PEG-coated gold nanoparticles attenuate β-adrenergic receptor-mediated cardiac hypertrophy

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Abstract: Gold nanoparticles (AuNPs) are widely used as a drug delivery vehicle, which can accumulate in the heart through blood circulation. Therefore, it is very important to understand the effect of AuNPs on the heart, especially under pathological conditions. In this study, we found that PEG-coated AuNPs attenuate β-adrenergic receptor (β-AR)-mediated acute cardiac hypertrophy and inflammation. However, both isoproterenol, a non-selective β-AR agonist, and AuNPs did not induce cardiac function change or cardiac fibrosis. AuNPs exerted an anti-cardiac hypertrophy effect by decreasing β-AR expression and its downstream ERK1/2 hypertrophic pathway. Our results indicated that AuNPs might be safe and have the potential to be used as multi-functional materials (drug carrier systems and anti-cardiac hypertrophy agents).

Keywords: AuNPs, cardiac hypertrophy, β-adrenergic receptor, ERK1/2 signaling pathway

Background

Along with the advances in nanotechnology, nanoparticles have been applied widely in biomedicine.1,2 In particular, gold nanoparticles (AuNPs) are a potential candidate for the development of diagnostic and therapeutic methods, due to their unique physical, chemical, optical, and pharmacological properties. For example, gold cores are inert, stable, biocompatible, and have low toxicity. In addition, AuNPs are easy to prepare and functionalize.3,4 Therefore, AuNPs have great potential for many biomedical applications, such as drug or protein delivery, gene transfection, cancer therapy, biomedical imaging, bio-labeling, and molecular diagnostic tools. Furthermore, the surface of AuNPs can be modified to improve specificity and safety of its application in clinical or research.5,6 Since AuNPs have shown great potential for wide application in diagnosis and treatment of diseases, it is important to evaluate the safety of AuNPs in the diseases.

Cardiac diseases have been the leading cause of death worldwide.7 AuNPs are widely used in the diagnosis and treatment of cardiac diseases.8-11 However, there have been only a few studies that examined the effect of AuNPs on the heart. So it is necessary to investigate the safety of AuNPs for the heart under both physiological and pathological conditions. Our previous study proved the safety of AuNPs under physiological conditions,12 so we focused on the safety of AuNPs for the heart under pathological conditions in the present study.

Cardiac hypertrophy is an important pathological basis for various heart diseases and is an independent risk factor for morbidity and mortality of heart failure.13 The safety of AuNPs in cardiac hypertrophy is still unclear. A key factor in cardiac
hypertrophy is the over-activation of β-adrenergic receptor (β-AR)\textsuperscript{14} and β-blockers have been one of the standardized therapeutic drugs for heart failure in clinical settings.\textsuperscript{15} Thus isoproterenol (ISO), the agonist of β-AR, has been widely used to establish the animal model of cardiac hypertrophy.\textsuperscript{16,17} ISO is reported to induce both chronic and acute cardiac hypertrophy in animal experiments. The chronic cardiac hypertrophy model is used to simulate the chronic progression of heart remodeling in some chronic cardiovascular diseases such as hypertension.\textsuperscript{18} The acute cardiac hypertrophy model reflects cardiac remodeling following acute cardiac injuries.\textsuperscript{19} The safety of AuNPs in ISO-induced chronic cardiac hypertrophy model has been demonstrated in our previous study.\textsuperscript{20} Thus, in the present study, the safety of AuNPs in ISO-induced acute cardiac hypertrophy model was investigated.

The results in this study indicated that the accumulation of AuNPs in the heart depends on their size and the accumulation of AuNPs in the heart attenuates β-AR-mediated acute cardiac hypertrophy and inflammation through decrease of β₁-AR expression and its downstream ERK1/2 hypertrophy signaling pathway. These novel findings will be helpful for the wider application of AuNPs in cardiac diseases.

**Methods**

**Characterization of the AuNPs**

Three different sizes (13, 30, and 50 nm) of PEG-coated AuNPs were used in this experiment (Nanocs Inc., New York, NY, USA). The morphology size and aggregation state of the AuNPs were evaluated using transmission electron microscopy (JEM-200CX; Jeol, Ltd., Tokyo, Japan) and Multiskan GO (Thermo Scientific, Ltd, Waltham, MA, USA). The PEG-coated AuNP suspension was sonicated for 5 min before use.

