Lansoprazole-Induced Osteoporosis via IP3R and SOCE Mediated Calcium Signaling Pathway

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Abstract

Background: Many clinical studies have shown a correlation between proton pump inhibitors (PPIs) and osteoporosis or fractures. The purposes of this study were to establish a murine model of chronic oral administration of PPIs to verify whether PPIs caused bone metabolic impairment, and to investigate the relevant molecular mechanism underlying the effects of PPIs on MC3T3-E1 mouse osteoblasts.

Methods: Lansoprazole-induced bone loss model was employed to investigate the damage effects of PPIs. In vivo, immunohistochemistry and HE staining, micro-CT analysis, blood biochemical tests were used to evaluate the effect of lansoprazole on bone injury in mice. In vitro, the effects and related signaling pathway of lansoprazole on MC3T3-E1 cells were investigated by CCK8, EDU kit, flow cytometry, laser confocal, patch clamp, PCR and Western blotting, etc.

Results: After 6 months of lansoprazole gavage in ICR mice, micro-CT results showed that compared with the vehicle group, the bone mineral density (BMD) of high-dose group was significantly decreased ($P < 0.05$), and the bone microarchitecture gradually degraded. Biochemical assay of bone serum found that blood calcium and phosphorus were both decreased ($P < 0.01$). We found that long-term administration of lansoprazole impairs skeletal function in mice. In vitro, we found that lansoprazole (LPZ) could cause calcium overload in MC3T3-E1 cells leading to apoptosis, and 2-APB, an inhibitor of IP3R calcium release channel and SOC pathway, effectively blocked calcium increase caused by LPZ, thus protecting cell viability.

Conclusion: Long-term administration of LPZ induced osteoporotic symptoms in mice, and LPZ triggered calcium elevation in osteoblasts in a concentration dependent manner, intracellular calcium ($[Ca^{2+}]$) persisted at a high concentration thereby causing endoplasmic reticulum stress (ERS) and inducing osteoblasts apoptosis.

Introduction

PPIs had long been recognized as the first-line drug for the treatment of gastric acid related diseases by irreversibly binding to H^+/K^+ -ATPase to inhibit gastric acid secretion (Chen et al. 2016). Generally, PPIs were considered to be a kind of medicine with good tolerance in clinical practice. However, more than 40% of the clinical cases belong to irrational drug use, and some continue to use PPIs for a long time regardless of the clinical indicators (Grant et al. 2006). Up to now, the majority of PPIs include omeprazole, lansoprazole, pantoprazole, rabeprazole, esomeprazole and ilaprazole (Ito and Jensen 2010; Gyawali 2017). In 2010, FDA warned that patients taking PPIs for more than a year or higher doses may increase the risk of the hip, wrist, and spine fractures. Clinical meta-analyses had also shown that PPIs use increased the risk of hip, spine, or vertebral fracture (Ito and Jensen 2010; Yu et al. 2011; van der Hoorn et al. 2015; Chen et al. 2016; Poly et al. 2018), patients treated with PPIs for two years were more likely to have a hip fracture ($OR = 1.30$, $CI = 1.21-1.39$) (Corley et al. 2010). Besides, animal experiments had pointed out that pantoprazole had a negative effect on bone metabolism in young male rats for 12

weeks(Matuszewska et al. 2016), and H⁺/K⁺-ATPase β subunit KO mice had lower BMD and bone mineral content (BMC)(Fossmark et al. 2012), and pantoprazole (100 mg/kg/day) could affect fracture healing in mice(Histing et al. 2012; Menger et al. 2020). However, there are few studies on the molecular mechanism of the effect of PPI on osteocytes *in vitro*.

Bone homeostasis was maintained by a balance between bone resorption mediated by osteoclasts (OC) and bone formation mediated by osteoblasts (OB), respectively(Yin et al. 2019). So far, literature associated with PPIs and bone loss focused more on the relationship between PPIs and OC, few on PPIs and OB(Yuan et al. 2010; Jo Y 2015). Since new bone formation depended largely on osteoblasts, any factors that promote osteoblast apoptosis could increase the risk of osteoporosis(Guo et al. 2014; Elias and Targownik 2019). Unlike before, we focused on OB's role in the skeletal system injury induced by PPIs.

