EXPERIMENTAL PROCEDURES

Chemicals and Reagents. A 25-mer phosphorodiamidate morpholino oligo (PMO) against CPR (3'–GTACCCCCTGAGAGTGCTTCTGTGG–5') and its 3'-amine derivative and corresponding invert oligo and in vitro transfection reagent Endoporter (EP) were purchased from Gene Tools (Philomath, OR, USA). Improved minimal essential medium (Richter's modified) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Fetal bovine serum was obtained from Equitech-Bio, Inc. (Kerrville, TX, USA). Avidin and the amine reactive biotinylation reagents NHS-PEG_{12}-Biotin, Sulfo-NHS-SS-Biotin, and Sulfo-NHS-LC-LC-Biotin were obtained from Thermo Fisher Scientific Inc, (Hanover Park, IL, USA). Rat CPR antibody raised in rabbit was purchased from Stressgen (Ann Arbor, MI, USA), and peroxidase conjugated goat anti-rabbit secondary antibody was obtained from Invitrogen (Carlsbad, CA, USA). All other chemicals were of the highest grade available and obtained from commercial sources.

Biotinylation and Avidin Conjugation. Four different linkers (L1, L2, L3, and L4; Figure 2) were used for biotinylation of PMOs. Whereas the biotinylated PMO with L1 linker was purchased from Gene Tools, biotinylation with L2, L3, and L4 was carried out in house using NHS-PEG_{12}-Biotin, Sulfo-NHS-SS-Biotin, and Sulfo-NHS-LC-LC-Biotin, respectively, according to the manufacturer's instructions. Briefly, biotinylation reagents were mixed with 3'-amine PMO at a molar ratio of ~14:1 (biotinylation reagent: 3'-amine PMO), and the mixture was kept at room temperature for 30 minutes. The biotinylated oligos were then purified using 2 mL Zeba Desalt Spin Columns (Thermo Fisher Scientific). Finally, the purified oligos were quantified spectrophotometrically at 265 nm and stored at 4^{o}C.

To prepare avidin-biotin oligo conjugates, avidin was dissolved in sterile phosphate buffered saline and mixed with biotinylated oligos at a molar ratio of 1:1. The mixture was kept at room temperature for 15 minutes before use.

Characterization of Biotinylated PMOs. Biotinylated PMOs with linkers 1-4 (Figure 2) were characterized for their degree of biotinylation using HPLC before and after passing the biotinylated samples through a commercially-available monomeric avidin affinity column (Monomeric Avidin Agarose Resin, Thermo Fisher Scientific Inc). Biotinylated samples (1.75 ml) were subjected to the avidin column and the column was washed six times each time with 2 ml of phosphate buffer, according to the manufacturer instructions. A small aliquot (20 µl) of each fraction was stored before combining all six fractions. The combined fractions were freeze-dried and redissolved in 1.75 ml of water. The avidin precolumn and postcolumn samples were then subjected to quantitation using HPLC with a size exclusion column (PolySep GFC 3000, Phenomenex, Torrance, CA) and mobile phase of 0.1 M phosphate buffer (pH 7.4): acetonitrile (60:40, v/v), delivered at a flow rate of 1 ml/min. The PMOs were quantitated at a wavelength of 258 nM. The degree of biotinylation was then estimated using the following equation:

$$\%\text{Biotinylation} = \frac{\text{Precolumn Conc.} - \text{Postcolumn Conc.}}{\text{Precolumn Conc.}} \times 100$$

Cell Line. Rat liver epithelial (WB-F344) cells (*1, 2*) were grown in improved minimal essential medium (Richter's modified) containing 10% fetal bovine serum, insulin (1 µM), 20 mM HEPES buffer, and gentamicin (0.005%). Approximately 70-80% confluent cells were used for evaluating the effects of antisense oligos on CPR expression.