**Establishment of animal model**

Our investigation was approved by the Biomedical Research Ethics Committee of Peking University (LA 2010-048) and strictly adhered to the American Physiological Society’s “Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training”. Male FVB/N mice (10–12 weeks) were obtained from laboratory animal department of Peking University Health Science Center. Mice were housed in groups of four and maintained on a 12 h dark/light cycle in a room with controlled temperature (25°C±2°C). Mice had free access to food and water. Cardiac hypertrophy model was established by subcutaneous injection of ISO (200 mg/kg/day, dissolved in saline [Sigma-Aldrich, St Louis, MO, USA]) for 3 consecutive days.

There were five groups in total (eight to ten mice in each group): i) control group: daily administration of saline for 3 days; ii) ISO group: daily subcutaneous administration of ISO (200 mg/kg/day) for 3 days; iii) ISO +13 nm AuNPs group: daily subcutaneous administration of ISO (200 mg/kg/day) for 3 days, 540 μg/kg AuNPs of 13 nm were injected into the tail vein on the same days after ISO injections; iv) ISO +30 nm AuNPs group: daily subcutaneous administration of ISO (200 mg/kg/day) for 3 days, 540 μg/kg AuNPs of 30 nm were injected into the tail vein on the same days after ISO injections; v) ISO +50 nm AuNPs group: daily subcutaneous administration of ISO (200 mg/kg/day) for 3 days, 540 μg/kg AuNPs of 50 nm were injected into the tail vein on the same days after ISO injections. The content of AuNPs of all three sizes (13, 30, and 50 nm) was 0.01% Ag/mL. The volume of injection was adjusted to 5 μL/g of mice weight. The PEGylated AuNP suspension was sonicated for 5 min before use to make the AuNPs disperse adequately. The treatment of animals in each group is shown in Table 1.

**Echocardiographic analysis**

Echocardiographic analysis was performed 1 day after the last injection. Mice were anesthetized with 1.5% isoflurane (Baxter International Inc., Deerfield, IL, USA). Echocardiographic images were obtained by the Visualsonics high-resolution Vevo 770 system (VisualSonics, Inc., Toronto, ON, Canada). Two-dimensional short-axis views were obtained at the level of the papillary muscle. The diastolic left ventricular posterior wall thickness (LVPW;d) and systolic left ventricular posterior wall thickness (LVPW;s) were measured to calculate the ejection fraction (EF) and fractional shortening (FS). All measurements were averaged from three consecutive cardiac cycles. Cardiac systolic function was represented by the values of EF and FS. Doppler echocardiograms were captured via an apical four chamber view. Transmitral flow Doppler was obtained through mitral flow center and two characteristic E wave and A wave were obtained. Tissue Doppler images were obtained through the mitral annulus and Doppler velocities E’ and A’ were obtained. E/A, E’/A’,

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<th>Group</th>
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<td>ISO +50 nm AuNPs</td>
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Abbreviations: AuNPs, gold nanoparticles; ISO, isoproterenol; Con, control; d, days.
and E/E’ ratios were calculated to evaluate cardiac diastolic function. The average of three consecutive cardiac cycles was taken for each parameter. Echocardiography procedures were performed in accordance with the guideline of American Society of Echocardiography.

Quantitative histological analysis
Mice were anesthetized and sacrificed after echocardiography analysis. The hearts were excised and weighed immediately after being washed with cold PBS. The cardiac tissues for histological and immunohistochemistry analysis were fixed with 4% paraformaldehyde for 12 h, dehydrated in 20% sucrose for 24 h, and then embedded in paraffin. Serial sections (5 μm thick) were stained with hematoxylin and eosin stain (H&E) for morphological analysis and picrosirius red was used for the detection of fibrosis. For morphometric analysis, photographs of left ventricular sections cut from the same location of each heart were observed under 4×, 200×, and 400× magnification, respectively (Leica Microsystems Imaging Solutions Ltd., Wetzlar, Germany). Myocyte cross-sectional area was measured by Image Pro Plus using the photographs under 400× magnification. Interstitial fibrosis was visualized with picrosirius red staining, and the cardiac fibrosis volume fraction was calculated as the ratio of the stained fibrotic area to the total myocardial area.

Western blot analysis
The cardiac tissues were cracked by tissue homogenizer and ultrasonic unit. After centrifugation (12,000 rpm, 15 min, 4°C), the protein content of supernatant was determined by BCA protein quantitative method. The loading quantity of samples was 30 μg. Samples were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After being blocked, blots were probed with the appropriate primary antibodies overnight at 4°C, then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody. Bands were visualized with a super Western blot sensitivity chemiluminescence detection system (Thermo Fisher Scientific). The conditions of primary and secondary antibodies used for immunoblot analysis are summarized in Table 2.