Naseri *et al*/ showed that both omeprazole and lansoprazole could induce arterial relaxation in a time-dependent manner, and this effect could be associated with their regulation of intracellular calcium(Naseri and Yenisehirli 2006). Schillinger found that pantoprazole could affect the uptake of Ca²⁺ in the sarcoplasmic reticulum (SR) by inhibiting SERCA, thus reducing the transient amplitude of calcium and myocardial contractility(Schillinger et al. 2007; Sato et al. 2017). Yurtsever and Aydin speculated that omeprazole and lansoprazole may inhibit Rho-kinase thus affected Ca²⁺ regulation or block the calcium channels to inhibit muscle contraction(Aydin et al. 2003; Yurtsever et al. 2011). Aydan(Yenisehirli and Onur 2006) considered lansoprazole (100-300 μM) could inhibit Ca²⁺ entry through voltage-gated channels. Combined with these findings, it seemly suggested that PPIs may have an effect on intracellular calcium homeostasis. In addition, Ca²⁺-ATPase (PMCA), SERCA, Na⁺/K⁺-ATPase, and gastric H⁺/K⁺-ATPase all belong to P-type ATPases and share high homology with each other(Toyoshima and Cornelius 2013). In view of the above, we speculated that LPZ could inhibit SERCA in OB and led to calcium homeostasis disorder. The aim of this study was to explore the effect of LPZ on [Ca²⁺]_i changes and viability in MC3T3-E1 cells *in vitro* and to detect whether LPZ caused OP in mice *in vivo*. The combination of *in vitro* and *in vivo* experiments provided a potential mechanism for the deleterious effects of PPIs on the skeleton system.

Materials And Methods

Animals model

ICR mice (SPF grade), 18 to 22g, aged 6-8 weeks, half male and half female, provided by Nanjing Jiangning District Qinglongshan Animal Farm. The quality certificate of experimental animals No. 201824637, production license No. SCXK (SU) 2017-0001.

Drugs

Lansoprazole (purity 99.45%): Wuhan Yuancheng Co-founder Technology Co., Ltd; Sodium carboxymethyl cellulose (CMC-Na), viscosity: 600-3000 mPa.s, USP grade: Shanghai Macklin Biochemical Co., Ltd.

Drug administration

Mice were given lansoprazole orally for 6 months. All the mice were placed in separate cages and fed under standard conditions, and fed with standard laboratory rodent chow and water. During the experiments, mice were maintained at a constant temperature of 26 °C and kept on a 12 hours light-dark cycle. After 3 days of adaptive feeding, the mice were randomly divided into three groups: the lansoprazole high dose group (1000 mg/kg), the lansoprazole low dose group of (250 mg/kg), and the control group (0.5% CMC-Na). The dosage (unit: mL) = $0.02 \times m$ (unit: g). After feeding for 6 months, the mice in each group were randomly selected to determine the bone mineral density and serum biochemical indexes.

Micro-computed tomography (Micro-CT)

Ten mice were randomly selected from each group and were sent to the Animal Experimental Center of Nanjing Medical University. After anesthesia, mice were employed SkyScan1176 *in vivo* Micro-CT scan the right femur. Using 12.59 μ M pixel size, we set on an X-ray at 50 kV and 455 μ A to scan the distal metaphysis of the femur. Regions of interest (ROI) of cancellous bone were taken from layer 50 to layer 100 distant from the growth plate.

Femur biomechanical examination

Six mice were randomly selected from each group, the complete right femur of the mice was taken after put to death under anesthesia, and the maximum bone load (maximum breaking force) and the bone structural strength (maximum crushing force) of the femur samples were measured by the YLS-16A small animal bone strength instrument. Apparatus maximum applied pressure: 25 kg; test bone length range: 20~75 mm; minimum reading: 0.001 kg.

Bone serum biochemical indicators analysis

After the last administration, peripheral whole blood samples of mice were obtained and collected into 1.5 mL plastic centrifuge tubes. Blood samples were centrifuged at 3000 rpm for 10 min, and the supernatant was taken and the serum inorganic phosphorus (S-IP), serum calcium (S-Ca) and serum alkaline phosphatase (S-ALP) were measured by automatic biochemical analyzer.

Cell culture

MC3T3-E1 cells were cultured in α -MEM medium (GIBCO, 11095080) supplemented with 10% fetal bovine serum (FBS) (Biological, 4-001-1ACS), 100 mg/dl glucose, and 1% antibiotics (100 U/ml of penicillin G

and 100 mg/ml of streptomycin) (GIBCO, 15140-122) at 37 °C in a humidified atmosphere of 5% CO₂, and cultured for future experiments at 70%–80% confluency.

Cell viability

CCK-8 (Beyotime, C0038) and EDU kit ((Beyotime, C0078S) were used to study the effects of PPIs on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were seeded in 96-well plates (density: 8000 cells/well) till cell proliferation reached about 70% to 80% density and treated with LPZ. MC3T3-E1 cells were incubated for 24 h in α-MEM/10% FBS containing different concentrations of LPZ (5, 10, 20, 50, 100 and 200 μM), and the cells in the vehicle group were cultured in α-MEM/10% FBS containing 0.1% DMSO (v/v) (Sigma, D2650). After that, cultures were washed with PBS, the medium in each well was replaced by 100 μL of α-MEM without FBS and containing 10% working solution of CCK-8 was added. The mixture was incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO₂ and measured at 450 nm using a Microplate Reader. The cells in the control group were cultured in α-MEM containing 0.1% DMSO.

