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ORIGINAL RESEARCH PAPER

Anti-hypercalcemic effect of orally administered recombinant *Saccharomyces cerevisiae* expressing salmon calcitonin on hypercalcemic rats

Pingnan Sun · Xuecheng Zhang · Xiaonan Zang · Xiaoling Zhou · Yunsong Chen · K. K. I. U. Arunakumara · Bin Liang

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Abstract Oral delivery of salmon calcitonin (sCT) to rats via a recombinant *Saccharomyces cerevisiae* was assessed. A synthetic sCT gene was cloned and expressed in *S. cerevisiae* yAGA2-sCT. Recombinant salmon calcitonin (rsCT) expression was detected by flow cytometry. The resorption activity of osteoclasts was inhibited by 3×10^{-6} M rsCT. Oral administration of 5 g lyophilized yAGA2-sCT/kg to hypercalcemic rats decreased serum calcium from 2.8 ± 0.02–2.7 ± 0.02 mM.

Keywords Hypercalcemia · Osteoclast · *Saccharomyces cerevisiae* · Salmon calcitonin

Introduction

Delivery of drugs to animals by ingesting a recombinant *Saccharomyces cerevisiae* has been developed (Blanquet et al. 2004). The drug produced by yeast transformants is known as a 'biodrug' (Blanquet et al. 2001). This concept was demonstrated using recombinant *S. cerevisiae* expressing the plant cytochrome P450 73A1 (an important enzyme in various detoxication systems) in the artificial gastrointestinal tract. Furthermore, freeze-dried *S. cerevisiae* showed a high tolerance to digestive conditions of artificial gastrointestinal tract (Blanquet et al. 2004). However, using recombinant *S. cerevisiae* as peptide drug delivery system has not yet been reported.

Salmon calcitonin (sCT), a peptide consisting of 32 amino acids, possesses an *N*-terminal disulfide bridge and a *C*-terminal prolinamide residue. The anti-resorptive effect of salmon cacitonin on mammalian bones has led its widely applications in treating diseases such as hypercalcemia, Paget's disease and osteoporosis (Zaidi et al. 2002). However, the half-life of sCT after intravenous administration is less than 2 h (Beveridge et al. 1976). For treating hypercalcemia, sCT has thus been replaced by bisphosphonates in view of their long-term effect (Pecherstorfer et al. 2003). In contrast to bisphosphonates, sCT remains as an alternative in treating hypercalcemia for relieving of osteogenic pain, in particular, when safety considerations have become important (Tuysuz et al. 1999).

The present investigation was aimed at applying *S. cerevisiae* as sustained-release sCT delivery system. Here, recombinant salmon calcitonin

P. Sun · X. Zhang (✉) · X. Zang · X. Zhou · Y. Chen · K. K. I. U. Arunakumara
College of Marine Life Sciences, Ocean University of China, 5 Yushan Road, Qingdao 266003, P.R. China
e-mail: xczhang@ouc.edu.cn

B. Liang
Navy 401 Hospital of PLA, Qingdao 266003, P.R. China

(rsCT) was expressed on the surface of *S. cerevisiae* EBY100 strain by the vector modified from plasmid pYD1 (Boder and Wittrup 1997). The in vitro bioactivity of rsCT expressed by yAGA2-sCT was detected by rat's osteoclastic resorption expriment. In vivo, orally administered 5 g lyophiled yAGA2-sCT/kg to hypercalcemic rats decreased the serum calcium level. Recombinant *S. cerevisiae* could thus be considered as a new drug delivery system for sCT in hypercalcemia therapy.

Materials and methods

Materials

Primers were synthesized and sequenced in Shanghai Invitrogen, by which the Pyd1 Yeast Display Vector Kit and antibodys were also provided. Vitamin D3 (1,25-dihydroxy-cholecalciferol) was purchased from Sigma. Restriction enzymes were from Takara Co. (Dalian, China). Miacalcic (sCT injection, 5000 IU/mg) was obtained from Novartis Pharma Schweiz AG and native enterokinase (26 kDa, pI 5.2) was from Roche Co. A salmon calcitonin-ELISA kit and Wistar rats were respectively purchased from Diagnostic Systems Laboratories, Inc and Chinese Qingdao Medicament Inspection Institution.

