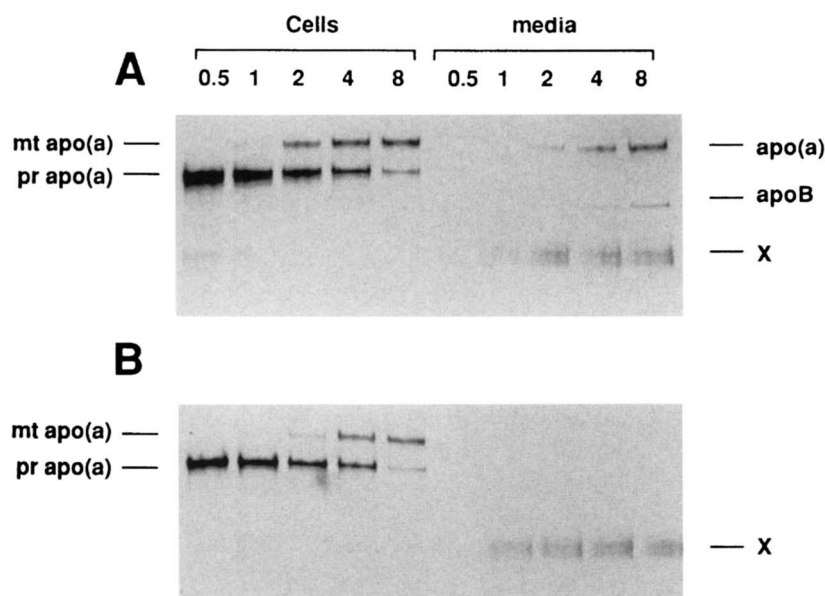
Fig. 4. Immunoprecipitation of apo[a] after pulse-chase labeling. Hepatocyte cultures were treated with 10 mM neomycin (B) for 24 h and then were labeled for 10 min with ^{35}S methionine and cysteine. The cultures were chased for periods of 0.5 to 8 h in unlabeled medium containing neomycin. Control cultures (A) were treated in the same manner except the medium contained no neomycin. Apo[a] was immunoprecipitated from the cell lysates and culture media and analyzed by 3–10% gradient SDS-PAGE and fluorography. The positions of the precursor (pr) and mature (mt) forms of apo[a] are indicated as is the position of nonspecifically precipitated protein (X).

decreased by 12-fold in the neomycin-treated cultures. When total immunoprecipitated apo[a] was taken into account, the percentage of total apo[a] being secreted was 9.5% and 55.5% for neomycin and control cultures, respectively; which is equivalent to an 83% inhibition of apo[a] secretion. This effect was specific for apo[a], as the level of apoB immunoprecipitated from the culture medium of treated and untreated cultures was very similar (Fig. 3). These data suggest that the greatest effect of neomycin was not on synthesis or maturation of apo[a], but possibly some event involved in secretion or release from the cell surface.

To further explore the mechanism by which neomycin inhibits the secretion of apo[a], pulse-chase labeling experiments were performed. Cultures were treated with 10 mM neomycin for 24 h, labeled for 10 min with ^{35}S methionine and cysteine, and chased in unlabeled medium containing neomycin for times between 30 min and 8 h. Apo[a] was immunoprecipitated from the cell lysates and culture media and analyzed by SDS-PAGE. Comparison of the level of apo[a] immunoprecipitated from the cell lysates following 30 min of chase indicated that, in this experiment, the level of apo[a] synthesis was reduced by approximately 40% in the neomycin-treated cultures in comparison to the untreated cultures (**Fig. 4**). This level of inhibition was similar to the reduction observed for total trichloroacetic acid-precipitable counts (20–30% reduction) and the reduction in synthesis apoB (20–30%) in the same experiment. Analyses of apo[a] synthesis in pulse-chase experiments with shorter chase periods (0–30 min) confirmed that the reduction was due to a decrease in apo[a] synthesis rather than increased degradation. Thus, the effects of neomycin on apo[a] synthesis do not appear to be specific for apo[a]

and are most likely related to the general inhibition of all protein synthesis at this concentration of neomycin.

The mature form of apo[a] became apparent after 2 h of chase in both untreated and neomycin-treated cultures. After 8 h of chase, the predominant cell-associated form of apo[a] was the mature form (Fig. 4). Thus, the kinetics of maturation and the percentage of apo[a] being converted to the mature form were similar in the presence and absence of neomycin. In the absence of the drug, secretion of apo[a] into the culture medium could be detected after 2 h of chase, and continued to increase throughout the chase period. However, no secretion of apo[a] was observed in the neomycin-treated cultures over this time period.

