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Original Research

Aptamer-Conjugated Calcium Phosphate Nanoparticles for Reducing Diabetes Risk via Retinol Binding Protein 4 Inhibition

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ABSTRACT

Objectives: Inhibition of the binding of retinol to its carrier, retinol binding protein 4, is a new strategy for treating type 2 diabetes; for this purpose, we have provided an aptamer-functionalized multishell calcium phosphate nanoparticle.
Methods: First, calcium phosphate nanoparticles were synthesized and conjugated to the aptamer. The cytotoxicity of nanoparticles releases the process of aptamer from nanoparticles and their inhibition function of binding retinol to retinol binding protein 4.
Results: After synthesizing and characterizing the multishell calcium phosphate nanoparticles and observing the noncytotoxicity of conjugate, the optimum time (48 hours) and the pH (7.4) for releasing the aptamer from the nanoparticles was determined. The half-maximum inhibitory concentration (IC_{50}) value for inhibition of retinol binding to retinol binding protein 4 was 210 femtomolar (fmol).
Conclusions: The results revealed that the aptamer could prevent connection between retinol and retinol binding protein 4 at a very low IC_{50} value (210 fmol) compared to other reported inhibitors. It seems that this aptamer could be used as an efficient candidate not only for decreasing the insulin resistance in type 2 diabetes, but also for inhibiting the other retinol binding protein 4-related diseases.
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Mots clés :
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RÉSUMÉ

Objectifs : L'inhibition de la liaison du rétinol à son transporteur, la protéine 4 de liaison au rétinol, est une nouvelle stratégie de traitement du diabète de type 2. À cet effet, nous avons fourni une nanoparticule multicoquille de phosphate de calcium fonctionnalisée par l'aptamère.
Méthodes : D'abord, les nanoparticules de phosphate de calcium ont été synthétisées et conjuguées à l'aptamère. La cytotoxicité des nanoparticules déclenche le processus de libération de l'aptamère des nanoparticules et leur fonction d'inhibition de la liaison du rétinol à la protéine 4 de liaison au rétinol.
Résultats : Après la synthèse et la caractérisation des nanoparticules multicoquilles de phosphate de calcium et l'observation de la non-cytotoxicité du conjugué, nous avons déterminé la durée optimale (48 heures) et le pH (7,4) pour libérer l'aptamère des nanoparticules. La valeur de concentration inhibitrice semi-maximum (CI_{50}) pour l'inhibition de la liaison du rétinol à la protéine 4 de liaison au rétinol était de 210 femtomoles (fmol).
Conclusions : Les résultats ont révélé que l'aptamère pourrait prévenir la liaison entre le rétinol et la protéine 4 de liaison au rétinol à une très faible valeur CI_{50} (210 fmol) comparativement aux autres inhibiteurs rapportés. Il semble que cet aptamère pourrait être utilisé comme un candidat efficace non seulement pour diminuer l'insulinorésistance lors de diabète de type 2, mais également pour inhiber les autres maladies liées à la protéine 4 de liaison au rétinol.
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Introduction

Type 2 diabetes has become a major challenge for public health worldwide. Aging, urbanization, increasing rates of obesity, physical inactivity and modern lifestyles are the factors that contribute to increased insulin resistance and the prevalence of this chronic disease (1). One of the reliable markers for the prediction of insulin resistance is retinol-binding protein 4 (RBP4). In recent years, it has been revealed that RBP4 is secreted by adipose tissue. Therefore, RBP4 is related to adiposity, insulin resistance and type 2 diabetes (2). The molecular mechanism of RBP4 in inducing insulin resistance is controversial and complex. However, studies have shown that increasing RBP4 concentration in serum disrupts the insulin pathways in glucose metabolism, and this leads to hyperglycemia and, consequently, to type 2 diabetes (3). But it has also been shown that elevation of RBP4 induces adipose tissue inflammation and impairs glucose tolerance and insulin sensitivity, which cause insulin resistance (4).

RBP4 is the principal carrier of retinol in the human plasma, and it forms a complex with transthyretin (TTR), a homotetramericthyroxin transport protein. This complex is thought to prevent glomerular filtration of RBP4. Binding retinol to RBP4 induces conformational changes that cause greater affinity between the retinol-RBP4 complex and TTR (retinol-RBP4-TTR) than RBP4 and TTR (RBP4-TTR) (5). Changing RBP4 activity (the ability of RBP4 to bind with retinol and TTR), altering the structural stability of RBP4 in tissues or the half-life in circulation could be targets for the treatment of insulin resistance (6). It seems that the inhibition of binding retinol to RBP4 can decrease the affinity of RBP4 to TTR, which may result in renal clearance of RBP4 and the lowering of serum RBP4 (7). Many synthetic retinoid compounds have been reported as retinol antagonists, which disrupt the interaction between RBP4 and its binding partner (TTR), and that leads to lower serum levels of RBP4 (8,9).

Aptamers, a new generation of antagonists, are a special class of nucleic acid molecules (single-stranded DNA or RNA oligonucleotides) that can bind to their targets with high affinity and specificity due to their tertiary structures (10). Since 1990, aptamers have been used in medicine as therapeutics, for example, pegaptanib sodium injection (Macugen), which targets age-related macular degeneration. Many aptamers, now in differing stages of clinical development, have been discovered to affect a variety of diseases. Thus, in the future, we can expect more therapeutic aptamers to be available on the market for clinical applications (10–13).