Reverse transcription-quantitative polymerase chain reaction

After being treated with LPZ for 24 h, the total RNA of MC3T3-E1 cells was extracted with Trizol reagent (Thermo Fisher, 15596018). Subsequently, the concentration of RNA in samples was determined by TECAN Grating-type multifunctional microplate detector, and the ratio should be between 1.8 - 2.0. Next reverse transcription of RNA into cDNA under the reaction conditions of 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 5 min. After that, the samples were stored in -20 °C refrigerator for future use. Polymerase chain reaction conditions were set as follows: 1, 95 °C for 10 min to pre-denature the sample; 2, 95 °C for 15 s and 60 °C for 1 min to alternate for 40 cycles. GAPDH was used as an internal reference, and the fold change value was used to express the relative expression of genes. The primers used in this experiment were listed in Table 1.

Table 1. Primers sequence for PCR

Genes	Forward (5'-3')	Reverse (3'-5')
OCN	AGACTCCGGCGCTACCTTGG	CGGTCTTCAAGCCATACTGGTCTG
ALP	TCATTCCCACGTTTTTCACATTC	GTTGTTGTGAGCGTAATCTACC
Colla1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Runx2	ATGCTTCATTGCGCTCACAAA	GCACTCACTGACTCGGTTGG
Caspase-12	TGGCCCATGAATCACATCTAAT	TGGACAAAGCTTCAGTGTATCT
ATF4	TGGCTGGCT GTGGATGG	TCCCGGAGAAGGCATCCT
GAPDH	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC
ATP2B1	TCATTCCCACGTTTTTCACATTC	GTTGTTGTGAGCGTAATCTACC

Quantification of apoptosis using flow cytometry

Cell apoptosis was detected by Annexin V-FITC and PI staining (Vazyme, A211-02). MC3T3-E1 cells were treated with LPZ (5, 10, 20, 50 μM) for 12 h. After treatment, cells were washed with pre-cooling PBS twice. The cells were digested with 0.25% trypsin (without EDTA) then centrifuged, and the centrifuged cells were washed twice with PBS again. After that, 500 μL Annexin V binding solution, 5 μL Annexin V-FITC staining solution, and 5 μL pyridine iodide (PI) were added to each tube of cells respectively. Cells in the control group were treated with only binding solution without staining solution. Double-labeling has performed at room temperature for 10 min in the dark before flow cytometric analysis. The cells in the control group were cultured in a normal medium containing 0.1% DMSO.

Calcium analysis

$[\text{Ca}^{2+}]_i$ was measured using a calcium-sensitive fluorescent indicator fluo-3/AM (Keygen Biotech, KGAF023-1). MC3T3-E1 cells were cultured in the confocal vessel and loaded with 5 μM fluo-3AM for 30 min at Hank's balanced salt solution (HBSS) (Gibco, C14175500BT). After loading, cells were washed softly three times with HBSS then cells were incubated with calcium-containing HBSS (Gibco, 24020-133) or calcium-free HBSS and detected in real-time under laser confocal microscopy (Zeiss, LSM 5) within 1 h, recording started when the fluorescence intensity of the cells remained stable. Cells were then promptly treated with LPZ, 2-APB (ApexBio, B6643), Ryanodine (ApexBio, B5092), Thapsigargin (TG) (Sigma, T9033), Verapamil (ApexBio, B1687), and BTP-2 (ApexBio, B7542). Mag-fluo-4/AM (AAT Bioquest, 20401) is a fluorescent probe for labeling endoplasmic reticulum Ca^{2+} ($[\text{Ca}^{2+}]_{\text{ER}}$), and Rhod-2/AM (APEX-BIO, C3276) is a fluorescent probe for labeling mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{mit}}$) with bright and stable fluorescence. Next MC3T3-E1 cells were loaded 2.5 μM Mag-Fluo-4-AM with 2 μM ER-tracker (Beyotime, C1041) or 5 μM Rhod-2 AM with Mito-tracker green (1 μM) (Beyotime, C1048) for 20 min at room temperature to detect the ER and mitochondrial Ca^{2+} changes. The waves of fluorescence intensity of the indicator represent the change of Ca^{2+} concentration. Besides, changes in calcium after long-term drug treatment were analyzed by flow cytometry. The cells in the control group were cultured in a normal medium containing 0.1% DMSO.

Patch clamp measurement of sodium-calcium exchanger

The electrode is made of hard glass with a microelectrode puller (HEKA) for two times. The resistance value of the electrode is 4 - 8 $\text{M}\Omega$. After filling the liquid in the electrode into water. Single cell with neat edges, no particles on the surface and no contraction are selected. The three-dimensional manipulator is adjusted to move the tip of the electrode to the cell surface, and negative pressure is applied slightly. After high resistance sealing is formed, fast capacitance is compensated and the negative pressure is sucked to break the cell membrane to form a whole cell mark recording mode. All experiments were carried out at room temperature (25 $^{\circ}\text{C}$). Stimulation parameters were Constant -40 mV 250 ms, ramp 60 mV 1000 ms, Ramp -140 mV 2000 ms, Ramp -40 mV 1000 ms, constant -40 mV 250 ms. After recording the I_{NCX} current on the cells, the I_{NCX} current was stable for 10 min, and the prepared test drug solution was given, so that

the final drug concentration in the cell pool was 0, 10, 50, 100 μM in turn, which was stable for 20 min after each administration, and the I_{NCX} current of the cells was recorded after the drug diffusion was uniform.

