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Selective Inhibition of α_{1B}-Adrenergic Receptor Expression and Function Using a Phosphorothioate Antisense Oligodeoxynucleotide

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ABSTRACT

To investigate α_{1B}-adrenoceptor function, we developed a phosphorothioate antisense oligodeoxynucleotide (AO) to inhibit the expression of the α_{1B}-adrenoceptor subtype in DDT_1 MF2 cells. We measured the cellular uptake of the AO and its effect on α_{1B}-adrenoceptor mRNA expression, protein density, and coupling to phospholipase C. Cells treated with either a control oligodeoxynucleotide (CO) or medium alone served as control groups. Confocal microscopy demonstrated that DDT_1 MF2 cells internalized carboxyfluorescein-labeled (FAM) AO within 30 min. Analysis of cellular lysates showed that approximately 50% of the intracellular FAM-AO was present as an intact 18-mer for up to 48 hr. Incubation of cells with AO for 48 hr decreased α_{1B}-adrenoceptor density ([^{3}H]prazosin B_{max}) versus control groups by 12% (1 μM AO) and 72% (10 μM AO). In time course experiments, AO (10 μM) reduced α_{1B}-adrenoceptor density by 28, 64, and 68% versus controls after 24, 48, and 72 hr of exposure, respectively. α_{1B}-Adrenoceptor mRNA concentration (measured by RT-PCR) was reduced by 25% in cells treated for 48 hr with 10 μM AO versus controls. AO pretreatment (10 μM, 48 hr) reduced the maximum response to agonist-stimulated [^{3}H]inositol phosphate accumulation. The maximal response of the full agonist norepinephrine was reduced by 30% after AO treatment, and by 73% for the partial agonist naphazoline. In contrast, AO did not affect histamine-stimulated total [^{3}H]inositol phosphate accumulation. Thus, AO effectively reduced α_{1B}-adrenoceptor subtype expression and function *in vitro*, suggesting a potential to selectively inhibit α_{1B}-adrenoceptor function *in vivo*.

α_1-Adrenergic receptors are a subfamily of G protein-coupled receptors that mediate the actions of catecholamines. Based on cloning and pharmacological data, it is known that α_1-adrenergic receptors can be classified into three subtypes (α_{1A}-, α_{1B}-, and α_{1D}-adrenergic receptors). We (Scofield *et al.*, 1995) and others (Perez *et al.*, 1994; Price *et al.*, 1994) have shown that the genes for each of the subtypes are expressed in discrete, tissue-specific patterns. Each of the α_1-adrenergic receptor subtypes has been shown to mediate distinct physiological functions. For example, the α_{1B}-subtype mediates activation of glycogenolysis in rat liver (Garcia-Sainz and Macias-Silva, 1995). The α_{1A}-subtype is involved in the contraction of human prostate smooth muscle (Forray *et al.*, 1994), and contraction of rat aorta seems to be mediated at least in part by the α_{1D}-subtype (Buckner *et al.*, 1995; Piascik *et al.*, 1995). Despite these examples, a major challenge to the determination of the function of each of the α_1-adrenergic receptor subtypes is the paucity of available pharmacological tools to distinguish among them. Competitive antagonists such as 5-methyl-urapidil can be used experimentally to distinguish the α_{1A}-adrenergic receptor subtype from the other two subtypes (Gross *et al.*, 1988). Unfortunately, antagonists with good selectivity for the α_{1B}- and α_{1D}-adrenergic receptors are currently lacking. Some studies have reported that the alkylating agent chloroethylclonidine can distinguish between the α_{1A}- and α_{1B}-adrenergic receptor subtypes (Minneman *et al.*, 1988); however, chloroethylclonidine irreversibly alkylates both the α_{1B}- and the α_{1D}-adrenergic receptors nonselectively (Hirasawa *et al.*, 1997; Xiao and Jeffries, 1998).

Techniques that block receptor protein expression may provide an alternative means to study individual α_1-adren-

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ABBREVIATIONS: AO, antisense oligodeoxynucleotide; CO, control oligodeoxynucleotide; FAM, carboxyfluorescein; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ergic receptor subtype function. Since its first use by Zamecnik and Stephenson (1978), AO technology has been shown to effectively block protein synthesis of specified target genes *in vitro* and *in vivo* (Hunter *et al.*, 1995). In this study, we report the use of AO methods to inhibit the expression of an α_1-adrenergic receptor subtype. We have designed and synthesized an 18-base phosphorothioate AO directed against the translation start-site of the hamster α_{1B}-adrenergic receptor mRNA, and investigated its effectiveness *in vitro* using a cell culture model (DDT_1 MF2) that expresses a high density of the α_{1B}-adrenergic receptor subtype. We have measured the intracellular distribution and kinetics of cellular uptake of the α_{1B}-adrenergic receptor AO in DDT_1 MF2 cells, and its ability to reduce 1) α_{1B}-adrenergic receptor density, 2) the steady state α_{1B}-adrenergic receptor mRNA concentration, and 3) α_1-adrenergic receptor-stimulated accumulation of inositol phosphates.