Although these data imply that neomycin inhibits release of apo[a] from the cell surface, the possibility still existed that in neomycin-treated cultures mature apo[a] was not reaching the cell surface, but was being retained in some post-ER compartment. To address this possibility, susceptibility to digestion with protease was used as a marker for cell surface exposure of apo[a]. Hepatocyte cultures were treated with 10 mM neomycin for 24 h. The medium was removed, the cells were treated with trypsin for 20 min on ice, and apo[a] in the cell lysate was analyzed by immunoblot. In cultures not exposed to trypsin, a prominent band was observed for the mature form of apo[a] (**Fig. 5**). The size of the mature form was dramatically reduced by protease treatment, but a large protected apo[a] fragment was still apparent. Essentially all of the mature form of apo[a] was susceptible to protease treatment, and no difference was observed between the untreated and neomycin-treated cultures. These results demonstrate that neomycin does not affect the transport of apo[a] to the cell surface, and
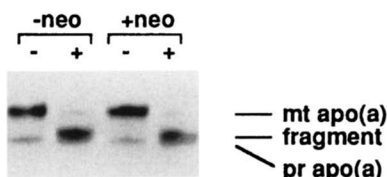
Fig. 5. Immunoblot analysis of apo[a] after treatment of cells with trypsin. Hepatocyte cultures were maintained in medium with (+neo) or without (-neo) 10 mM neomycin for 24 h. The medium was removed and the cells were chilled to 0°C and were incubated with (+) or without (-) trypsin (1 μg/ml) for 20 min. Apo[a] present in the cell lysate was analyzed by immunoblot as described in Materials and Methods. The mature (mt) and precursor (pr) forms of apo[a] are indicated as is the proteolytic fragment resulting from trypsin treatment of the cell surface-associated mature form of apo[a].

thus the inhibition of secretion must be due to the lack of release of apo[a] from the cell surface.

Deoxystreptamine antibiotics inhibit apo[a] secretion

To better understand the mechanism by which neomycin inhibits apo[a] secretion, a series of related antibiotics was evaluated to determine whether a common structural motif capable of inhibiting apo[a] secretion could be identified. Neomycin is a member of the class of antibiotics designated aminoglycoside-aminocyclitol antibiotics (37). Two major subclasses of aminoglycoside-aminocyclitol antibiotics have been defined: the streptadine antibiotics of which streptomycin is a member and the deoxystreptamine antibiotics. The deoxystreptamine antibiotics are divided into subgroups depending on whether the deoxystreptamine ring is modified in the 1,2 or 1,3 positions (37). Neomycin, ribostamycin, and paromomycin are among the 1,2 modified deoxystreptamines (**Fig. 6,** top row), while
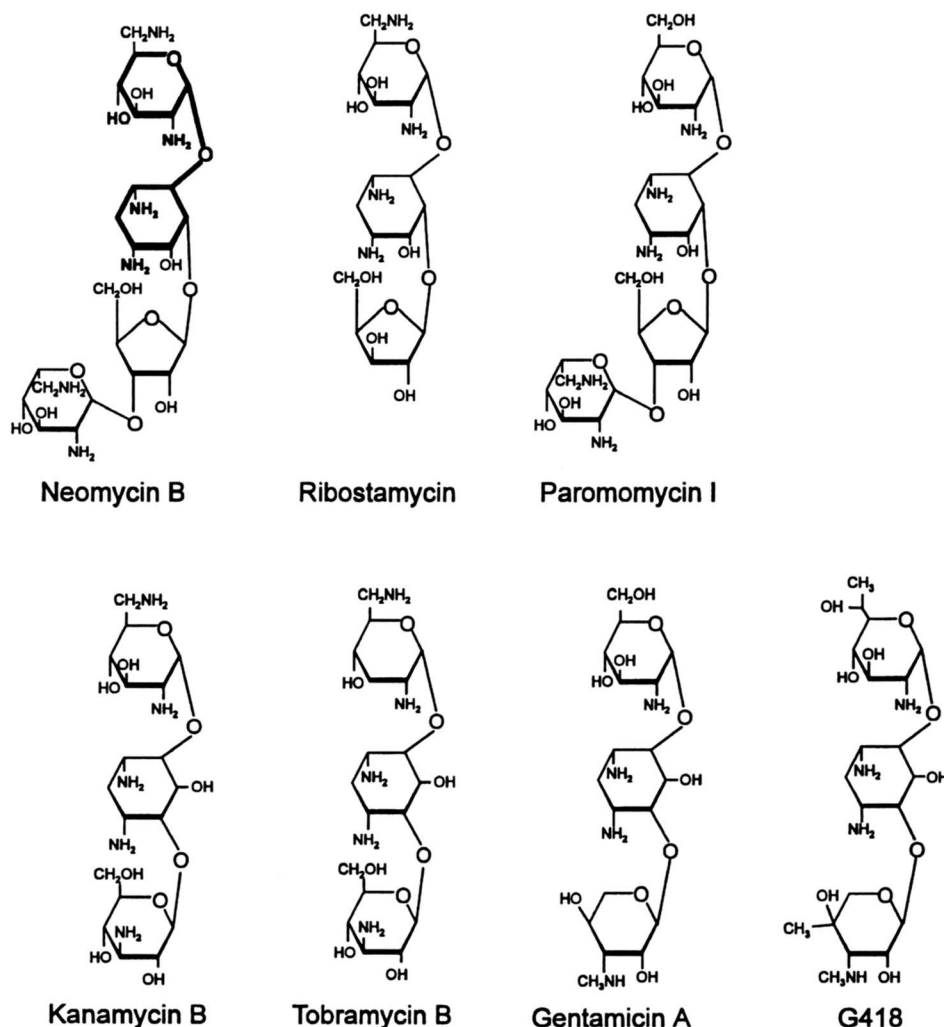