Western blot analysis

MC3T3-E1 cells were treated with 0.1% DMSO (the vehicle group), 10 μM LPZ, 25 μM 2-APB, and 2 μM TG for 24h. The whole protein was extracted using RIPA buffer, then denatured for 10 min at 95 $^{\circ}\text{C}$, equal amounts of protein (10 μg) were separated on a 10% - 12% SDS-PAGE gel and transferred to PVDF (Merck millipore, ISEQ00010) membrane. The membrane was rinsed and blocked with 5% non-fat milk in TBST buffer at room temperature for 2 h and then incubated overnight at 4 $^{\circ}\text{C}$ with the specific primary antibodies including Grp78 (Servicebio, GB11098), CHOP (Cellsignaling, L63F7), Caspase-12 (CST, 2202), Calpain-2 (abcam, ab126600), Cleaved-Caspase-3 (CST, 9661), ATF4 (SAB, 32007), OPG (BTL3466), Rankl (BTL5404T), ALPL (proteintech, 11187-1-AP), OSTCN (SAB,23319), GAPDH (SAB, 21612), Actin β (BTL338), Bax (proteintech, 50599-2-Ig), Bcl-2 (ab59348), CytC (Servicebio, GB11080). After that, the membranes were washed 3 $\times$ 10 min with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Servicebio, GB23303) for 1 h at room temperature. The membranes were washed 3 $\times$ 10 min with TBST buffer, and the blots were visualized with enhanced chemiluminescence reagent and were analyzed with ImageJ software.

Statistics.

Measurement data were expressed as mean $\pm$ SD. One-way ANOVA followed by Tukey's test was used for comparison among groups, and $P < 0.05$ was considered statistically significant (*: $P < 0.05$, **: $P < 0.01$).

Results

Effect of LPZ on bone density, serum biochemical indexes and femoral biomechanics property in mice

After continuous gavage administration for 6 months, there was no statistical difference in mouse weight among groups. Compared with the CMC-Na group, the low dose group and high dose group mice showed a decreasing trend in the maximum bone load (maximum breaking force) and bone structure strength (maximum crushing force) in femoral samples of material mechanical parameters in a dose-dependent manner (Fig. 1A, B). Furthermore, the low dose group (250 mg/kg) and high dose group (1000 mg/kg) mice showed a decreasing trend in femoral BMD, and there was a significant difference between the control group and high dose group (Fig. 1C). Bone serum biochemical results showed that serum-ALP (S-ALP) decreased slightly, serum-calcium (S-Ca) and serum-phosphorus (S-IP) decreased significantly (Fig. 1D, E, F). Similarly, compared with the CMC-Na group, trabecular bone micro-architecture was gradually thin and loose (Fig. 2).

Effect of LPZ on MC3T3-E1 cells viability

After discovering that LPZ had a damaging effect on mouse bone tissue, MC3T3-E1 cells, as a common osteoblast cell line, we used it to explore the mechanism of LPZ causing bone damage. First, we used an EDU kit to determine whether the LPZ promoted/inhibited MC3T3-E1 cells proliferation. The proportion of proliferating cells decreased significantly ($P < 0.05$) when cells were treated with LPZ at concentrations of 5, 10, 20, 50 μM (Fig. 3A, C). Next, we further explored whether the elevation of $[\text{Ca}^{2+}]_i$ by LPZ could injure MC3T3-E1 cells through the apoptotic mechanism, MC3T3-E1 cells were incubated in α -MEM/10% FBS containing different concentrations (0, 5, 10, 20, and 50 μM) of LPZ and or none pre-incubated 10 μM BAPTA/AM for 30 min respectively. The apoptosis rate in the LPZ treatment group was increased in a dose-manner (Fig. 3B, D). Furthermore, LPZ could inhibit the viability of MC3T3-E1 cells in a dose-dependent manner (Fig. 3E). Above all, these results suggested that LPZ inhibited the viability of MC3T3-E1 cells