Experimental Procedures

Materials. Norepinephrine, naphazoline, histamine, prazosin-HCl, phentolamine mesylate, mepyramine, and Hoechst dye (No. 33258) were purchased from Sigma (St. Louis, MO). *myo*-[^{3}H]Inositol and [^{3}H]prazosin were purchased from (DuPont NEN, Boston, MA). DMEM and inositol-free DMEM were purchased from GIBCO BRL Products (Gaithersburg, MD).

Cell culture. DDT_1 MF2 cells were maintained in a humidified incubator at 5% CO_2 and 95% O_2. For radioligand binding experiments, cells were maintained in high glucose DMEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. For assays of accumulation of total [^{3}H]inositol phosphates, cells were maintained in inositol-free DMEM under the same conditions. All experiments were performed in 100% confluent monolayers, passage number 18–30.

Oligodeoxynucleotide synthesis. All chain-extension syntheses were performed as previously described (Desjardins and Iversen, 1995). The AO was synthesized to target the hamster α_{1B}-adrenergic receptor mRNA translational start site, with the sequence: 5′-CAGATCGGGATTCATTTT-3′. The CO was designed with two bases mismatched plus two bases transposed relative to the AO construct: 5′-CAGACTGGGATTGTTTTT-3′. The oligodeoxynucleotides were high performance liquid chromatography-purified, dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM $Na_2HPO_4 \cdot 7H_2O$; (Mg^{2+}- and Ca^{2+}-free, pH 7.4), sterilized by filtration and the concentrations determined spectrophotometrically, by dividing the absorbance of the respective oligodeoxynucleotides obtained at 260 nm by their respective molar extinction coefficients at 260 nm.

Cellular uptake of fluorescently labeled AO. The AO was fluorescently labeled with FAM and used to determine the kinetics of cellular uptake and/or cellular distribution in DDT_1 MF2 cells. The AO was synthesized with FAM at the 5′-position as reported previously (Iversen *et al.*, 1992). Time course uptake studies using the FAM-AO were performed in DDT_1 MF2 cells incubated with 10 μM FAM-AO for intervals of 0.5, 1, 6, 12, 24, and 48 hr. After incubation, the cells were washed twice with PBS and fixed with 4% buffered formalin, and cell nuclei were counterstained using 0.2 mM bisbenzimide (Hoechst stain, No. 33258). The distribution of the intracellular FAM-AO was examined with an Olympus confocal microscope station (BH2-RFCA) using HazeBuster software (VayTech, Fairfield, IA).

The integrity of the intracellular FAM-AO was also determined in DDT_1 MF2 cells incubated with 10 μM FAM-AO for 0.5-, 1-, 6-, 12-, 24-, and 48-hr intervals. After incubation, the monolayers were washed three times with PBS, and cells were lysed in 0.5 ml of buffer containing formamide and 0.05 M EDTA (1:5, v/v). The cell lysates were then collected and applied (25 μl) to a 6% acrylamide gel using a STRECH-373 DNA sequencer (Applied Biosystems, Foster City, CA). To determine the stability of the FAM-AO throughout the incubation times, the 373 DNA sequencer GeneScan was used to compare the mobility of each time course band with that of the native AO (18-mer) to quantify the area and height of each DNA band, represented as peaks. The amount of intact 18-mer recovered from the lysates at each time point was plotted as counts of areas under the curve obtained from the peaks corresponding to those of the native AO 18-mer.

