Volume 288, number 1,2, 30–32 FEBS 10035 August 1991

ADONIS 001457939100746N

Enhanced cellular uptake of biotinylated antisense oligonucleotide or peptide mediated by avidin, a cationic protein

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Received 11 June 1991

The cellular uptake of a model antisense oligonucleotide complementary to 21 bases of the bovine GLUT-1 glucose transporter mRNA and a model vasopressin peptide that were biotinylated, was markedly stimulated by the presence of avidin, a cationic protein. Conversely, the bacterial homologue of avidin, streptavidin, which is a slightly acidic protein, did not facilitate cellular uptake. The avidin-mediated uptake of biotinylated derivatives was competitively inhibited by another cationic protein, protamine, with a K_i of 5 μg/ml; was saturable, temperature- and time-dependent; and was associated with endocytosis. The use of the avidin-biotin system provides a new approach to increasing the cellular uptake of antisense oligonucleotides or peptides.

Drug delivery; Blood-brain barrier; Glucose transporter; Vasopressin

1. INTRODUCTION

Antisense oligonucleotides or peptides are potential highly specific chemotherapeutic agents for the treatment of cancer, viral infections, and other disorders [1,3]. However, a major problem limiting the therapeutic efficacy of these agents in vivo is the minimal cellular uptake of the highly charged oligonucleotide or peptide compounds. It may be advantageous to conjugate the antisense oligonucleotide to a transport vector via high affinity binding that is stable in the circulation but is labile in cells. Such a binding underlies the avidin–biotin interaction, as previous studies have shown the avidin–biotin binding reaction, ($K_d = 10^{-15}$ M) [4], is stable in the circulation [5]. In addition, avidin is a cationic protein with an isoelectric point (pI) of 10 [4], and cationic proteins are taken up by cells via an absorptive-mediated endocytosis mechanism [6]. Therefore, the present studies examine the feasibility of using avidin–biotin technology to enhance cellular uptake of antisense oligonucleotides or peptides.

2. MATERIALS AND METHODS

2.1. *Materials*

The model antisense oligonucleotide used in these studies is a 21-mer complementary to the bovine GLUT-1 glucose transporter mRNA [7], and it corresponds to nucleotides −9 to +12 (+1 represents the first nucleotide of the methionine initiation codon), wherein the thymine base that pairs to −10 of the mRNA is replaced by 6-amino uracil (aU) suitable for biotinylation near the 5′-end of the antisense oligonucleotide. The oligonucleotide, with the sequence 5′-GGaUGGGCTCCATGGCCGCGCT-3′, was custom-synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX). The model vasopressin analogue, [desamino-Cys[1],D-Lys[8]]lysine vasopressin (DDLVP) was custom-synthesized by Peninsula Laboratories (Belmont, CA). Sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-biotin) and *N*-hydroxysuccinimidobiotin (NHS-biotin) were purchased from Pierce Chemical Co. (Rockford, IL). [^{125}I]iodine, [^{3}H]biotin (sp. act. 45 mCi/μmol), and [γ-^{32}P]ATP were purchased from DuPont-NEN (Boston, MA). Avidin, streptavidin, α-methylmannoside (αMM), salmon protamine (Grade 4), and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Millex GV 0.22 μ filters were obtained from Millipore Corp. (Bedford, MA).

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2.2. *Synthesis of biotinylated oligomers*

The antisense oligonucleotide was biotinylated with NHS-biotin [8]. Following biotinylation, the product was purified by Sephadex G-25 gel filtration and labeled at the 5′-end with polynucleotide T_4 kinase using [γ-^{32}P]ATP, and this labeling resulted in a final product with a specific activity of 2.6 μCi/pmol. The labeled oligonucleotide was re-purified through a Sephadex G-25 column, avidin was added, and the avidin/bio-DNA complex was purified by Sephadex G-75 column chromatography prior to experiments, where bio-DNA = biotinylated oligonucleotide. The DDLVP was purified to homogeneity by C18 reverse-phase high-performance liquid chromatography (HPLC), radiolabeled with [^{125}I]iodine and choramine T, followed by purification through a C18 Sep Pak extraction cartridge, and was biotinylated with NHS-SS-biotin [9].