Calcium responses after LPZ treatment in MC3T3-E1 cells

1. LPZ induced calcium increase through ER Ca^{2+} release

We used confocal laser scanning microscopy technology to investigate calcium transient after LPZ treatment in MC3T3-E1 cells in real-time. We found that calcium fluorescence significantly increased in MC3T3-E1 cells treated with 50 μM LPZ (Fig. 4A). Moreover, the elevation of $[\text{Ca}^{2+}]_i$ was slightly higher in calcium-contained solution than in calcium-free solution (Fig. 4B), but there was no significant difference between the two groups (Fig. 4C), which indicated that $[\text{Ca}^{2+}]_i$ originated from both intracellular calcium release and extracellular calcium influx, and intracellular calcium release was mainly required for LPZ-induced $[\text{Ca}^{2+}]_i$ response in MC3T3-E1 cells. Thapsigargin (TG) is an inhibitor of SERCA with high specificity. MC3T3-E1 cells were pre-incubated with TG in calcium-free solution to empty calcium storage in ER, and then treated with 50 μM LPZ, but there was almost no longer calcium transient in MC3T3-E1 cells (Fig. 4D). These findings suggested that LPZ-mediated calcium signaling in MC3T3-E1 cells was mainly from ER calcium release. Furthermore, we pre-incubated BAPTA/AM to chelate intracellular free Ca^{2+} , and found MC3T3-E1 cells survival viability was significantly up-regulated ($P < 0.05$). LPZ inhibited the viability of MC3T3-E1 cells, however, the viability of MC3T3-E1 cells preincubated with BAPTA/AM was significantly higher than those without BAPTA/AM preincubation, which indicated that $[\text{Ca}^{2+}]_i$ increase was an important factor for LPZ-induced cells impairment, and BAPTA/AM could effectively protect cell viability by blocking this process (Fig. 4E).

2. LPZ-induced calcium increases through IP3R and SOCE pathway

Cells were pre-incubated with 2-APB (25 μM) or Ryanodine (20 μM) in calcium-free medium, blocking the IP3R Ca^{2+} release channel and SOC Ca^{2+} entry pathway, or Ryr Ca^{2+} release channel respectively. Then cells were treated with 10 μM LPZ (clinically relevant concentrations). It turned out that Ryanodine had little effect on $[\text{Ca}^{2+}]_i$ activated by LPZ (Fig. 5B), but 2-APB almost made the calcium fluorescence intensity consistent with the vehicle group (Fig. 5A). In addition, in order to confirm which calcium

channel is responsible for the calcium influx, MC3T3-E1 cells pre-incubated with verapamil (10 μ M), a L-type calcium channel blocker(Jung et al. 2015), or BTP-2 (20 μ M), store-operated calcium (SOC) channel in calcium-contained medium, then cells treated with 10 μ M LPZ. It was found that BTP-2 could partly restrain calcium fluorescence enhancement (Fig. 5D), however, verapamil had little influence on $[Ca^{2+}]_i$ (Fig. 5C). Taken together, these results above revealed LPZ-mediated calcium transient mainly from ER released by IP3R channel and could promote the activation of SOCE.

BTP-2 in Ca^{2+} -contained medium (n = 3).

3. LPZ induced a decrease in $[Ca^{2+}]_{ER}$ and an increase $[Ca^{2+}]_{mito}$

Furthermore, to investigate whether LPZ had an effect between ER and mitochondria, MC3T3-E1 cells were loaded with Mag-fluo4/AM and Rhod-2/AM probe to identify the calcium levels in ER and mitochondria respectively. As we expected, ER Ca^{2+} fluorescence decreased after LPZ (10 μ M) was added, meanwhile Ca^{2+} in mitochondria slightly increased. (Fig. 6)

Assessment of calcium in flow cytometry

1. Elevated calcium in long term LPZ exposed MC3T3-E1 cells

Then we used flow cytometry to investigate the intracellular calcium changes after long-term treatment (24 h) of LPZ in MC3T3-E1 cells. MC3T3-E1 cells were pre-incubated with TG (2 μ M) in calcium-free medium, or pre-incubated with BTP-2 (20 μ M) in calcium-contained medium. Then MC3T3-E1 cells were added with 50 μ M LPZ for another 1 h. It was found that $[Ca^{2+}]_i$ was significantly higher either in calcium-contained or free medium than the vehicle group (0.1% DMSO). Furthermore, the level of calcium elevation in MC3T3-E1 cells pre-incubated with BTP-2 under calcium-contained conditions was not statistically different from that cells treated with LPZ only under calcium-free conditions. However, taking vehicle as reference, the elevation of $[Ca^{2+}]_i$ was significantly lower when MC3T3-E1 cells were pre-incubated with TG under calcium-free conditions (Fig. 7).

2. $[Ca^{2+}]_i$ remained at a high level after LPZ exposed in MC3T3-E1 cells

We selected TG (2 μ M) and LPZ (50 μ M) in calcium-contained medium for 24 h. It was found that the intracellular calcium level remained high in the LPZ group, but the intracellular calcium level was low in the TG group, and there was no significant difference between the TG and the vehicle group. These reminded us that LPZ may have an inhibitory effect on SERCA. When MC3T3-E1 cells were treated with TG, TG could not make $[Ca^{2+}]_i$ remain at a high level for a long time, and Ca^{2+} would be expelled by PMCA and NCX on the plasma membrane in time. However, LPZ could keep $[Ca^{2+}]_i$ at a high level for a long time, LPZ might inhibit calcium efflux transporters. Ca^{2+} stress seems to be more associated with long-term stress conditions, thus persistent Ca^{2+} elevation triggered cell apoptosis. We hypothesized that LPZ

also had an inhibitory effect on PMCA and/or NCX, so that LPZ-treated cells remained calcium overloading after 24 h (Fig. 8). (n = 3)