Fig. 6. Structure of various deoxystreptamine antibiotics. The structures of the antibiotics used in this study are indicated. The structures in the top row have 1,2 modified deoxystreptamine rings, while those in the bottom row have 1,3 modified deoxystreptamine rings. The structure in common to all of the antibiotics is indicated in bold for neomycin.
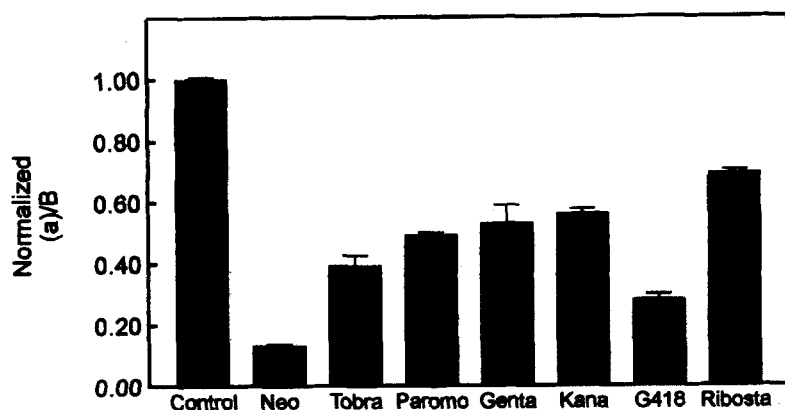
Fig. 7. Deoxystreptamine antibiotics inhibit the secretion of apo[a]. Hepatocyte cultures were treated for 48 h with various deoxystreptamine antibiotics; Control (untreated), Neo (10 mM neomycin B), Tobra (100 mM tobramycin B), Paromo (10 mM paromomycin 1), Genta (10 mM gentamicin A), Kana (100 mM kanamycin B), G418 (0.1 mM geneticin) and Ribosta (10 mM ribostamycin). The culture media were analyzed by ELISA for apo[a] and apoB. The data were normalized by calculating the ratio of secreted apo[a] to secreted apoB and then dividing all values by the ratio obtained for control cultures. The standard error of the mean is indicated by a thin line at the top of each bar.

kanamycin, gentamicin, G418, and tobramycin are within the 1,3 modified subgroup (Fig. 6, bottom row).

Each of the compounds was tested for the ability to inhibit the secretion of apo[a] from baboon hepatocyte cultures. Cultures were treated for 48 h with 1, 10, and 100 mM concentrations of each of the antibiotics, and secretion of apo[a] was measured by ELISA. As the antibiotics displayed widely different levels of toxicity, the results were normalized to obtain meaningful data. Normalization was performed by calculating the ratio of secreted apo[a] to secreted apoB and then dividing all values by the ratio obtained for the untreated controls. Each of the compounds displayed some specific inhibition of apo[a] secretion in comparison to apoB secretion and untreated cultures (**Fig. 7**), indicating that some structural feature common to each of the antibiotics was involved. Comparison of the structures of each of the compounds revealed a common structural motif comprised of the deoxystreptamine ring and the aminoglycoside ring structure (bold structure on neomycin in Fig. 6). This minimum structure is similar to neamine. Analysis of the effects of neamine on the secretion of apo[a] and normalization of the values using secretion of apoB revealed a similar inhibition of apo[a] secretion as observed with neomycin.