Generation of plasmid and strain

The sCT gene was synthesized with the biased codons of *S. cerevisiae* (Sharp et al. 1988). Double-strand sCT DNA was generated by annealing together the complement single-strand DNA sCT1 (5′-CGGGATCCTGTTCTAACTTGTC TACCTGTGTTTTGGGTAAGTTGTCTCAA GAATTGCACAAGTTGCAAACCT-3′) and sCT2 (5′-CGGGATCCTTATCATTAACCTGG AGTACCAGAACCAGTGTTAGTTCTTGGG TAAGTTTGCAACTTGTGAGCGTC-3′). The assembled products were filled in with the Klenow enzyme (Horton et al. 1993). Plasmid pAGA2-V5 was generated by inserting the *URA3* gene from *S. cerevisiae* to 5′ and 3′ flanks of the expression cassette of pYD1 vector. The BamhI-digested sCT DNA was inserted into plasmid pAGA2-V5 to yield plasmid pAGA2-sCT. Plasmids were transformed into the *S. cerevisiae* strain EBY100 (*trp1 leu2 Δ1 his3 Δ200 pep4::HIS2 prb Δ1.6R can1 GAL*) as described previously by Gietz and Woods (2002). Recombinants transformed with pAGA2-V5 and pAGA2-sCT was named as yAGA2-V5 and yAGA2-sCT respectively.

Protein expression and analysis

The transformants were cultured and induced for 12 h under the instruction of the Pyd1 Yeast Display Vector Kit manual. Cells were previously washed with phosphate buffered saline (PBS) containing 0.5% (w/v) BSA and incubated for 1 h at 4°C with the primary antibody staining reagent. The cells were washed again with PBS and incubated for 1 h at 4°C with goat-anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC). Then the labeled cells were resuspended in 200 μl PBS and 5 μl cell suspension were spot on a slide for microscopic observation under UV light. The remaining labeled cells were analyzed on a Coulter Epics XL flow cytometer at the Biotechnology Center of Ocean University of China.

Yeast culture and freeze-drying conditions

The yAGA2-sCT and yAGA2-V5 strains were pre-cultured at 30°C in YNB-CAA broth (containing 2% glucose) and induced in YNB-CAA broth (containing 2% galactose) according to the instructions of the Pyd1 Yeast Display Vector Kit. These cells were harvested at the beginning of their stationary growth phase (Hall and Webb 1975) and subsequently freeze-dried for 24 h in a standard freeze-dryer, followed by heating at 23°C to minimize the residual moisture content (Cerrutti et al. 2000).

Release of rsCT from yAGA2-sCT strain

In order to release rsCT, 10 mg lyophilized yAGA2-sCT strain was dissolved in 500 μl buffer containing 50 mM Tris/HCl (pH 5.6) and 5 mM $CaCl_2$ and incubated with 1 U native enterokinase

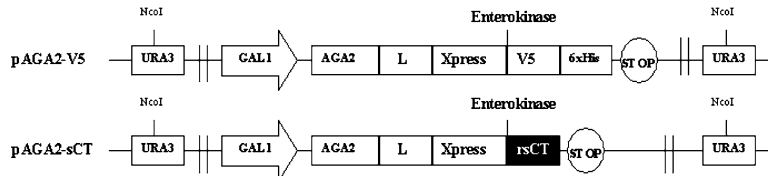

Fig. 1 Schematic representation of the expression cassette of vector pAGA2-V5 and pAGA2-sCT. V5 = V5 epitope, Xpress = Xpress epitope, L = linker region, rsCT = recombinant salmon calcitonin

at 21°C for 24 h. The digested products were centrifuged for 10 min at 5000*g*. The supernatants of enterokinase-digested product were abbreviated as 'EKS'. The rsCT concentration of yAGA2-sCT EKS was analyzed using DSL-10-3600 ELISA Kit. The same procedure was followed to yield yAGA2-V5 EKS.