Radioligand binding studies. The effect of the AO on α_{1B}-adrenergic receptor density was determined in saturation binding experiments performed on membranes from DDT1 MF2 cells grown in 75-cm^2 flasks to confluency. For time course experiments, cells received 10 μM of either AO or CO or medium alone, and were incubated for 24, 48, or 72 hr. For concentration-response experiments, cells were incubated for 48 hr with medium alone, or with 1 or 10 μM of the oligodeoxynucleotides. After two washes with ice-cold PBS, the cells were harvested by scraping and were collected into 50-ml centrifuge tubes. After a 5-min centrifugation at 3,000 $\times$ *g* at 4°, the pellets were resuspended in 50 mM Tris·HCl, 2 mM EDTA (pH 7.4), and disrupted using a glass-Teflon homogenizer. The homogenate was centrifuged twice at 26,000 $\times$ *g* for 20 min at 4°, and the pellets obtained were reconstituted in 50 mM Tris·HCl, 2 mM EDTA, yielding a protein concentration of 0.25–0.50 mg/ml. [^{3}H]Prazosin saturation binding assays were performed in 50 mM Tris·HCl buffer (pH 7.4, 1-ml incubation volume, 50 μg of membrane protein). Eight concentrations of [^{3}H]prazosin (0.015–2 nM, specific activity 77.9 Ci/mmol) were added to the membrane aliquots and incubated for 30 min at 37°. Nonspecific binding was defined with 10 μM phentolamine. Incubations were terminated by rapid filtration through glass-fiber filters (Schleicher & Schuell No. 32) followed by three 5-ml washings with ice-cold incubation buffer. Binding experiments were always performed in freshly prepared cell membranes. Protein concentrations were determined as described by Lowry *et al.* (1951) using bovine serum albumin as the standard. The values reported for B_{max} (maximal receptor density) and K_D were obtained by nonlinear regression analyses of eight-point saturation plots conducted in duplicate. To study the possibility of the AO binding to the α_{1B}-adrenergic receptor protein itself, saturation binding experiments were performed in DDT_1 MF2 cell membranes that had been preincubated for 30 min (37°) with 10 μM of the AO before the saturation binding assay.

Quantitation of mRNA. The effect of the AO on the α_{1B}-adrenergic receptor steady state mRNA concentration was studied by quantitative competitive RT-PCR. α_{1B}-Adrenergic receptor steady state mRNA from DDT1 MF2 cells was quantified as previously described by our laboratory (Scofield *et al.*, 1995) using RT combined with a competitive PCR.

Phosphoinositide hydrolysis. To determine the effect of the AO on α_{1B}-adrenergic receptor function, agonist-induced total inositol phosphate accumulation was measured in 24-well plates (2×10^6 cells/plate) in cells incubated for 48 hr with 10 μM AO or CO or medium alone. The cells were labeled with *myo*-[^{3}H]inositol at 3 μCi/ml for 24 hr in inositol-free DMEM. The monolayers were then washed twice with HEPES-buffered Krebs buffer (20 mM HEPES, 4 mM $NaHCO_3$, pH 7.4, 37°) and preincubated at 37° for 30 min in the same buffer containing 20 mM LiCl iso-osmotically substituted for NaCl. Antagonists were added during the preincubation period when appropriate. Agonists were added in 100-μl aliquots for 10 min at 37°. Incubations were stopped by the addition of 1 ml of ice-cold chloroform:methanol solution (1:2, v/v). After a 2-hr incubation at −20°, the cell monolayers were collected and sonicated for 5 sec at maximum power using a sonic Dismembrator (model 300; Fisher Scientific, Pittsburgh, PA) to obtain cellular lysates. After combining each lysate with 0.5 ml of water, the samples were centrifuged at 6000 $\times$ *g* for 10 min to separate the phases, and the total [^{3}H]inositol phosphate fraction was extracted by column chromatography as previously described (Berridge *et al.*, 1983; Jeffries *et al.*, 1988). To

reduce interassay variability, [^{3}H]inositol phosphate accumulation studies were always performed for all experimental groups in a single assay with a particular drug. Agonist additions were performed in duplicate, and two wells per plate were used for cell counts.

Statistical analysis. Radioligand saturation binding parameters (B_{max} and K_D) were obtained with the curve-fitting program Prism (GraphPAD Software, Inc., San Diego, CA). Comparisons among group means were determined by analysis of variance, and differences among groups were determined using the Newman-Keuls multiple comparison test.

Results

Stability of the FAM-AO in DDT$_1$ MF2 cells. Fig. 1 shows the kinetics of FAM-AO uptake in DDT$_1$ MF2 cell lysates obtained from cells incubated with 10 μM FAM-AO for 0.5, 1, 6, 12, 24, and 48 hr. The amount of intact 18-mer reached plateau by 12 hr of incubation and was maintained at a steady state throughout the rest of the study. The results showed that for each of the incubation times indicated, intact 18-mer FAM-AO was recovered from the intracellular compartment (compared with a positive control 18-mer run on the same gel). The range of percentages of intact 18-mer AO recovered over the incubation times was: 0.5 hr, 33%; 1 hr, 18%; 6 hr, 44%; 12 hr, 49%; 24 hr, 51%; 48 hr, 47%. There was a longer size fragment (corresponding to an 25-mer) recovered from the cell lysates at each of the incubation times, whose percentage as a percent of total counts was: 0.5 hr, 17%; 1 hr, 25%; 6 hr, 14%; 12 hr, 10%; 24 hr, 9%; 48 hr, 3%.