In Vitro Studies. To characterize the dose-response of CPR antisense, cells were incubated in the presence of 0.0, 0.1, 1, 10, and 100 μM concentrations of unmodified PMO antisense for 48 hours using 2 μM EP as in vitro transfection agent. Afterward, cells were lysed in a lysis buffer (50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, and Sigma protein inhibitor cocktail), and total protein was measured by the Bradford assay. The lysate was then used for CPR protein analysis using western blotting method, which is described later in this section.

To determine the effect of biotinylation of PMO on its antisense effect, PMO biotinylated with L1 (PMO-L1-Biotin), L2 (PMO-L2-Biotin), L3 (PMO-L3-Biotin), or L4 (PMO-L4-Biotin) was incubated with WB cells for 48 h in the presence of EP, as described above. Additionally, unmodified PMO and invert PMO were used for comparison. The concentration of the PMOs was 5 μM in all the cases. After 48 h of incubations, cells were processed as described above.

Finally, to determine the effectiveness of avidin for delivery of the biotinylated PMO, avidin conjugated biotinylated PMOs (5 μM) with L1 (PMO-L1-Biotin:Avidin), L2 (PMO-L2-Biotin:Avidin), L3 (PMO-L3-Biotin:Avidin), or L4 (PMO-L4-Biotin:Avidin) linkers were incubated with WB cells in the absence of the transfection reagent EP. Additionally, the effects of avidin alone (5 μM) and unmodified PMO alone on the expression of CPR were tested in the absence of EP. After 48 h, the cells were processed as described above.

In Vivo Studies. Male Sprague-Dawley rats (75-100 g) were purchased from Charles River Laboratories (Indianapolis, IN, USA) and maintained on a 12-h light/dark cycle in our institutional animal facility. They were allowed free access to food and water throughout the study. Animal handling protocol was approved by Institutional Animal Care Committee.

Rats were divided into three groups of Control (n = 8), Avidin (n = 4), and PMO (n = 4). The PMO group was treated with 5 mg/kg/day doses of PMO-L4-Biotin:Avidin (dose volume of 5 ml/kg) for 3 days, injected into the sublingual vein under isoflurane anesthesia. The Control and Avidin groups were injected with vehicle or avidin (40 mg/kg/day). On day 4, the liver was isolated after perfusion with 15 ml of cold saline and used for preparation of microsomes.

Preparation of Liver Microsomes. Microsomes were prepared according to the established ultracentrifugation methods (*3*). The final pellet was dispersed in a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl, 1 mM EDTA, and, 20% glycerol and stored at -80^{o}C. Protein content in the microsomes was determined by Bradford assay using bovine serum albumin as standard.

Cytochrome P450 Reductase Activity. Microsomal CPR activity was measured according to minor modifications of a kinetic method reported before (*4*). Our total reaction volume was 1 ml, consisting of microsomes (15 µg), oxidized cytochrome c (50 µM), and NADPH (200 µM) in phosphate buffer.

Western Blot Analysis. The CPR content of liver microsomes after in vivo studies and cell lysates after in vitro studies were determined using Western blot analysis. Briefly, 5 or 10 µg of microsomal or cell lysate proteins, respectively, were resolved by 10% SDS polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were then probed with rat CPR primary antibody raised in rabbit (1:2500 dilution), followed by peroxidase conjugated anti-rabbit secondary antibody (1:5000 dilution). Band intensity was quantified using VersaDoc Imager system and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). In the cell lysate samples, beta actin expression was used as the protein standard.

Data and Statistical Analysis. The relationship between the inhibitory effects of PMO on CPR protein expression (E) and the PMO concentrations (C) was fitted to the following equation using nonlinear regression analysis:

$$E = E_{\max} - \frac{I_{\max} \cdot C}{IC_{50} + C}$$

where E_{max}, I_{max}, and IC_{50} represent the maximum expression of CPR, maximum inhibition of CPR expression by PMO, and the concentration of PMO producing half of I_{max}, respectively.

All the results are expressed as mean ± S.D. The differences among different groups were analyzed using one way analysis of variance with Tukey's *post hoc* analysis. A P value of <0.05 was considered as statistically significant.

References

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