DISCUSSION

A number of compounds have been reported to reduce Lp[a] levels in vivo, most notably are neomycin, niacin, and anabolic steroids (35, 38–41). Whether these compounds have a direct effect on the synthesis and secretion of apo[a] and/or the biogenesis of Lp[a] or whether the reduction in in vivo levels of Lp[a] are due to a secondary effect of the compound has not been addressed due to the lack of suitable culture systems for the analysis of Lp[a] biogenesis. We have utilized primary cultures of baboon hepatocytes to examine Lp[a] biogenesis and have previously demonstrated that

apo[a] is synthesized as a lower molecular weight precursor that has a prolonged residence time in the ER (28, 32) and that following maturation apo[a] becomes associated with the cell surface (31). LDL can interact with the cell surface form of apo[a] and induce the formation and release of an Lp[a] particle. Thus, this system is particularly well suited for the analysis of compounds that may directly affect Lp[a] biogenesis.

In this study, we examined the effects of neomycin on Lp[a] biogenesis. The data indicate that neomycin markedly inhibits the secretion of apo[a]. The inhibition of secretion could be partially ascribed to an inhibition of protein synthesis by neomycin. The general inhibition of protein synthesis was observed for apo[a] as well as apoB and apoA-I. However, neomycin also had a very specific effect on the secretion of apo[a]. A series of experiments including steady-state labeling, pulse-chase labeling, and protease sensitivity demonstrated that the greatest effect of neomycin on the inhibition of secretion of apo[a] was not at the level of synthesis, intracellular degradation, or transport to the cell surface. The greatest effect of neomycin on the secretion of apo[a] was to inhibit the release of mature apo[a] from the cell surface. Although neomycin inhibits the release of apo[a] from the cell surface, it does not enhance the binding of exogenously added apo[a] (data not shown). This would suggest that the interaction of newly synthesized apo[a] with the hepatocyte cell surface as it exits the secretory pathway differs in some manner from the interaction of apo[a] added to the culture medium, or alternatively, that apo[a] synthesized and secreted from untreated cultures differs from apo[a] synthesized in the presence of neomycin in some manner important for interaction and release from the hepatocyte cell surface. The interaction of apo[a] with the cell surface is dependent upon the lysine and proline binding pockets of the apo[a] kringle repeats (31), and neomycin partially inhibits the ability of proline to release apo[a] from the cell surface (data not shown). Thus, neomycin appears to specifically alter the interaction between newly syn-

thesized apo[a] and its cell surface binding site. At this time, the nature of the binding site for apo[a] on the hepatocyte cell surface, or even whether the cell surface binding site for apo[a] is a single entity, is not known. Lp[a] can interact with many components of the cell surface and extracellular matrix. Our previous studies have indicated that heparin sulfate proteoglycan and the plasminogen receptor are not required for the binding of apo[a] to the hepatocyte cell surface (31). Lp[a] also binds to other glycosaminoglycans (42), fibronectin (43), fibrin (44), and the LDL receptor (45, 46), all of which are found on the cell surface. Whether the cell surface binding site for apo[a] is a molecule known to interact with Lp[a] would be questionable, as the interaction of LDL with cell surface apo[a] to induce the formation of Lp[a] also results in the release of the newly formed lipoprotein particle from the cell surface.

The mechanism by which neomycin inhibits the release of apo[a] from the hepatocyte cell surface is not known, but several possibilities can be postulated based on known properties of neomycin. The effects of neomycin on absorption of cholesterol from the intestine are related to the ability of neomycin to interact with bile acids and neutral sterols (47, 48). This property alone could perturb the cell surface in such a manner as to affect the interaction of apo[a] with its putative hepatocyte cell surface receptor. However, the interaction of neomycin with membranes is more extensive and specific than originally recognized. Neomycin interacts with inositol phospholipids, with the highest affinity for phosphatidylinositol 4,5 biphosphate. These interactions have been shown to perturb membrane structure (49–52) and to inhibit the metabolism of inositol phospholipids (53–57).

Inhibition of phosphatidylinositol turnover can have pleotropic effects on the cell. Phosphatidylinositol 4,5 biphosphate is a component of the plasma membrane and is cleaved by phospholipase C to form the second messengers inositol 1,4,5 triphosphate and diacylglycerol. Inositol triphosphate induces the release of intracellular stores of Ca^{2+} and diacylglycerol activates protein kinase C. Neomycin is routinely used as an inhibitor of processes dependent on these important secondary messengers. Activation of protein kinase C has numerous effects on cellular metabolism as does the release of intracellular stores of Ca^{2+}, including changes in the cell surface, induction of DNA synthesis, and actin reorganization. Studies with neomycin have also demonstrated that it directly inhibits the interaction of certain ligands with their receptors. Neomycin blocks the interaction of platelet-derived growth factor (PDGF), herpes simplex virus 1, and thrombin with their respective receptors (58–61). The actions of neomycin to inhibit PDGF and thrombin-induced changes have been ascribed to both inhibition of phosphatidylinositol turnover and inhibition of receptor binding. Whether neomycin acts through more than one mechanism in some systems must be considered. Neomycin has been shown to inhibit other hormone-induced effects including those induced by bradykinin (57), aldosterone (62), eclosion hormone (63). Whether these inhibitions are due to direct interference of receptor binding by the hormones or inhibition of phosphatidylinositol turnover is not known.