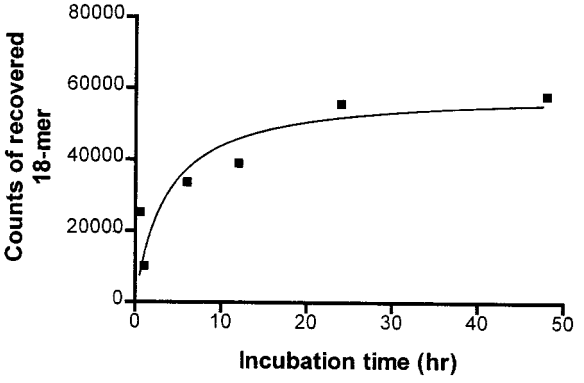

Fig. 1. Plot of intact 18-mer FAM-AO recovered from DDT$_1$ MF2 cell lysates after incubation at 10 μM, 37°, for 0.5-, 1-, 6-, 12-, 24-, and 48-hr intervals. *Points*, peak counts of intact 18-mer AO recovered from the cellular lysates that correspond to the same size fragment as that of the intact 18-mer α_{1B}-adrenergic receptor FAM-AO. A representative plot from three separate experiments is shown.

Cellular distribution of the FAM-AO. The cellular distribution of the FAM-AO in DDT$_1$ MF2 cells was examined by confocal microscopy (Fig. 2). After 30 min of exposure to the FAM-AO (10 μM), the majority of fluorescence was located in cell nuclei. In addition to this nuclear pattern of distribution, a punctate localization of fluorescence in the cytoplasm of DDT$_1$ MF2 cells was observed after 12 hr of incubation with the fluorescently labeled AO. FAM-AO treatment did not reduce cellular viability.

Radioligand binding. Incubation for 48 hr with the AO reduced α_{1B}-adrenergic receptor density in DDT1 MF2 cells in a concentration-dependent fashion (Fig. 3). After incubation with 1 μM AO, the B_{max} of [^{3}H]prazosin was decreased by 12.4% versus CO-treated cells and 18.2% versus medium-treated cells. Preincubation with 10 μM AO significantly reduced the α_{1B}-adrenergic receptor density by 73.5 and 72% of CO and medium alone, respectively. Neither AO or CO significantly altered the K_D value of [^{3}H]prazosin compared with medium-treated controls (medium alone, 0.30 $\pm$ 0.10 nM; CO, 0.30 $\pm$ 0.12 nM; AO, 0.14 $\pm$ 0.05 nM). The temporal effects of the AO in α_{1B}-adrenergic receptor density are shown in Fig. 4. There was a time-dependent decrease in α_{1B}-adrenergic receptor density with 10 μM AO that reached plateau at 48 hr of incubation (24% at 24 hr, 64% at 48 hr, and 68% at 72 hr, relative to medium alone). In contrast, preincubation with 10 μM CO had no effect in α_{1B}-adrenergic receptor density in any experiments relative to untreated cells. Acute treatment (30 min) of DDT$_1$ MF2 cell membranes with 10 μM AO or CO did not affect the affinity or the B_{max} values of [^{3}H]prazosin, indicating that the oligodeoxynucleotides did not functionally interact with the α_{1B}-adrenergic receptor protein (data not shown).