Quantitative real-time polymerase chain reaction (PCR)
Total RNA was isolated from heart tissues using Trizol Reagent (Thermo Fisher Scientific). The complementary DNA was synthesized using the kit (017317; Promega Corporation, Fitchburg, WI, USA). Relative quantitation by real-time PCR was performed using SYBR Green to detect PCR products in real time with ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Thermo Fisher Scientific). Primer sequences were as follows: EIF5: 5′-CAGAAACACAAAGCGAAGACAG-3′, 5′-GCTTCAGAGACAACCTTCC-3′; TNF-α: 5′-CCACGTCGTAGACAAACCA-3′, 5′-ACAAGGTACCAACCATCGGC-3′; IL-1β: 5′-TGCCACCCTTTGACAGT-3′, 5′-AGTATG-3′; 5′-AAGGTCACAGGGAAAGAC-3′; IL-6: 5′-CGGCCCTCCTACTTCACA-3′, 5′-ATGTCAGAAGCCTGCTAGCT-3′; β-AR: 5′-GCCCTTTCCCTACAGGTT-3′, 5′-ACTTGGG-3′; CollagenI: 5′-TGAACTTGCCTAGCAACATACA-3′, 5′-CTTCTGCTACAGAATTGAG-3′, 5′-GGGGCGTATGACCTCATTT-3′, 5′-AGCCAAGGCTTCTTCGAC-3′, 5′-AGGACAGGCTTCTTCGAC-3′; CollagenIII: 5′-GGACCTCAATCGTC-3′, 5′-CAGGACTGCCTTGATTC-3′. PCR was performed under the following conditions: 95°C for 2 min, followed by 40 amplification cycles (95°C for 15 s, 60°C for 1 min). The CT (threshold cycle) values obtained for genes of interest were normalized to concurrent measurement of EIF5 mRNA level, and fold changes were compared to the control.

Inductively coupled plasma-mass spectrometry (ICP-MS)
The concentrations of AuNPs in tissues were assessed by quantitative inductively coupled plasma mass spectrometry (ICP-MS). Cardiac tissues (approximately 30 mg each) were digested in aqua fortis (nitric acid:hydrochloric acid 3:1). After adjusting the solution volume to 2 mL using 2% nitric acid and 1% hydrochloric acid (1:1), Au content assays were performed using an ELAN DRC-e ICP-MS instrument (PerkinElmer Inc., Waltham, MA, USA).

Statistical analysis
Data were summarized as means ± SEM. Differences in data between groups were compared using Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) with one-way ANOVA.
followed by Dunnett’s test. Data with $P<0.05$ were considered statistically significant.

**Results**

**The accumulation of AuNPs in the mouse heart**
To investigate the effect of AuNPs on $\beta$-AR-mediated cardiac remodeling, experiments were designed as shown in the working flow chart, Figure 1A. Firstly, the accumulation of AuNPs was determined by ICP-MS in mouse heart. As shown in Figure 1B, accumulation of AuNPs in the heart was size-dependent. The 13 nm AuNPs showed the highest accumulation in heart ($1,438\pm236.9$ ng/g) which was extremely high in comparison with that of 30 nm ($68.76\pm17.33$ ng/g) and 50 nm ($18.21\pm4.052$ ng/g).

**Effects of the AuNPs on cardiac hypertrophy**
The ratio of heart weight to tibia length (HW/TL), the ratio of heart weight to body weight (HW/BW), LVPW;d, myocyte cross-sectional area, ANF, and BNP are all major indicators of cardiac hypertrophy. As shown in Figure 2A and B, ISO markedly increased LVPW;d and AuNPs (13, 30, and 50 nm) reversed this process. Similarly, AuNPs (13, 30, and 50 nm) attenuated HW/BW (Figure 2C) and HW/TL (Figure 2D) increased significantly by ISO. Moreover, AuNPs (13, 30, and 50 nm) reduced myocyte cross-sectional area (Figure 2E and F) increased by ISO. AuNPs (13, 30, and 50 nm) also decreased the mRNA expression of ANF (Figure 2G) and BNP (Figure 2H).

**Effects of the AuNPs on cardiac fibrosis**
Chronic long-term ISO stimulation could cause fibrosis.\(^{21}\) Our results indicated that acute short-term ISO stimulation did not induce obvious fibrosis. Furthermore, AuNPs did not affect cardiac fibrosis either (Figure 3A and B). Collagen I and Collagen III are also fibrosis markers. The mRNA expressions of Collagen I (Figure 3C) and Collagen III (Figure 3D) were consistent with the previously mentioned conclusion.