NCX current changes

In osteoblasts, PMCA and NCX are responsible for calcium balance. We examined the effect of LPZ on NCX transporter using the patch clamp technique. It was shown that the 10 μM LPZ increased the reverse current density from -3.29 ± 1.00 pA/pF (0 μM) to -5.62 ± 1.46 pA/pF by 70.91%, the current density was 18.9 ± 5.96 pA/pF by 475.10% at 50 μM , and the current density 100 μM was -5.21 ± 2.33 pA/pF by 58.52% at 100 μM (Fig. 9). Taken together, patch clamp results showed that 10, 50, and 100 μM lansoprazole could significantly strengthen the reverse current (forward mode) of Na^+ - Ca^{2+} exchange current in MC3T3-E1 cells ($P < 0.05$). It indicated that at the concentration of 10, 50, 100 μM LPZ, Ca^{2+} could be transported out through Na^+ - Ca^{2+} exchangers. We hypothesized that lansoprazole might stimulate Ca^{2+} overload to prompt an increase in sodium-calcium exchanger activity in order to uphold a low intracellular calcium balance.

Effects of LPZ on MC3T3-E1 mRNA expression levels

MC3T3-E1 cells were treated with 0.1% DMSO (the vehicle group), 10 μM LPZ, 25 μM 2-APB, and 2 μM TG for 24 h, and then we detected the effects of LPZ on MC3T3-E1 cells genes related to differentiating and ER stress apoptosis pathway. We found that LPZ could reduce the mRNA expression levels of differentiating and mature in MC3T3-E1 cells (ALP, OCN, Runx2, Col1 α) and increase the mRNA expression levels of ER stress markers (Caspase12, ATF4). (Fig. 10).

Expression of ERS and osteoblast functional protein

Previous studies had reported that Caspase-12, GRP78, and CHOP are the particular mediators of ERS, and Bcl-2 is an anti-apoptotic protein, while Bax is a pro-apoptotic protein (Chiu et al. 2018). TG could induce a rapid calcium release from ER and promote ER stress. We treated the TG group as positive control (Chen NX 2000 May). In this study, compared with the vehicle group, LPZ increased ERS-mediated caspase-12, Grp78, ATF4, and CHOP protein levels, and promoted the expression of cleave-caspase-3, a terminal cleavage enzyme during the process of apoptosis. However, pre-incubation of 2-APB lessened ER stress protein expression and reduced the elevation of Bax/BCL-2 ratio induced by LPZ. It is well documented that Bcl-2 also regulates endoplasmic reticulum calcium homeostasis (Chiu et al. 2018). Beyond these, the expression of calpain-2, a calcium-dependent ER stress protein, was up-regulated in LPZ and TG group, 2-APB could alleviate the expression of calpain-2, which means that LPZ caused a rise in intracellular calcium at the protein level and ER stress. Moreover, the expression of MC3T3-E1 osteoblast functional protein ALP, OPG/Rankl, OCN were reduced in the LPZ group and the TG group compared with the vehicle group, which illustrated that LPZ impaired the function of MC3T3-E1 osteoblasts, and affected the differentiation, secretion, and mineralization process, and 2-APB could protect the viability of MC3T3-E1 cells after the treatment of LPZ for 24h. LPZ could cause ERS in

MC3T3-E1 cells in a dose-dependent manner, and BAPTA could mitigate the damage caused by LPZ to OBs (Fig. 11).

Ca-ATPase activity assay

At the same time, the activity of Ca-ATP enzyme in MC3T3-E1 cells was determined by phosphorus assay, and the results showed that the activity of Ca-ATP enzyme decreased in LPZ group (Fig. 12). After treatment with 10 μ M LPZ, the expression of Ca^{2+} -ATP enzyme (ATP2B1) gene in plasma membrane decreased in a time-dependent manner. Although the kit measured the activity of all Ca-ATP enzymes, it also provided some reference for us. It is suggested that LPZ had a negative effect on calcium pump activity of MC3T3-E1 cells.

HE staining and immunohistochemical staining in bone

In vivo, HE staining showed that the control group, the trabecular bone microstructure was dense, with full cancellous bone and few vacuoles; In the low dose group, trabecular bone (cancellous bone) was loosely arranged and partially broken, with a decrease in thickness and some vacuoles in the bone marrow; In the high dose group, trabecular bone (cancellous bone) was fractured, with thinner thickness and with worsened structural integrity, increased separation and more vacuoles in the medulla (Fig. 13A). To test whether ER stress (ERS) occurred in mice, we used immunohistochemical staining to detect the expression of CHOP, an ER stress marker, in distal femoral of different groups. Immunohistochemical results showed that after long-term intragastric administration, the expression of CHOP was gradually increasing with the increase of LPZ dose. It was suggested that long-term LPZ administration may have a negative effect on bone tissue and caused ER stress in the distal femur (metaphysis), the osteoblasts on the trabecular bone surface also decreased, influencing bone remodeling. It indicated that endoplasmic reticulum stress occurred in the bone tissues of mice (Fig. 13B, C). In conclusion, *in vivo* and *in vitro* experiments show that LPZ could lead to increased apoptosis of bone cells and thus bone injury by endoplasmic reticulum stress.