The inhibition of the release of apo[a] from the hepatocyte cell surface is the first example in which neomycin inhibits the release of a ligand from its binding site. The mechanism by which neomycin inhibits the release of apo[a] from the cell surface can be experimentally approached in several ways including cell surface binding studies and the use of other inhibitors of phosphatidylinositol turnover. In an initial effort to define the portion of the neomycin structure responsible for inhibiting the release of apo[a] from the cell surface, we examined a series of related deoxystreptamine antibiotics. All deoxystreptamine antibiotics examined inhibited the release of apo[a] from the cell surface. The minimal common structure of these compounds encompasses the deoxystreptamine ring as well as the aminoglycoside ring which is similar to the structure for neamine. Neamine was also capable of inhibiting secretion of apo[a]. The use of smaller molecules will be advantageous in studies to determine the mechanism by which these compounds inhibit the release of apo[a] from the cell surface.

Neomycin has been shown to reduce serum levels of LDL cholesterol in vivo (34), and neomycin, especially in combination with niacin, was shown to reduce plasma levels of Lp[a] (35). Previous studies suggested that the primary mechanism of the reduction of LDL cholesterol by neomycin was the inhibition of adsorption of cholesterol from the intestine (34). This effect may be due to the ability of neomycin to precipitate bile acids and neutral sterols in micelles (47, 48). The mechanism by which neomycin reduces Lp[a] levels is not understood. The same mechanism of action of neomycin on Lp[a] as for LDL is unlikely, as no significant effect of bile acid sequesterants and HMG-CoA reductase inhibitors was observed for Lp[a] (reviewed in 38).

The observation that neomycin inhibits the release of apo[a] from the cell surface in baboon hepatocytes in vitro invokes speculation on whether release from the hepatocyte cell surface is the mechanism operative in the reduction of Lp[a] levels in vivo. The fact that orally administered neomycin is poorly absorbed and the relatively high levels of neomycin required for an in vitro effect might suggest that insufficient neomycin would be available to induce the effect observed in vivo. A

first-pass phenomenon of the liver might increase the local effective concentration of neomycin. In addition, the effect of neomycin on the release of apo[a] from the hepatocyte cell surface in vitro is only partially reversed during a short washout period (data not shown). At this time, it is not possible to evaluate the potential of neomycin to affect apo[a] on the hepatocyte cell surface in vivo, but the in vitro observations invite speculation that cell surface association of apo[a] may play a role in Lp[a] biogenesis.∎