Osteoclastic resorption on bone wafers

Bovine diaphyseal cortical bone wafers ($4 \times 4 \times 0.3$ mm) were prepared as substrates. The osteoclasts were isolated from the long bones (femurs and tibias) of 4–6 days old euthanized rat pups and suspended in the isolation media as described previously by Lakkakorpi et al. (1989). The supernatant (100 µl) of the osteoclasts suspension was added to a 96-well plate containing one bone wafer in each well. After 24 h, 10 µl EKS of yAGA2-V5, 10 µl EKS of yAGA2-sCT or 10 µl Miacalcic (containing 10^{-8} M sCT) were added to different wells respectively. The areas of osteoclast resorption pits on the bone wafer were measured using image analysis software (Adobe Photoshop, USA) as previously described by Hirayama et al. (2001). Six bone wafers were analyzed per group.

The experimental hypercalcemia model

The male Wistar rats were orally administered with 2 µg vitamin D3/kg after 16 h fasting to induce the hypercalcemia as described proviously by Tsuruoka et al. (2000).

Statistics analysis

Data were expressed as the mean ± S.E. Comparisons were performed with ANOVA and Student's *t*-test as appropriate. $P < 0.05$ was regarded as significant.

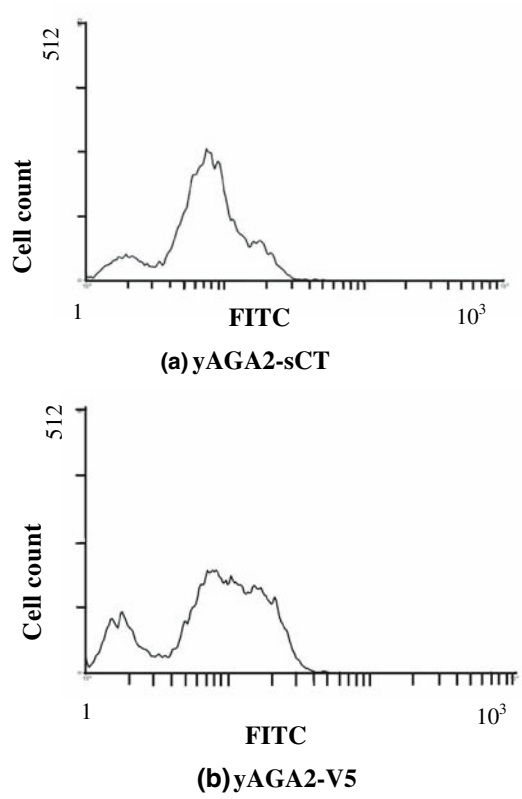

Fig. 2 Expression of the yeast cells displaying the foreign protein analyzed by the flow cytometer. (**a**) The yAGA2-sCT cells were labeled with rabbit-anti-calcitonin antibodies followed by secondary labeling with goat-anti-rabbit-FITC. (**b**) The yAGA2-V5 cells were labeled with the rabbit-anti-V5 antibodies followed by secondary labeling with goat-anti-rabbit-FITC. Labeled yeast cells were analyzed on a Coulter Epics XL flow cytometer collecting 10,000 cells gated on light scatter (size) to prevent analysis of the clumps. FITC = fluorescein isothiocyanate