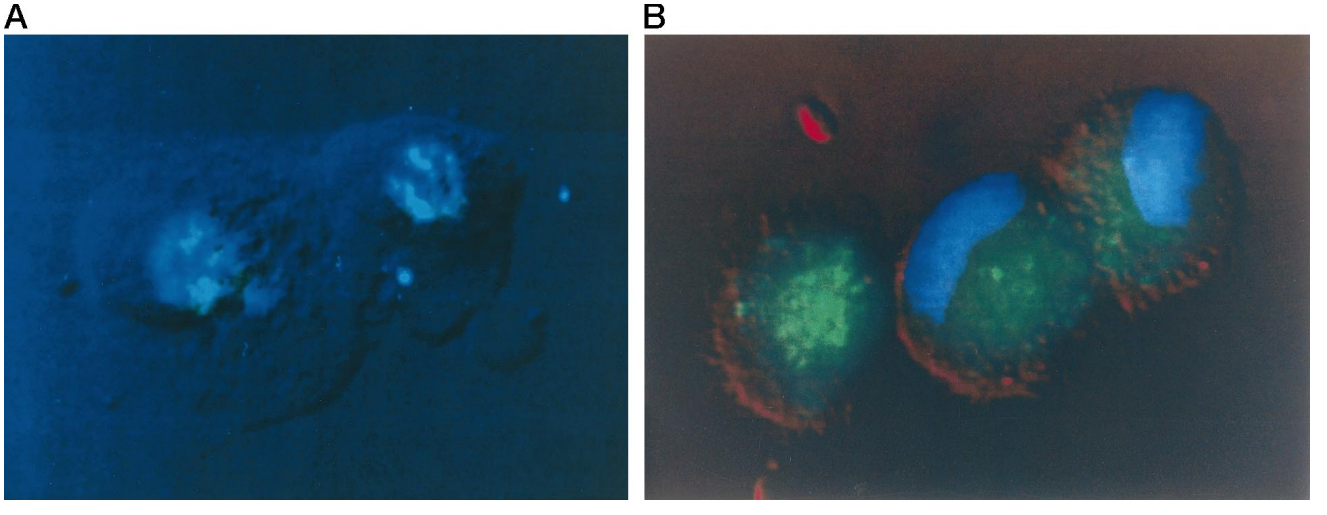

Fig. 2. Cellular uptake and/or localization of 10 μM FAM-AO in DDT$_1$ MF2 cells after 30 min (A) and 12 hr (B) of incubation. The cells were fixed in 4% formalin and mounted on glass coverslips, and their phase-contrast and confocal fluorescent images at 40× were superimposed after digital image deconvolution as described in experimental procedures. After 30 min of incubation, fluorescence accumulation was found predominantly in cell nuclei (*light color*), whereas at 12 hr of incubation the fluorescence localization was punctate and largely cytosolic (*green color*). At 12 hr, the cellular nuclei were stained with Hoechst dye No. 33258 (*blue color*) to distinguish nuclear versus cytosolic fluorescence localization.

Quantitative competitive RT-PCR. AO (10 μM) pretreatment for 48 hr significantly reduced the steady state concentration of α_{1B}-adrenergic receptor mRNA by 25% compared with medium-treated controls (Fig. 5) as measured by competitive RT-PCR. The reduction in α_{1B}-adrenergic receptor expression observed with 1 μM AO was relatively small (7.3% of medium alone) and did not reach statistical significance. CO did not significantly change α_{1B}-adrenergic receptor mRNA concentration versus untreated cells at either 1 or 10 μM.

Phosphoinositide hydrolysis. Fig. 6 shows concentration-response curves for norepinephrine- and naphazoline-stimulated total [^{3}H]inositol phosphate accumulation in DDT_1 MF2 cells, plotted as counts/min. The nonstimulated (basal) labeling of [^{3}H]inositol phosphates did not differ among any of the experimental groups. Norepinephrine produced a concentration-related increase in total [^{3}H]inositol phosphate accumulation in DDT_1 MF2 cells. Pretreatment of these cells with CO (10 μM, 48 hr) had no effect on the norepinephrine concentration-response curve. Naphazoline acted as a partial agonist in DDT_1 MF2 cells, producing a maximal response that was 60% of that of norepinephrine. CO pretreatment did not affect the naphazoline concentration-response curve. The responses to both norepinephrine and naphazoline could be blocked by the α_1-adrenergic receptor antagonist prazosin at 10^{-6} M (data not shown). Prior treatment with 10 μM AO for 48 hr significantly reduced the maximum response to norepinephrine by 30%, and to naphazoline by 74% relative to controls. The EC_{50} values for norepinephrine were not different among experimental groups (medium alone, 0.24 $\pm$ 0.10 μM; CO, 0.44 $\pm$ 0.06 μM; AO, 0.47 $\pm$ 0.13 μM). Similarly, the EC_{50} values for naphazoline controls were not significantly different among treatments (medium alone, 0.24 $\pm$ 0.34 μM; CO, 0.23 $\pm$ 1.16 μM; AO, 5.6 $\pm$ 1.35 μM).

To establish the specificity of the AO-induced inhibition of α_{1B}-adrenergic receptor function, we measured accumulation of [^{3}H]inositol phosphates in response to histamine (10^{-4} M) in cells pretreated with AO or CO or medium alone for 48 hr. Histamine acts on histamine H-1 receptors in DDT_1 MF2 cells to stimulate phospholipase C activity (Dickenson and Hill, 1994). In these experiments, we used twice the concentration of CO and AO (20 μM) as in previous experiments. Histamine resulted in a 1.5-fold increase in total [^{3}H]inositol phosphate accumulation over base-line accumulation, a response that was fully antagonized by the selective histamine H-1 receptor antagonist mepyramine (10^{-6} M, data not shown). Neither of the oligodeoxynucleotides produced a significant effect on the histamine-induced [^{3}H]inositol phosphate accumulation in DDT_1 MF2 cells (Fig. 7). In contrast, in parallel experiments using norepinephrine (10^{-5} M), the AO nearly abolished the norepinephrine-stimulated response, resulting in 90% inhibition relative to treatment with medium alone.