REFERENCES

1. Berg, K. 1963. A new serum type system in man-Lp system. *Acta. Pathol. Microbiol. Scand.* **59:** 369–382.
2. Utermann, G. 1989. The mysteries of lipoprotein[a]. *Science.* **246:** 904–910.
3. Scanu, A. M., and G. M. Fless. 1990. Lipoprotein [a]. Heterogeneity and biological relevance. *J. Clin. Invest.* **85:** 1709–1715.
4. Liu, A. C., and R. M. Lawn. 1994. Lipoprotein[a] and atherogenesis. *Trends Cardiovasc. Med.* **4:** 40–44.
5. Valentine, R. J., P. A. Grayburn, G. L. Vega, and S. M. Grundy. 1994. Lp[a] lipoprotein is an independent, discriminating risk factor for premature peripheral atherosclerosis among white men. *Arch. Intern. Med.* **154:** 801–806.
6. Rainwater, D. L., and R. E. Lanford. 1989. Production of lipoprotein[a] by primary baboon hepatocytes. *Biochim. Biophys. Acta.* **1003:** 30–35.
7. Kraft, H. G., H. J. Menzel, F. Hoppichler, W. Vogel, and G. Utermann. 1989. Changes of genetic apolipoprotein phenotypes caused by liver transplantation. Implications for apolipoprotein synthesis. *J. Clin. Invest.* **83:** 137–142.
8. Tomlinson, J. E., J. W. McLean, and R. M. Lawn. 1989. Rhesus monkey apolipoprotein[a]. Sequence, evolution, and sites of synthesis. *J. Biol. Chem.* **264:** 5957–5965.
9. Laplaud, P. M., L. Beaubatie, S. C. Rall, Jr., G. Luc, and M. Saboureau. 1988. Lipoprotein [a] is the major apoB-containing lipoprotein in the plasma of a hibernator, the hedgehog (*Erinaceus europaeus*). *J. Lipid Res.* **29:** 1157–1170.
10. Lawn, R. M., N. W. Boonmark, K. Schwartz, G. E. Lindahl, D. P. Wade, C. D. Byrne, K. J. Fong, K. Meer, and L. Patthy. 1995. The recurring evolution of lipoprotein[a]—insights from cloning of hedgehog apolipoprotein[a]. *J. Biol. Chem.* **270:** 24004–24009.
11. Utermann, G., H. J. Menzel, H. G. Kraft, H. C. Duba, H. G. Kemmler, and C. Seitz. 1987. Lp[a] glycoprotein phenotypes. Inheritance and relation to Lp[a]-lipoprotein concentrations in plasma. *J. Clin. Invest.* **80:** 458–465.
12. Gaubatz, J. W., K. I. Ghanem, J. Guevara, Jr., M. L. Nava, W. Patsch, and J. D. Morrisett. 1990. Polymorphic forms of human apolipoprotein [a]: inheritance and relationship of their molecular weights to plasma levels of lipoprotein [a]. *J. Lipid Res.* **31:** 603–613.
13. Lackner, C., J. C. Cohen, and H. H. Hobbs. 1993. Molecular definition of the extreme size polymorphism in apolipoprotein[a]. *Hum. Mol. Genet.* **2:** 933–940.
14. Marcovina, S. M., Z. H. Zhang, V. P. Gaur, and J. J. Albers. 1993. Identification of 34 apolipoprotein[a] isoforms: differential expression of apolipoprotein[a] alleles between American Blacks and Whites. *Biochem. Biophys. Res. Commun.* **191:** 1192–1196.
15. McLean, J. W., J. E. Tomlinson, W. J. Kuang, D. L. Eaton, E. Y. Chen, G. M. Fless, A. M. Scanu, and R. M. Lawn. 1987. cDNA sequence of human apolipoprotein [a] is homologous to plasminogen. *Nature.* **330:** 132–137.
16. Hixson, J. E., M. L. Britten, G. S. Manis, and D. L. Rainwater. 1989. Apolipoprotein [a] (apo[a]) glycoprotein isoforms result from size differences in apo[a] mRNA in baboons. *J. Biol. Chem.* **264:** 6013–6016.
17. Lackner, C., E. Boerwinkle, C. C. Leffert, T. Rahmig, and H. H. Hobbs. 1991. Molecular basis of apolipoprotein [a] isoform size heterogeneity as revealed by pulsed-field gel electrophoresis. *J. Clin. Invest.* **87:** 2153–2161.
18. Fless, G. M., M. E. ZumMallen, and A. M. Scanu. 1986. Physicochemical properties of apolipoprotein [a] and lipoprotein [a-] derived from the dissociation of human plasma lipoprotein [a]. *J. Biol. Chem.* **261:** 8712–8718.
19. Kratzin, H., V. W. Armstrong, M. Niehaus, N. Hilschman, and D. Seidel. 1987. Structural relationship of an apolipoprotein [a] phenotype (570 kDa) to plasminogen: homologous kringle domains are linked by carbohydrate-rich regions. *Biol. Chem. Hoppe-Seyler.* **368:** 1533–1544.
20. Gaubatz, J. W., G. L. Cushing, and J. D. Morrisett. 1986. Quantitation, isolation, and characterization of human lipoprotein [a]. *Methods Enzymol.* **129:** 167–186.
21. Boerwinkle, E., C. C. Leffert, J. Lin, C. Lackner, G. Chiesa, and H. H. Hobbs. 1992. Apolipoprotein [a] gene accounts for greater than 90% of the variation in plasma lipoprotein [a] concentrations. *J. Clin. Invest.* **90:** 52–60.
22. Rainwater, D. L., G. S. Manis, and J. L. VandeBerg. 1989. Hereditary and dietary effects on apolipoprotein [a] isoforms and Lp[a] in baboons. *J. Lipid Res.* **30:** 549–558.
23. Krempler, F., G. M. Kostner, and K. Bolzano. 1980. Turnover of lipoprotein[a] in man. *J. Clin. Invest.* **65:** 1483–1490.
24. Rader, D. J., W. Cain, L. A. Zech, D. Usher, and H. B. Brewer, Jr. 1993. Variation in lipoprotein[a] concentrations among individuals with the same apolipoprotein[a] isoform is determined by the rate of lipoprotein[a] production. *J. Clin. Invest.* **91:** 443–447.
25. Azrolan, N., D. Gavish, and J. L. Breslow. 1991. Plasma lipoprotein[a] concentration is controlled by apolipoprotein[a] (apo[a]) protein size and the abundance of hepatic apo[a] mRNA in a cynomolgus monkey model. *J. Biol. Chem.* **266:** 13866–13872.
26. Albers, J. J., J. L. Adolphson, and W. R. Hazzard. 1977. Radioimmunoassay of human plasma Lp[a] lipoprotein. *J. Lipid Res.* **18:** 331–338.
27. White, A. L., D. L. Rainwater, J. E. Hixson, L. E. Estlack, and R. E. Lanford. 1994. Intracellular processing of apo[a] in primary baboon hepatocytes. *Chem. Phys. Lipids.* **67-68:** 123–133.
28. White, A. L., J. E. Hixson, D. L. Rainwater, and R. E. Lanford. 1994. Molecular basis for "null" lipoprotein[a] phenotypes and the influence of apolipoprotein[a] size on plasma lipoprotein[a] level in the baboon. *J. Biol. Chem.* **269:** 9060–9066.
29. Rainwater, D. L., G. S. Manis, and R. S. Kushwaha. 1986. Characterization of an unusual lipoprotein similar to human lipoprotein[a] isolated from the baboon, Papio sp. *Biochim. Biophys. Acta.* **877:** 75–78.
30. Rainwater, D. L., and G. S. Manis. 1988. Immunochemical characterization and quantitation of lipoprotein [a] in baboons. *Atherosclerosis.* **73:** 23–31.
31. White, A. L., and R. E. Lanford. 1994. Cell surface assembly of lipoprotein[a] in primary cultures of baboon hepatocytes. *J. Biol. Chem.* **269:** 28716–28723.