Discussion

In our previous experiments, we found that the peak concentration (C_{max}) of LPZ in mice was about 4000 ng/mL after intragastric administration of 250 mg/(kg•d) LPZ, which was almost close to the peak plasma concentration of clinical routine dose treatment (Sun et al. 2017). In this study, 250 mg/(kg•d) (low dose) and 1000 mg/(kg•d) (high dose) were selected as the doses for experiments *in vivo*. After six months, based on of no significant differences in body weight were observed in each group, the bone metabolic index, BMD, and bone structural strength of mice in the low and high dose groups showed a decreasing trend. These suggested that long-term use of LPZ could influence the balance of bone metabolism and the microstructural damage to bone tissue in mice *in vivo*. HE staining results reflected the morphological structure of trabecular bone in distal femoral metaphyses (Liu et al. 2017). There was an obvious morphological difference between the control group and the LPZ groups. Besides,

immunohistochemistry of the distal femur revealed endoplasmic reticulum (ER) stress occurred in the bone with increasing doses, conforming that ER stress plays a vital role in the pathogenesis of osteoporosis(Wu et al. 2014; Li et al. 2017).

In vitro, it was found that the toxic effect of LPZ is related to the increased $[Ca^{2+}]_i$. BAPTA-AM rescued the increase of MC3T3-E1 cells apoptosis rate triggered by LPZ, suggesting the increased Ca^{2+} levels engaged in LPZ-induced OB apoptosis. Ca^{2+} is an important second messenger. The regulation mode of calcium signal is diverse and the regulation mechanism is complex. The precise regulation of Ca^{2+} by calcium pumps, calcium channels, and sodium-calcium exchangers achieves the normal physiological activities of cells(Krebs et al. 2011). Subtle alterations in either link of calcium signaling regulation can cause serious consequences in osteoblasts(Liu et al. 2008; Yu et al. 2018). Calpain-2 is a Ca^{2+} dependent cysteine protease whose activity and function depend on intracellular Ca^{2+} levels(Bano and Ankarcrona 2018; Wang et al. 2018; Wang et al. 2019). In the present study, LPZ exposure increased the expression of calpain-2 and caspase-12 in MC3T3-E1 cells. Caspase-12, localized on the cytoplasmic side of the ER membrane, is cleaved and specifically activated during ERS(Zhu et al. 2018; Qiu et al. 2020), illustrating that the increased calcium levels in osteoblasts by LPZ led to the occurrence of ER stress.

Then we investigated the source of intracellular calcium by flow cytometry and confocal microscopy. It was found that 2-APB could significantly reduce the increase of Ca^{2+} fluorescence in calcium-free medium, and BTP-2 could partly restrain $[Ca^{2+}]_i$ rise in calcium-contained medium, implying the $[Ca^{2+}]_i$ were originated from IP3R-mediated ER Ca^{2+} release and the SOC pathway could be the main pathway of extracellular Ca^{2+} influx. Furthermore, we found a slight increase in the red fluorescence of mitochondria, and the green fluorescence value of ER decreased. It was suggested that LPZ can release ER calcium into the cytoplasm, causing calcium overload, and Ca^{2+} released into the cytosol by the IP3R channel can be taken up by neighboring mitochondria, a similar mechanism as that reported by Filadi R(Filadi and Pizzo 2019).

Patch clamp results showed that 10, 50 and 100 μ M LPZ could significantly enhance the reverse current (forward mode) of Na^+ - Ca^{2+} exchange current in MC3T3-E1 cells ($P < 0.05$). It indicated that at the concentration of 10, 50, 100 μ M LPZ, Ca^{2+} could be transported out through Na^+ - Ca^{2+} exchangers. We hypothesized that LPZ may stimulate Ca^{2+} overload to prompt an increase in sodium-calcium exchanger activity to maintain a low intracellular calcium balance(Sosnoski and Gay 2008; Bano and Ankarcrona 2018). PMCA is responsible for calcium excretion in parallel with NCX, the remarkable promoted calcium efflux function of NCX might indirectly reflect Ca^{2+} overload.