Discussion

The results of the present study demonstrate that AO techniques can be used to inhibit the expression of an α_1-adrenergic receptor subtype. In our studies, we showed that an 18-mer AO is taken up into intracellular sites by DDT_1 MF2 cells and that intact AO is still bioavailable after 48 hr of treatment. Antisense oligodeoxynucleotide but not CO reduced α_{1B}-adrenergic receptor density, steady state mRNA concentration, and α_{1B}-adrenergic receptor-stimulated [^{3}H]inositol phosphate production. Taken together, these data strongly suggest that the AO inhibited α_{1B}-adrenergic receptor synthesis in DDT_1 MF2 cells.

The findings in this study are in agreement with a growing body of evidence supporting the sequence-specific inhibition of cellular gene expression by phosphorothioate AO. However, the extensive use of AO technology has posed several questions concerning the specificity of antisense effects. Toxic effects of phosphorothioate AO have been observed at high oligodeoxynucleotide concentrations in cell culture conditions (Crooke, 1991). However, it is unlikely that nonspecific toxic effects account for the results obtained in our study, as the AO concentrations used fall within the range in which true antisense effects are observed (Stein and Cheng, 1993). The specificity of our AO for inhibition of α_{1B}-adrenergic receptor expression is evidenced by the results obtained from CO-treated cells. These cells received an equal concen-

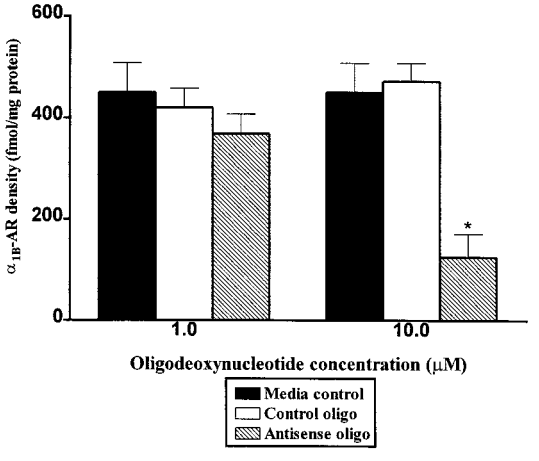

Fig. 3. α_{1B}-Adrenergic receptor density after 48 hr of incubation with medium alone or 1 μM and 10 μM CO or AO. *Bars*, mean $\pm$ standard error from three separate experiments; *, $p < 0.05$. Densities were measured as B_{max} values in saturation binding assays with [^{3}H]prazosin.

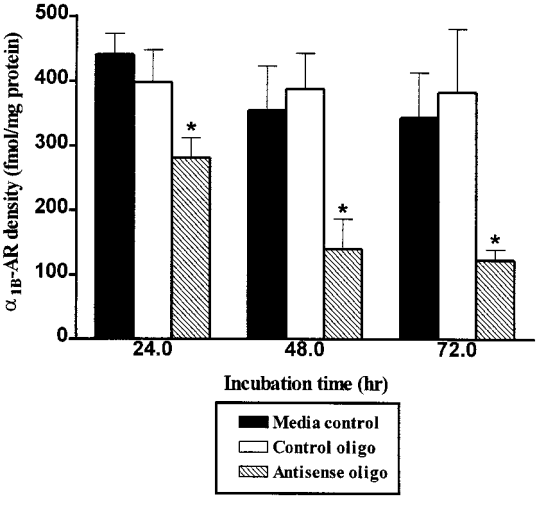

Fig. 4. Time course of α_{1B}-adrenergic receptor density after incubation with medium alone or 10 μM CO or AO. *Bars*, mean $\pm$ standard error from three separate experiments; *, $p < 0.05$. Densities were measured as B_{max} values in saturation binding assays with [^{3}H]prazosin.

tration of oligodeoxynucleotide, which was composed of the same number of bases as the antisense construct, but with two bases mismatched and two bases transposed versus the AO. After CO treatment, α_{1B}-adrenergic receptor density and α_{1B}-adrenergic receptor-stimulated [^{3}H]inositol phosphate accumulation were similar to those obtained from cells incubated with medium alone, suggesting that the AO demonstrates target hybridization selectivity for the α_{1B}-adrenergic receptor mRNA.