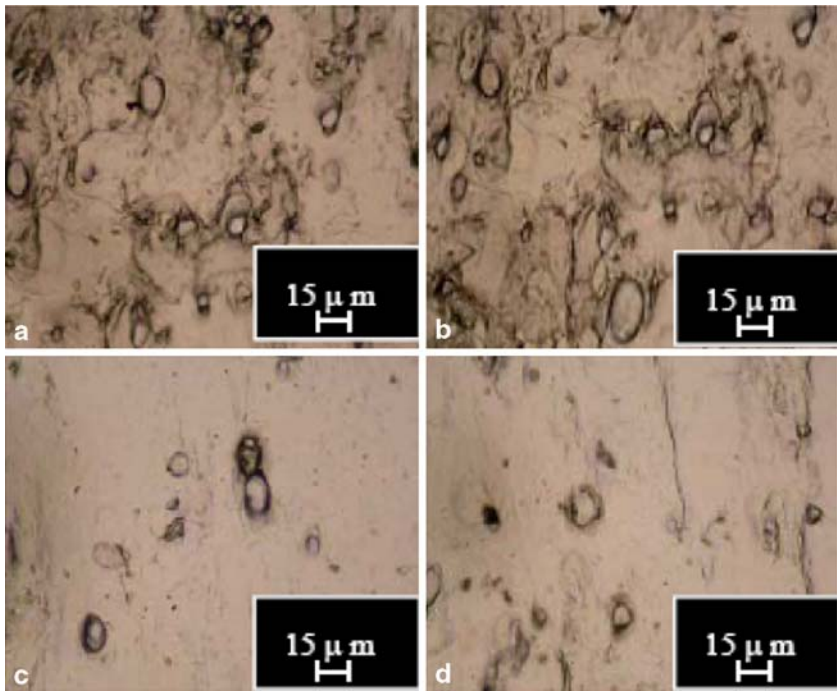

Fig. 3 Digital photographs of the bone wafer surfaces displaying the inhibition effect of rsCT on resorption activity of osteoclasts. The area of lacuna decreases as the osteoclasts resorption activity declines. Bone wafers cultured with osteoclasts were (**a**) without treatment, (**b**) treated with 10 μl EKS of yAGA2-V5, (**c**) treated with 10 μl Miacalcic containing 10^{-8} M sCT, (**d**) treated with 10 μl EKS of yAGA2-sCT containing 3×10^{-6} M rsCT. Original magnification = 100X. EKS = the supernatants of enterokinase-digested product. rsCT = recombinant salmon calcitonin. Photographs were made 7th day after the treatment

Results and discussion

Expression and detection of rsCT on the yeast cell surface

The synthesized sCT gene was cloned into the yeast display vector pAGA2-V5 to yield plasmid pAGA2-sCT (Fig. 1). The foreign protein (V5 epitope tags or rsCT protein) combined on the surface of yAGA2-V5 or yAGA2-sCT cells was labeled by FITC and recognized by the flow cytometer.

Not all the yeast transformants expressed the foreign protein (Fig. 2). Out of 10000 cells, the percentage of FITC-labeled yAGA2-sCT cells, automatically calculated by the flow cytometer, was 65% which was higher than that of yAGA2-V5 cells (52%). This indicated the expression level of rsCT protein was higher than that of protein V5, which might attribute to the usage of biased codons of *S. cerevisiae* in the sCT DNA Fig. 3.

Inhibitive effect of rsCT protein released from the yAGA2-sCT on the osteoclastic resorption

Bone wafer was dealt as quickly as possible to avoid the formation of artificial lacuna, and the mean area of lacuna in the bone wafer was 222 ± 34 μm^2. The bone wafers of control group became rough and the mean area of lacuna was 1506 ± 40 μm^2 after being exposed to the osteoclasts for 7 days (Fig. 3a). No significant reduction of the area (1381 ± 45 μm^2, $P = 0.081$) could be seen after adding 10 μl of yAGA2-V5 EKS to the osteoclasts solution compared to the control group (Fig. 3b), suggesting that the V5 protein released in yAGA2-V5 EKS had little influence on the bioactivity of osteoclasts. However, a significant area reduction was observed after adding 10^{-8} M sCT (diluted from Miacalcic) to the osteoclasts solution (689 ± 35 μm^2, $P < 0.01$, vs. control, Fig. 3c). Furthermore, adding 10 μl yAGA2-sCT EKS decreased the mean area of

lacuna significantly (696 ± 29 μm^2 $P < 0.01$, vs. control, Fig. 3d). This indicated that the rsCT protein released in yAGA2-sCT EKS could restrain the resorption bioactivity of osteoclasts as effectively as Miacalcic.

Anti-hypercalcemic effect of orally administered yAGA2-sCT strain on hypercalcemic rats

As can be seen in Fig. 4, the anti-hypercalcemic effect was not evident in the vehicle group (2.81 ± 0.02 mM), suggesting the oral administration of yAGA2-V5 could not prevent the formation of hypercalcemia in rats. Subcutaneous injection of 1.25 mg pamidronate/kg significantly inhibited the increase of the serum calcium (2.71 ± 0.03 mM, $P < 0.01$) in the positive control group compared to the vehicle group. Oral administration of lyophilized yAGA2-sCT strain at 5 g/kg also significantly inhibited the formation of hypercalcemia (2.69 ± 0.025 mM, $P < 0.01$). The results indicated that the orally administered 5 g yAGA2-sCT/kg caused long-term hypocalcemic effects in hypercalcemic rats. The long-term hypocalcemic effect was also reported in oral administration of the double liposomes containing 10 μg sCT/kg (Yamabe et al. 2003) or the pH-sensitive microsphere containing 100 μg human calcitonin/kg in rats (Lamprecht et al. 2004).