Two different types of biotin analogues were employed in these studies. The antisense oligonucleotide was biotinylated with NHS-biotin, and this biotinylation reaction results in the formation of an avidin–biotin conjugate that is resistant to thiol cleavage. The DDLVP was biotinylated with a cleavable biotin linker employing NHS-SS-biotin, and this results in the formation of a disulfide-based linker that allows for thiol-based cleavage of the biotinylated drug from the avidin vector [8–10].

2.3. *Binding studies with isolated brain capillaries*

Capillaries were isolated from fresh bovine brain cortex using a mechanical homogenization technique as described previously [6]. The isolated brain capillaries were incubated in Ringer-HEPES buffer (RHB) containing either [^{125}I]avidin (0.2 μCi/ml), [^{3}H]biotin (0.5 μCi/ml), [^{125}I]bio-SS-DDLVP (0.5 μCi/ml), or [^{32}P]bio-DNA (0.2 μCi/ml), along with various concentrations of unlabeled avidin,

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streptavidin, or α-methylmannoside (αMM). Unless otherwise indicated, all incubations were performed at 23°C for 30 min, and the amount of brain capillaries added per 0.45 ml incubation reaction corresponded to approximately 100 μg protein per tube. The data are reported as mean ± SE ($n = 3$ per point). Following incubation of capillaries for 5 s to 30 min at either 37°C or 4°C, endocytosis of [^{3}H]biotin-avidin was measured using an acid wash assay described previously [6].

2.4. *Avidin radio-iodination*

Avidin was iodinated with ^{125}I and chloramine T to a specific activity of 2.0 μCi/μg. The final product was 95% precipitable with trichloroacetic acid (TCA).

3. RESULTS

The brain capillary uptake of [^{3}H]biotin is negligible in the presence of Ringer-HEPES buffer alone (Fig. 1B). However, when avidin is added to incubation, the brain capillaries avidly take up the [^{3}H]biotin-avidin complex. This uptake is rapid and reaches equilibrium at 37°C by 5 min of incubation (Fig. 2), and approximately 40% of the uptake of the [^{3}H]biotin-avidin complex is resistant to a mild acid wash (Fig. 2).

In contrast to avidin, the addition of streptavidin to the incubation mixture results in no increased uptake of biotin (Fig. 1B). The differences between the cationic avidin and the slightly acidic streptavidin, (pI = 5–6) [11], underscore the importance of the cationic nature of avidin which initiates the absorptive-mediated endocytosis of the avidin-biotin complex by brain capillaries (Fig. 2). Similar to other cationic proteins such as cationized albumin or histone [6,12], the absorptive-mediated endocytosis of avidin by isolated brain capillaries is competitively inhibited by the polycationic protein, protamine, with a $K_i = 5$ μg/ml protamine (Fig. 2). 50 mM α-methyl-mannoside results in no significant inhibition of the uptake of the avidin–biotin complex by brain capillaries (Fig. 1B). The uptake by isolated brain capillaries of either the [^{125}I]avidin or [^{3}H]biotin bound to unlabeled avidin is shown in Fig. 1A. The uptake of the [^{3}H]biotin is approximately fourfold greater than the uptake of the [^{125}I]avidin, consistent with four biotin binding sites per avidin tetramer [4]. The concentration of avidin which results in 50% inhibition of uptake is approximately