Several studies have reported that phosphorothioate AO can produce biological effects that are not attributed to a true antisense mechanism of action (Krieg and Stein, 1995). For example, some phosphorothioate oligodeoxynucleotides can have higher affinity for hybridization to intracellular proteins compared with the mRNA species in question. Thus, it was possible that the effect of the AO in the present study was because of direct binding of the AO to the α_{1B}-adrenergic receptor protein. We excluded this possibility by demonstrating that 10 μM AO had no effect on α_{1B}-adrenergic receptor density or affinity when added 30 min to the cell membranes before the saturation binding experiment. It was also possible that the inhibitory effect on α_{1B}-adrenergic receptor-stimulated total [^{3}H]inositol phosphate accumulation observed with our AO could be interpreted as a direct antagonism of phospholipase C by the AO. This potential mechanism was excluded because stimulation of the histamine H-1 receptor in DDT$_1$ MF2 cells, which couples to phospholipase C (Dickenson and Hill, 1994), was unaffected by AO under conditions where AO inhibited the effect of norepinephrine by 90%. Based on our data with histamine, it seems likely that the AO inhibits α_{1B}-adrenergic receptor function through a decrease in α_{1B}-adrenergic receptor synthesis, and not because of a nonspecific reduction in phospholipase C activity.

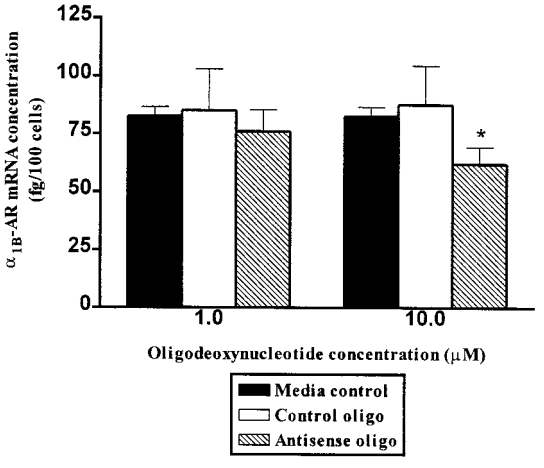

Fig. 5. α_{1B}-Adrenergic receptor mRNA concentration after a 48-hr incubation with medium alone or 1 μM and 10 μM CO or AO. *Bars*, mean ± standard error from three separate experiments; *, $p < 0.05$. α_{1B}-Adrenergic receptor mRNA concentrations were determined in competitive RT-PCR assays.

Phosphorothioate oligodeoxynucleotides are thought to be internalized by cells through adsorptive endocytosis and fluid-phase endocytosis events (Iversen *et al.*, 1992; Beltinger *et al.*, 1995), which may be triggered by the binding of the AO to receptor-like proteins in cell membranes (Hawley and Gibson, 1996; Gewirtz, 1996). After internalization, there seems to be a rapid nuclear localization (Gewirtz, 1996), followed by the accumulation of oligodeoxynucleotide molecules in endosomal or lysosomal compartments. Our results indicate that the α_{1B}-adrenergic receptor AO followed a pattern of distribution similar to that reported by others for phosphorothioates. In our study, approximately 50% of the AO remained intact after uptake in DDT$_1$ MF2 cells, with the variable appearance of a larger size fragment (25-mer) than the 18-mer AO during the course of incubation. Biotransformation of an antisense oligodeoxynucleotide that produced a mass greater than the parent compound has been reported previously in antisense studies (Phillips *et al.*, 1997), although the mechanism for its appearance was not determined. Nevertheless, the detection of intact 18-mer AO in cellular lysates suggests that a relatively large amount of active AO is present intracellularly even at 48 hr of incubation. The results from the AO stability experiments are not surprising, because AO was still highly effective (68% inhibition with respect to medium alone) in reducing α_{1B}-adrenergic receptor density after 72 hr of incubation.

One of the proposed mechanisms of action of AO involves the degradation of the target mRNA-antisense duplex by RNase H enzymes (Gewirtz *et al.*, 1996). The reduction in the concentration of α_{1B}-adrenergic receptor mRNA caused by AO treatment was lower (25% inhibition) than the 64% decrease in α_{1B}-adrenergic receptor density under the same conditions. These differences in results could be attributed to an inhibition of α_{1B}-adrenergic receptor translation, or up-regulation of α_{1B}-adrenergic receptor mRNA level at 48 hr after AO treatment, or that α_{1B}-adrenergic receptor mRNA