The dosage effect was observed in different dosages (0.1 mg/kg, 0.5 g/kg and 5 mg/kg) of lyophilized yAGA2-sCT strain. Anti-hypercalcemic effect of yAGA2-sCT was not observed at 0.1 mg/kg (2.82 ± 0.028 mM, $P > 0.05$). While at the dose of 0.5 g/kg and 5 g/kg, serum calcium level of the experimental rats was restricted to 2.75 ± 0.016 mM ($P < 0.05$) and 2.71 ± 0.032 mM ($P < 0.01$) respectively.

The mechanism of long-term hypocalcemic effect caused by orally administered yAGA2-sCT is still to be elaborated fully. Since yeast have high level of resistance to gastric and pancreatic secretions, the still-viable yAGA2-sCT could continuously release rsCT protein in vivo, under the action of the enterokinase in rats. The 'controlled release' of rsCT protein is thus achieved by this way.

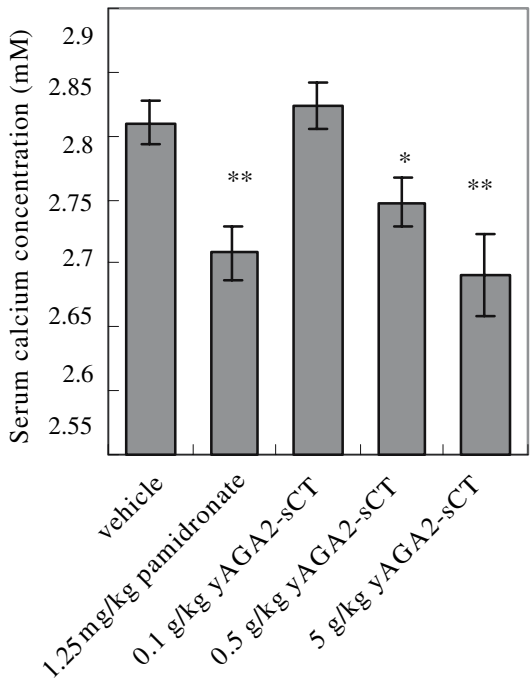

Fig. 4 Inhibition effect of yAGA2-sCT on the serum calcium levels in hypercalcemic rats. The hypercalcemic rats were administered various doses of yAGA2-sCT (0.1, 0.5, 5 g/kg) and the vehicle group were given 5 g/kg yAGA2-V5 by gastric gavages. In the positive control group, 1.25 mg/kg pamidronate were subcutaneously injected to hypercalcemic rats. Serum calcium levels were monitored at 12 h after administration. The results were expressed as mean ± S.E. $n = 6$. * $P < 0.05$, ** $P < 0.01$ compared with vehicle group

In the osteoclastic resorption experiment, the released rsCT protein added to the wells of the 96-well-plate was 3×10^{-6} M calculated by the ELISA method (data not shown). It was 300-fold higher than the concentration of sCT (10^{-8} M) used in the experiment, though their anti-resorptive effects were similar (Fig. 3c, d). The relatively low bioactivity of rsCT protein in yAGA2-sCT EKS might be mostly correlative to the non-amidated carboxy-terminus. The carboxy terminus is important for sCT to display its best potency, but this remains to be confirmed. Yoshina et al. (1996) found that Proline amide at the *C*-terminus of calcitonin was not essential for its biological activity in vivo, and Ivanov et al. (1987) found that bacterial calcitonin with a non-amidated *C* terminus was biologically active in rat

cells. However, the amidated sCT was generally considered to be of higher potency (McKee et al. 1998). Expressing peptidylglycine alpha-amidating monooxygenase together with sCT in *S. cerevisiae* seemed to be an alternative to improve the potency of sCT.

Saccharomyces cerevisiae is generally recognized as a safe microbe to humans and taking recombinant *S. cerevisiae* orally has been a promising way in drug delivery. The present findings also provided a potential new strategy to use living recombinant *S. cerevisiae* as a new drug delivery system for sCT in hypercalcemia therapy.

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