Recently, a 76-mer single-stranded DNA aptamer was reported; it binds specifically to human RBP4 protein (RBP4 binding aptamer) (RBA) (14). A molecular dynamic simulation study of RBP4-RBA interaction, which was also done by this research group, showed that RBA binding to RBP4 probably inhibits RBP4 interactions with retinol by detention of RBP4 active pocket residues (15). It seemed that in the case of proving the inhibition effect of RBA on binding retinol to RBP4, we could introduce a new therapeutic candidate for inhibition of retinol-RBP4 complex formation to cause disruption in RBP4 binding to TTR, which drives renal clearance of RBP4, lowering serum levels of RBP4 and, consequently, decreasing insulin resistance in persons with type 2 diabetes.

The specific challenges of therapeutic aptamers generally include nuclease-mediated degradation, rapid renal filtration and structural stability. Currently, to increase the structural stability and improve the half-life of aptamers, they are conjugated to nanoparticles extensively (16). One of the carriers of nucleic acids is calcium phosphate nanoparticles (CP-NPs), which are biocompatible and biodegradable and also are not subject to microbiologic degradation as are organic and polymeric carrier systems. The affinity of calcium of CP-NPs with the phosphate groups in nucleic acids is probably due to the electrostatic interaction between the negative charges of DNA and the positive charges of calcium ions. It has also been demonstrated that DNA can be protected against nuclease attacks if it is enclosed within CP-NPs with an additional shell of calcium phosphate (17). CP-NPs, with their fast degradation rate in cells, are appropriate for applications in drug delivery (18).

In the present study, the synthesis of RBA-conjugated multishell CP-NPs was described. The procedure was so mild that the function and structure of RBA were conserved. In addition, these prepared nanoparticles could inhibit the binding of retinol to RBP4 without showing toxicity, so they could be suggested as efficient candidates for decreasing insulin resistance in patients with diabetes.

Methods

Materials and reagents

Human RBP4 (a full-length protein) was provided by Abcam (Cambridge, Massachusetts, United States). A 76-mer RBA was purchased from Bioneer (Daedeok-gu, Daejeon, Republic of Korea). Calcium nitrate ($Ca[NO_3]_2$), aluminum chloride ($AlCl_3$), ammonium phosphate dibasic ($[NH_4]_2HPO4$), retinol (R7623), Dulbecco/Vogt modified Eagle minimal essential medium (DMEM), fetal bovine serum (FBS) and thiazolyl blue tetrazolium bromide (MTT) were prepared (Sigma, St. Louis, Missouri, United States). A fibroblast NIH-3T3 cell line was obtained from the Pasteur Institute (Tehran, Iran).

Calcium phosphate nanoparticle synthesis

CP-NPs were prepared according to the literature, with a slight modification (19). First, to control the size growth of CP-NPs, calcium was partially substituted by aluminum by adding $AlCl_3$ (2 mM) to the $Ca(NO_3)_2$ solutions (30 mM, pH 9). Briefly, aqueous solutions of $Ca(NO_3)_2$ were pumped (flow rate: 34 mL/min) into a vessel containing $(NH_4)_2HPO_4$ (3.74 mM, pH 9) at room temperature while stirring. After 30 seconds, 100 µL of the nanoparticles (pH 8) were taken up with a syringe and mixed rapidly with a solution of RBA (100 µL of 60 ng/mL) at room temperature to result in the CP-NPs' being coated with RBA (RBA/CP).

To change RBA/CP into multishell nanoparticles, the following steps were carried out. First, 0.5 mL of calcium nitrate (30 mM, pH 9) were added to the RBA/CP dispersion, followed by the addition of 0.5 mL $(NH_4)_2HPO_4$ (3.74 mM, pH 9). This process led to the deposition of calcium phosphate on the surface of the RBA/CP. The resulting CP/RBA/CP colloidal solution was stabilized by adding 100 µL of 60 ng/mL of RBA solution to form RBA/CP/RBA/CP nanoparticles. The final nanoparticles were centrifuged at 9000 rpm for 10 minutes to collect the nanoparticles and remove the unconjugated RBA in the supernatant. This washing step was repeated at least 3 times (20). The final CP-NP conjugates were stable and could be stored at 6°C. The size and zeta potential of the RBA/CP/RBA/CP nanoparticles were measured by dynamic light scattering (90 plus; Brookhaven National Laboratory, Upton, New York, United States).

RBA release

RBP4 was synthesized by hepatocytes and adipocytes and then secreted into the blood (21), so cells and blood circulation can be considered the main targets for releasing RBA to bind RBP4 and inhibit its function. The experiment was carried out at 2 different pHs: 7.4 and 5.0. The pH 7.4 reflects the physiologic pH of blood, and the pH 5.0 indicates the pH of the endosome or skin (22,23). The release of RBA was conducted by dispersing 100 µL of conjugates (RBA/CP/RBA/CP) in 0.8 mL PBS by gentle shaking (20 rpm) at 37°C (20 cycles per minute). After various time intervals (1, 2,

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