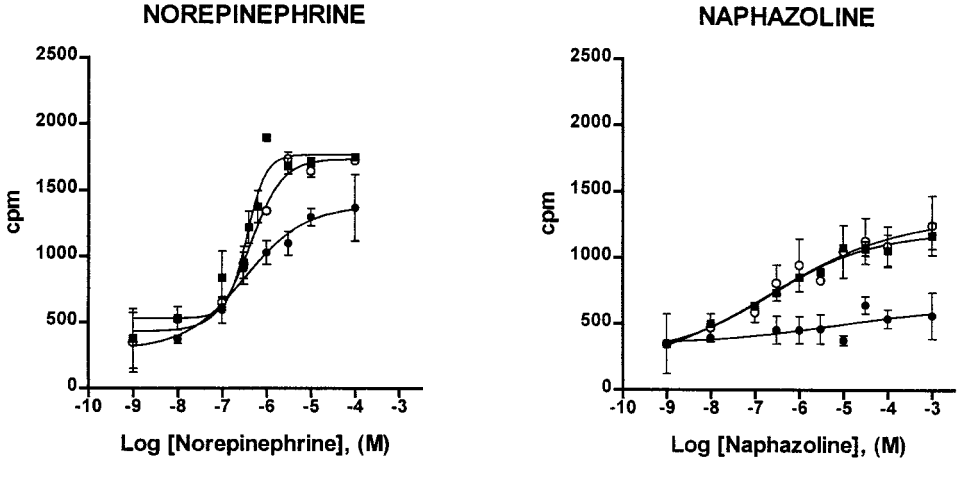

Fig. 6. Agonist-induced total [^{3}H]inositol phosphate accumulation in DDT$_1$ MF2 cells. Cells were incubated with 10 μM of either AO (●), CO (○), or medium alone (■) for 48 hr, and total [^{3}H]inositol phosphate accumulation was determined as described in Experimental Procedures. Basal (unstimulated) counts are included in each plot, and values are expressed as counts/min. *Points*, mean ± standard error from three separate experiments.

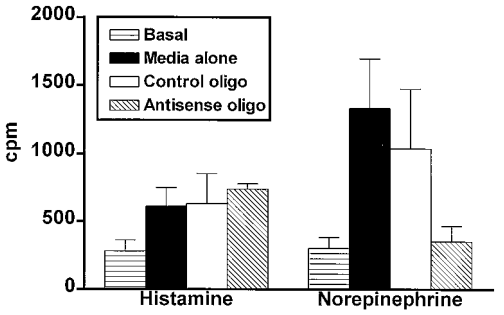

Fig. 7. Histamine (10^{-4} M)- and norepinephrine (10^{-5} M)-induced total [^{3}H]inositol phosphate accumulation in DDT_1 MF2 cells incubated with medium alone or CO or AO. For each treatment: *left bars*, mean unstimulated (basal) counts; *right bars*, mean counts after agonist stimulation. Cells were incubated with the respective oligodeoxynucleotides for a total of 48 hr (20 μM), and total [^{3}H]inositol phosphate accumulation was determined as described in Experimental Procedures. *Bars*, mean ± standard error from three separate experiments; *, $p < 0.05$.

and protein concentrations are not linearly related in DDT_1 MF2 cells. Regardless of the reason, the important finding of our study was that the AO reduced both α_{1B}-adrenergic receptor mRNA and protein expression.

In the present study, we found differences in the degree of inhibition by the AO in α_{1B}-adrenergic receptor-mediated accumulation of [^{3}H]inositol phosphates between norepinephrine and naphazoline. Norepinephrine acted as a full agonist with a higher intrinsic efficacy than did naphazoline in DDT_1 MF2 cells. AO reduced the maximal response to norepinephrine by only 30% in our functional assay despite a reduction of receptor protein by 64%. One likely explanation for this discrepancy is the presence of a small receptor reserve for norepinephrine. The fact that the maximum response to naphazoline was reduced by 73% by AO pretreatment supports this explanation. As a partial agonist, naphazoline cannot have spare receptors, thus, it would be predicted that one would observe similar degrees of inhibition by the AO of naphazoline-stimulated [^{3}H]inositol phosphate accumulation (73% inhibition) and receptor density (64% inhibition).

We have shown that our AO selectively inhibits the density, expression, and function of α_{1B}-adrenergic receptors in DDT_1 MF2 cells. AO approaches to inhibit the synthesis of other G protein-coupled receptors have proven successful, as in the case of opioid (Bilsky *et al.*, 1996), dopamine (Zhou *et al.*, 1994), neuropeptide Y (Wahlestedt *et al.*, 1993), and α_{2C}-adrenergic receptors (Lu and Ordway, 1997). Recently, AO targeted to α_1-adrenergic receptor subtypes have been used *in vivo* (Piascik *et al.*, 1997) and in primary culture (Liu *et al.*, 1997) in attempts to define their individual function.

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