32. White, A. L., D. L. Rainwater, and R. E. Lanford. 1993. Intracellular maturation of apolipoprotein[a] and assembly of lipoprotein[a] in baboon hepatocytes. *J. Lipid Res.* **34:** 509–517.

33. White, A. L., and R. E. Lanford. 1995. Biosynthesis and metabolism of lipoprotein[a]. *Curr. Opin. Lipidol.* **6:** 75–80.

34. Sedaghat, A., P. Samuel, J. R. Crouse, and E. H. Ahrens. 1975. Effects of neomycin on adsorption, synthesis and/or flux of cholesterol in man. *J. Clin. Invest.* **55:** 12–21.

35. Gurakar, A., J. M. Hoeg, G. Kostner, N. M. Papadopoulos, and H. B. Brewer, Jr. 1985. Levels of lipoprotein Lp[a] decline with neomycin and niacin treatment. *Atherosclerosis.* **57:** 293–301.

36. Lanford, R. E., K. D. Carey, L. E. Estlack, G. C. Smith, and R. V. Hay. 1989. Analysis of plasma protein and lipoprotein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium. *In Vitro Cell. Dev. Biol.* **25:** 174–182.

37. Rinehart, K. L. 1969. Comparative chemistry of the aminoglycoside and aminocyclitol antibiotics. *J. Infect. Dis.* **119:** 345–350.

38. Spinler, S. A., and M. J. Cziraky. 1994. Physiological function, association with athersclerosis, and effects of lipid-lowering drug therapy. *Ann. Pharmacother.* **28:** 343–351.

39. Sirtori, C. R., C. Manzoni, and M. R. Lovati. 1991. Mechanisms of lipid-lowering agents. *Cardiology.* **78:** 226–235.

40. Crook, D., M. Sidhu, M. Seed, M. O'Donnell, and J. C. Stevenson. 1992. Lipoprotein Lp[a] levels are reduced by danazol, an anabolic steroid. *Atherosclerosis.* **92:** 41–47.

41. Albers, J. J., H. M. Taggart, D. Applebaum-Bowden, S. Haffner, C. H. Chesnut, and W. R. Hazzard. 1984. Reduction of lecithin-cholesterol acyltransferase, apolipoprotein D and the Lp[a] lipoprotein with the anabolic steroid stanozolol. *Biochim. Biophys. Acta.* **795:** 293–296.

42. Bihari-Varga, M., E. Gruber, M. Rotheneder, R. Zechner, and G. M. Kostner. 1988. Interaction of lipoprotein Lp[a] and low density lipoprotein with glycosaminoglycans from human aorta. *Arteriosclerosis.* **8:** 851–857.

43. Salonen, E. M., M. Jauhiainen, L. Zardi, A. Vaheri, and C. Ehnholm. 1989. Lipoprotein [a] binds to fibronectin and has a serine proteinase activity capable of cleaving it. *EMBO J.* **8:** 4035–4040.

44. Loscalzo, J., M. Weinfeld, G. M. Fless, and A. M. Scanu. 1990. Lipoprotein [a], fibrin binding, and plasminogen activation. *Arteriosclerosis.* **10:** 240–245.