**Effects of the AuNPs on cardiac functions**
Assessment of cardiac function is important in cardiac diseases. The left ventricular EF and FS were the most important

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**Figure 1** Experimental flow chart and Au accumulation in heart.
**Notes:** (A) The experimental flow chart of the present study. (B) The Au content in mouse heart was determined with inductively coupled plasma mass spectrometry. *$P<0.001$. Data represent mean ± SEM.

**Abbreviations:** AuNPs, gold nanoparticles; ISO, isoproterenol; sc, subcutaneous; iv, intravenous; Con, control.
indicators to evaluate the cardiac contraction function. Results showed that the EF and FS were still normal after exposure to ISO. AuNPs (13, 30, and 50 nm) did not affect EF or FS (Figure 4A and B). E/A, E’/A’ and E/E’ were used to evaluate cardiac diastolic function. Similar to cardiac contraction function, ISO did not affect cardiac diastolic function obviously in this model. AuNPs (13, 30, and 50 nm) did not affect cardiac diastolic function either (Figure 4C–F).

Effects of the AuNPs on β-AR-mediated IL-6 mRNA expression

First, H&E staining showed that ISO induced inflammatory cell infiltration. However, the additional accumulation of AuNPs in the heart did not aggravate ISO-induced cardiac inflammation (Figure 5A). Furthermore, inflammatory cytokines TNF-α, IL-1β, and IL-6 were detected with real-time PCR. Consistent with cardiac hypertrophy, AuNPs (13, 30, and 50 nm) significantly inhibited IL-6 production increased by ISO (Figure 5B). In contrast to IL-6, ISO did not increase the production of TNF-α (Figure 5C) and IL-1β (Figure 5D).

Effects of the AuNPs on β-AR mRNA expression

It has been reported that chronic ISO stimulation downregulated the expression of both β1-AR and β2-AR.

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Figure 2 The effects of AuNPs on cardiac hypertrophy.
Notes: *P<0.05, **P<0.01, ***P<0.001. (A) Representative M-mode echocardiography images were taken to show left ventricular wall thickness. (B) Quantitative analysis of diastolic left ventricular posterior wall thickness. (C) Quantitative analysis of HW/BW ratio. (D) Quantitative analysis of HW/TL ratio. Data represent mean ± SEM. (E) Representative micrographs of myocyte cross-sectional area. (F) Quantitative analysis of myocyte cross-sectional area. (G) Quantitative analysis of ANF mRNA expression. (H) Quantitative analysis of BNP mRNA expression.

Abbreviations: AuNPs, gold nanoparticles; HW/BW, ratio of heart weight to body weight; HW/TL, ratio of heart weight to tibia length; ISO, isoproterenol; Con, control; LVAW/s, systolic left ventricular anterior wall thickness; LVID/s, left ventricular end-systolic inner-diameter; LVPW/s, systolic left ventricular posterior wall thickness; LVAW/d, diastolic left ventricular anterior wall thickness; LVID/d, left ventricular end-diastolic inner-diameter; LVPW/d, diastolic left ventricular posterior wall thickness.
the result showed that acute ISO stimulation upregulated β₁-AR but not β₂-AR mRNA expression (Figure 6A and B). AuNPs (13, 30, and 50 nm) inhibited the upregulation of β₁-AR mRNA expression significantly (Figure 6A) but had no influence on β₂-AR mRNA expression (Figure 6B).

**Effects of the AuNPs on β-AR-mediated ERK1/2 pathway activation**

It is well known that ERK1/2 MAPK pathway plays an important role in β-AR-mediated cardiac hypertrophy. The results indicated that AuNPs (13, 30, and 50 nm) decreased the phosphorylation of ERK1/2 induced by ISO obviously (Figure 7A and B), which is consistent with the function of AuNPs in inhibiting β-AR-mediated cardiac hypertrophy.

**Discussion**

In this study, we investigated the safety of AuNPs in the acute cardiac hypertrophy model induced by large dose of ISO. The result suggested that AuNPs could inhibit cardiac hypertrophy in this model. The mechanism may be related to the inhibition of β₁-AR expression and its downstream signaling pathway, such as inflammation and the phosphorylation of ERK1/2.