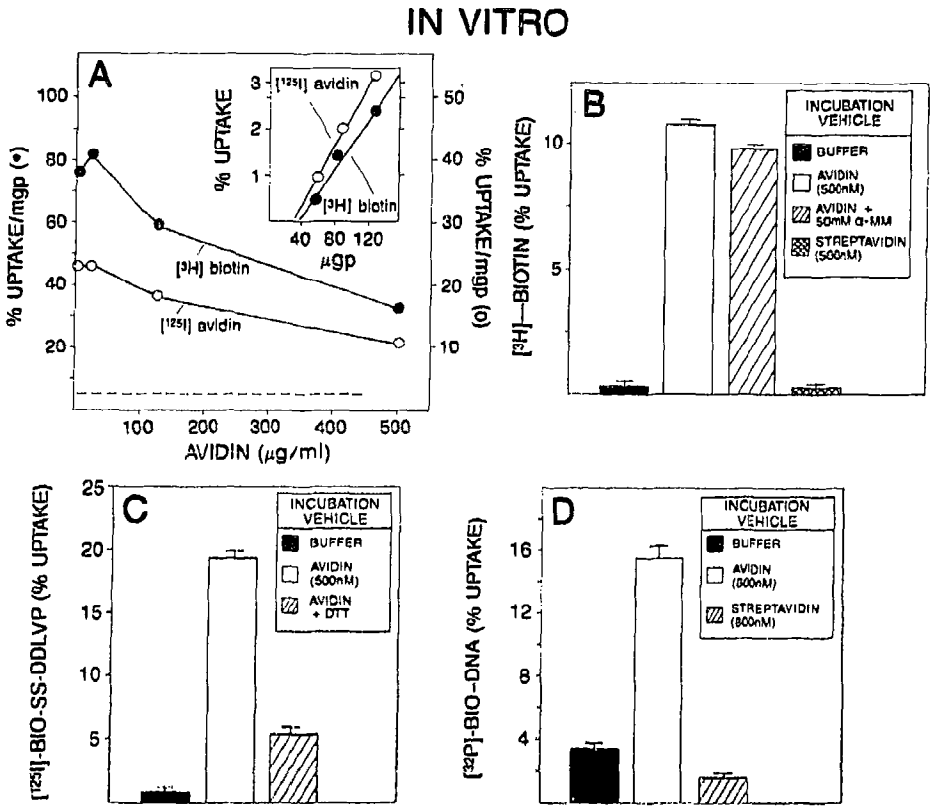

Fig. 1. Avidin-mediated uptake of biotin, biotinylated peptide, and biotinylated antisense oligonucleotide by isolated bovine brain capillaries in vitro. A. The percent uptake (per mg protein of isolated brain capillaries) of either [^{125}I]avidin or [^{3}H]biotin is plotted vs the concentration (1–500 μg/ml) of avidin in the incubation medium. (Inset) The uptake of either isotope is linear with respect to the amount of brain capillaries added to the incubation mixture. In these experiments, [^{125}I]avidin is present at a concentration of 0.1 μg/ml (1.6 nM) and [^{3}H]biotin is present at a concentration of 22 nM. B. The percent uptake of [^{3}H]biotin by isolated bovine brain capillaries is plotted vs the incubation vehicle containing either RHB buffer, avidin, avidin plus 50 mM α-methylmannoside (α-MM), or streptavidin. C. The percent uptake of [^{125}I]bio-SS-DDLVP by isolated brain capillaries is plotted vs the incubation vehicle, which contained either buffer, avidin, or avidin plus dithiothreitol (DTT) pre-treatment. D. The percent uptake of [^{32}P]biotinylated oligonucleotide (bio-DNA) by isolated bovine brain capillaries is plotted vs the incubation vehicle, which contains either buffer, avidin, or streptavidin.