45. Armstrong, V. W., B. Harrach, H. Robenek, M. Helmhold, A. K. Walli, and D. Seidel. 1990. Heterogeneity of human lipoprotein [a]: cytochemical and biochemical studies on the interaction of two Lp[a] species with the LDL receptor. *J. Lipid Res.* **31:** 429–441.

46. Harkes, L., G. Jurgens, A. Holasek, and T. J. C. Van Berkel. 1988. In vivo studies on the binding sites for lipoprotein [a] on parenchymal and non-parenchymal rat liver cells. *FEBS Lett.* **227:** 27–31.

47. Thompson, G. R., J. Barrowman, L. Gutierrez, and R. H. Dowling. 1971. Action of neomycin on the intraluminal phase of lipid absorption. *J. Clin. Invest.* **50:** 319–323.

48. Thompson, G. R., M. MacMahon, and P. Claes. 1970. Precipitation of neomycin compounds of fatty acids and cholesterol from mixed micellar solutions. *Eur. J. Clin. Invest.* **1:** 40–47.

49. Forge, A., G. Zajic, S. Davies, N. Weiner, and J. Schacht. 1989. Gentamicin alters membrane structure as shown by freeze-fracture of liposomes. *Hearing Res.* **37:** 129–139.

50. Riaz, M., N. D. Weiner, and J. Schacht. 1989. Separation of liposome populations differing in phosphoinositide content by chromatography on immobilized neomycin. *J. Pharm. Sci.* **78:** 172–175.

51. Au, S., N. D. Weiner, and J. Schacht. 1987. Aminoglycoside antibiotics preferentially increase permeability phosphoinositide-containing membranes: a study with carboxyfluorescein in liposomes. *Biochim. Biophys. Acta.* **902:** 80–86.

52. Williams, S. E., and J. Schacht. 1986. Binding of neomycin and calcium to phospholipids and other anionic compounds. *J. Antibiot.* **39:** 457–462.

53. Tysnes, O. B., A. J. Verhoeven, and H. Holmsen. 1987. Neomycin inhibits agonist-stimulated polyphosphoinositide metabolism and responses in human platelets. *Biochem. Biophys. Res. Commun.* **144:** 454–462.

54. Carney, D. H., D. L. Scott, E. A. Gordon, and E. F. LaBelle. 1985. Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. *Cell.* **42:** 479–488.

55. Van Rooijen, L. A., and B. W. Agranof. 1985. Inhibition of phosphoinositide phosphodiesterase by aminoglycoside antibiotics. *Neurochem. Res.* **10:** 1019–1024.

56. Gabev, E., J. Kasianowicz, T. Abbott, and S. McLaughlin. 1989. Binding of neomycin to phosphatidylinositol 4,5,biphosphate (PIP2). *Biochim. Biophys. Acta.* **979:** 105–112.

57. Slivka, S. R., and P. A. Insel. 1988. Phorbol ester and neomycin dissociate bradykinin receptor-mediated arachidonic acid release and polyphosphoinositide hydrolysis in Madin-Darby canine kidney cells. Evidence that bradykinin mediates noninterdependent activation of phospholipases A2 and C. *J. Biol. Chem.* **263:** 14640–14647.

58. Vassbotn, F. S., A. Ostman, A. Siegbahn, H. Holmsen, and C-H. Heldin. 1992. Neomycin is a platelet-derived growth factor (PDGF) antagonist that allows discrimination of PDGFα and β receptor signals in cells expressing both receptor types. *J. Biol. Chem.* **267:** 15635–15641.

59. Langeland, N., H. Holmsen, J. R. Lillehaug, and L. Haarr. 1987. Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. *J. Virol.* **61:** 3388–3393.

60. Langeland, N., A. M. Oyan, H. S. Marsden, A. Cross, J. C. Glorioso, L. J. Moore, and L. Haarr. 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. *J. Virol.* **64:** 1271–1277.

61. Tysnes, O., E. Johanessen, and V. M. Steen. 1991. Neomycin does not interfere with the inositol phospholipid metabolism, but blocks binding of alpha-thrombin to intact human platelets. *Biochem. J.* **273:** 241–243.

62. Christ, M., C. Meyer, K. Sippel, and M. Wehling. 1995. Rapid aldosterone signaling in vascular smooth muscle cells: involvement of phospholipase C, dicylglycerol and protein kinase C alpha. *Biochem. Biophys. Res. Commun.* **213:** 123–129.

63. Shibanaka, Y., H. Hayashi, M. Takai, and N. Fujita. 1993. Eclosion hormone activates phosphatidylinositol hydrolysis in silkworm abdominal ganglia during adult metamorphosis. *Eur. J. Biochem.* **211:** 427–430.