Our study indicated that AuNPs (13, 30, and 50 nm) inhibited the increase of β₁-AR mRNA expression induced by ISO but not β₂-AR, which is consistent with the result showing that β-AR-mediated cardiac hypertrophy is primarily due to the activation of β₁-AR. Recent studies have proven that AuNPs could affect the synthesis and function of biological macromolecules. For example, AuNPs could interfere with the synthesis of ribosomal protein. Further, AuNPs could conjugate to G-protein and affect the activity of G-protein. These studies suggest that AuNPs may regulate the expression of β₁-AR and its downstream signaling pathway which reflects the activity of β₁-AR.

Many studies have suggested that inflammation is involved in cardiac hypertrophy. Pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α promote cardiac hypertrophy. In this study, the mRNA expression of IL-1β and TNF-α did not increase, but the mRNA expression of IL-6 increased after acute ISO treatment. Some studies have...
indicated that ISO could increase the expression of IL-6, IL-1β, and TNF-α.31,32 However, it has also been reported that ISO treatment downregulated TNF-α production and caused no change in IL-6 production.33 This suggested that inflammation is a complicated process, and different inflammatory cytokines may have different reactions after β-receptor activation. In addition, the dose of ISO and the time course of administration may also have an influence on the inflammatory cytokines’ production. The specific mechanisms still need to be researched more. It has also been reported that AuNPs of 21 nm could reduce IL-6 mRNA level in the fat.34 Consistent with the previous study, in our study, AuNPs (13, 30, and 50 nm) inhibited ISO-increased IL-6 mRNA expression which contributed to the β-AR-mediated cardiac hypertrophy.

ERK1/2 signaling pathways are important regulators of β1-AR-mediated cardiac hypertrophy.35–37 In the present study, we found that AuNPs inhibited the phosphorylation of ERK1/2 induced by ISO. In addition, recent work has suggested that ERK1/2 signaling is regulated, at least in part, by oxidative stress38,39 and that antioxidants can function to block the activation of ERK1/2 both in vitro and in vivo.40,41 While some other studies have shown that AuNPs could suppress oxidative stress and elevate the antioxidant defense system in vivo.42–45 So AuNPs may inhibit the phosphorylation of ERK1/2 partly through the inhibition of oxidative stress.

AuNPs (13, 30, 50 nm) inhibited ISO-induced cardiac hypertrophy in the present study, but AuNPs have no effect on either the systolic or diastolic functions. It is noteworthy that both the systolic and diastolic functions did not change.

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Figure 5 The effects of AuNPs on inflammation in the heart.
Notes: (A) Representative images showing H&E staining of heart sections. The arrows refer to the infiltrated inflammatory cells. The scale bars of 12.5× images are 2 mm, of 250× images are 100 μm, and those of 500× images are 50 μm. (B) The mRNA expression of IL-6 in the heart tissue. (C) The mRNA expression of TNF-α in the heart tissue. (D) The mRNA expression of IL-1β in the heart tissue. *P<0.05, **P<0.01. Data represent mean ± SEM.
Abbreviations: AuNPs, gold nanoparticles; H&E, hematoxylin and eosin; ISO, isoproterenol; Con, control; ns, no significance.

Figure 6 The effects of AuNPs on β-adrenergic receptor (β-AR) mRNA expression in the heart.
Notes: (A) The mRNA expression of β1-AR in the heart tissue. (B) The mRNA expression of β2-AR in the heart tissue. *P<0.05, **P<0.01. Data represent mean ± SEM.
Abbreviations: AuNPs, gold nanoparticles; ISO, isoproterenol; Con, control; ns, no significance.
after ISO treatment. The possible reason is that cardiac remodeling is in a compensation stage, so both the systolic and diastolic functions are not damaged in this stage.

There are some differences between the results of all the studies about the toxicity of AuNPs. It is likely due to their modifications, functional attachment of their surfaces, shapes, and sizes.46,47 For example, known as a modifying polymer, PEG decreases immunogenicity and increases stability of drugs in the circulatory system. Therefore, the PEG coating reduces the chance of heart toxicity induced by AuNPs.48–50

Taken together, our results showed that AuNPs inhibited cardiac hypertrophy mediated by β-AR, and this effect depends on a complex mechanism involving inhibition of β,-AR expression and its downstream effectors IL-6 and ERK1/2 (Figure 8). These results raise the hope that AuNPs might be used as multi-functional materials (drug carrier systems and anti-cardiac hypertrophy agents) for cardiac diseases’ treatment.

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Disclosure
The authors report no conflicts of interest in this work.

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