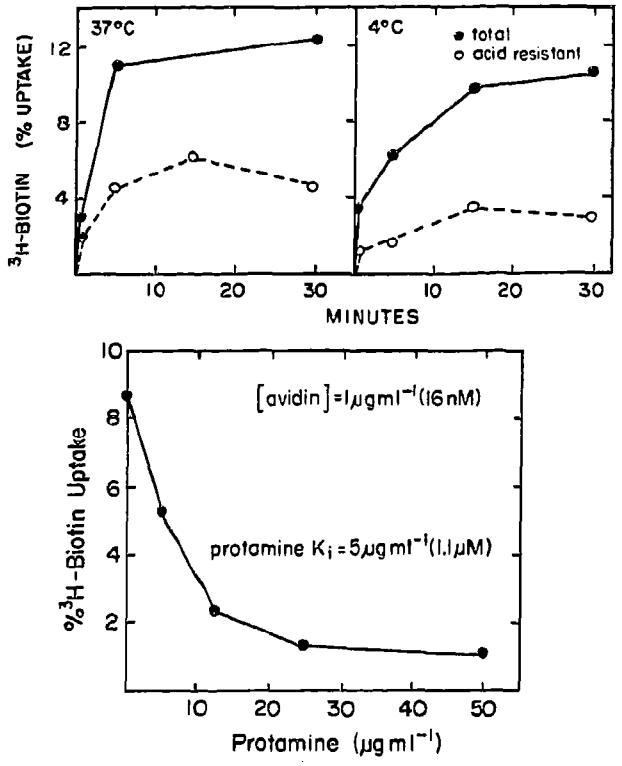

Fig. 2. (Top) The percent uptake of [^{3}H]biotin in the presence of 500 nM avidin by isolated bovine brain capillaries is plotted versus the incubation time at either 37°C or 4°C. Both total binding and binding that is resistant to a mild acid wash is shown. (Bottom) The percent uptake of [^{3}H]biotin in the presence of 1 μg/ml avidin (16 nM) is inhibited by increasing concentrations of unlabeled salmon protamine, and the concentration of protamine that gives 50% inhibition is 5 μg/ml (1.1 μM) protamine.

350 μg/ml of 5 μM avidin. The horizontal line in Fig. 1A represents the nonspecific uptake for an extracellular marker such as [^{3}H]sucrose or [^{3}H]albumin.

In Fig. 1C, the uptake by brain capillaries of [^{125}I]bio-SS-DDLVP is negligible in the presence of Ringer's buffer, but is increased in the presence of 500 nM avidin. In contrast, the pre-treatment of the [^{125}I]bio-SS-DDLVP-avidin complex with 25 mM dithiothreitol (DTT) prior to addition to the isolated brain capillary preparation results in a decrease in the capillary uptake of the [^{125}I]DDLVP peptide. The decreased uptake caused by the DTT pre-treatment is due to cleavage of the disulfide bond, which separates the biotinylated DDLVP peptide from the avidin vector. In Fig. 1D, the uptake of [^{32}P]bio-antisense oligonucleotide by isolated brain capillaries is relatively low, and this uptake is increased by the addition of 800 nM avidin to the incubation mixture. Conversely, there is no significant increase in uptake when 800 nM streptavidin is added to the incubation. The modest uptake of radioactivity in the presence of buffer alone may represent uptake of [^{32}P]nucleotide or phosphate ion generated by capillary nuclease or phosphatase degradation of the antisense oligonucleotide.

4. DISCUSSION

These studies show that avidin undergoes absorptive-mediated binding and endocytosis by isolated bovine brain capillaries, and the basis for this uptake is the cationic nature of the avidin protein. The evidence for this is threefold. First, the uptake is competitively inhibited by another cationic protein, protamine, with a K_i of 5 μg/ml (Fig. 2), which is similar to the K_i characterizing the protamine inhibition of brain capillary uptake of other cationic proteins, such as cationized albumin or histone [6,12]. Second, the bacterial homologue of avidin, streptavidin, which has a 38% amino acid homology with avidin [13], but is a slightly acidic protein [11], does not facilitate cellular uptake of biotin (Fig. 1). Third, the mannose-rich carbohydrate [14] moiety of avidin does not trigger the saturable uptake of avidin by isolated brain capillaries since high concentrations of α-methylmannoside result in no significant inhibition of the uptake of the avidin-biotin complex by brain capillaries (Fig. 1).

The present studies suggest that the avidin-biotin technology may be used to enhance cellular delivery of antisense oligonucleotides and peptides. In addition, the avidin cDNA has been cloned [15], and the production of avidin fusion constructs, wherein the avidin gene is fused to that of an organ-specific transport vector, may allow for targeted delivery of biotinylated antisense oligonucleotides or peptides.

Acknowledgements: The authors are indebted to Jody L. Buciak and Jing Yang for valuable technical assistance and to Sherri J. Chien for skillful preparation of the manuscript. This work was supported by NIH Grant RO1-AI-28760.

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