Finally, the expressions of ER stress-related proteins and osteoblast functional proteins were detected by western blot. MC3T3-E1 cells were pre-incubated with 2-APB (20 μ M) to confirm the main pathway of raised $[Ca^{2+}]_i$. 2-APB has independent targets including IP3R-dependent ER Ca^{2+} release and store-operated Ca^{2+} (SOC) channels in osteoblasts. TG was used as a positive control. In this study, we found that LPZ promoted the activation of caspase 12, Bax/Bcl-2, and caspase-3 induced by ER stress(Wu and

Kaufman 2006; Sato et al. 2015; Pihan et al. 2017). Then we examined some ER stress markers, Bip/Grp78, ATF4, CHOP(Nakamura et al. 2013), we found LPZ increased the expression of Grp78 and CHOP in MC3T3-E1 cells, which indicated that lansoprazole indeed causes ER stress in osteoblasts. However, when cells were preincubated with 2-APB, the expression of Grp78, CHOP, caspase-12, Bax/Bcl-2, and caspase-3 induced by LPZ were suppressed. It was demonstrated that Ca^{2+} release from ER through IP3R channel and Ca^{2+} influx from SOCE pathway contributed to developing ER stress in osteoblasts. Besides, after 24 h of treatment with 10 μM LPZ, the ratio of OPG/Rankl decreased. The OPG/RANKL ratio decides whether bone turnover is prone to the bone formation or bone resorption(Yuan et al. 2008). We speculated that LPZ may destroy the dynamic balance between OB bone formation and OC bone resorption, stimulating bone resorption rather than bone formation. Some animal experiments were consistent with our results that different kinds of PPIs can cause a decrease of OPG/Rankl ratio both in cells and animals' levels(Fossmark et al. 2012; Hoff et al. 2020). 2-APB could rescue the damage of MC3T3-E1 cells induced by LPZ, the expression of osteoblastic differentiation markers ALP, OCN were increased(Chou et al. 2005). Calcium signaling is important to bone remodeling and bone healing, imbalanced Ca^{2+} homeostasis can affect osteoblast activity and further affect bone formation(Han et al. 2018). At the same time, we noticed the treatment of LPZ for 24 h enhanced the expression of CytC, suggesting that mitochondrial dysfunction might be a possible mechanism in apoptosis in MC3T3-E1 cells induced by LPZ.

Conclusion

This study was the first to explore LPZ induced osteoporosis on the local level, using changes in $[\text{Ca}^{2+}]_i$ as an entry point. LPZ could inhibit MC3T3-E1 cells viability and triggered a significant decrease in BMD, S-Ca, S-IP levels in mice, inducing osteoporotic symptoms. LPZ could cause persistent elevation of $[\text{Ca}^{2+}]_i$ mediated by IP3R and SOCE pathway and promote ER stress by inhibiting PMCA and SERCA, thus promote apoptosis and eventually cause a decrease in MC3T3-E1 cells viability and function (Fig. 14). PPIs could disturb Ca^{2+} homeostasis in ER, increase $[\text{Ca}^{2+}]_i$, activate Calpain-2/Caspase-12 and Grp78/ATF4/CHOP pathway, which finally trigger osteoporosis.

Abbreviations

PPIs: proton pump inhibitors; LPZ: lansoprazole; BMD: bone mineral density; PMCA: plasma membrane Ca^{2+} -ATPase; SERCA: sarcoplasmic reticulum Ca^{2+} -ATPase;TG: Thapsigargin; S-IP: serum inorganic phosphorus; S-Ca: serum calcium; S-ALP: serum alkaline phosphatase

Declarations

Author contributions

Lu-Ning Sun, Yun Liu, Yong-Qing Wang contributed to the conception and design of the experiment. Material preparation, data collection, and analysis were performed by Meng-Yuan Ma, Shi-Yu Sun, Zeng-qing Ma, Yu Wang, Li-Yuan Yu, Xu-Ping Qian. The first draft of the manuscript was written by Zi-Ping Cheng. Jie-Yang Liu commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University (IACUC-1910006).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

Figure 1

After 6 months of lansoprazole gavage in mice, male/female were scanned by micro-CT on the right femur to calculate BMD (n = 10); the rest mice chosen randomly were sacrificed by taking blood to detect bone serum biochemical indicators (n = 10). (A, B) Femoral biomechanical strength properties. C BMD of the right femur of mice. (D, E, and F) Bone serum biochemical parameters.

A **B** **CMC-Na****250mg/Kg****1000mg/Kg**

Figure 2

Representative microcomputed tomography (Micro-CT) images of the distal femurs. A Micro-CT images of femur-specimens (n = 7). B Micro-CT images of cancellous bone structure near the growth plate.

Figure 3

Cell proliferation viability and apoptosis was detected by CCK-8 Kit, EDU kit and flow cytometry. A EDU cell proliferation assay of MC3T3-E1 treated with different concentrations of LPZ. B Apoptosis of osteoblasts pretreated with LPZ (0, 5, 10, 20, 50 μ M) for 12 h. C Count of cells in EDU kit. D Quantitative analysis of apoptotic rates. E Cell viability of MC3T3-E1 cells after LPZ treatment. *: $P < 0.05$, **: $P < 0.01$ ($n = 3$).

Figure 4

Calcium regulation of MC3T3-E1 cells under confocal microscopy. A Representative confocal images (40 $\times$). B Quantification of Fluo-3 fluorescence. C Area under the curve of Fluo-3 fluorescence. D Fluo-3 fluorescence after preincubation with TG ($n = 3$). E Cell viability of MC3T3-E1 cells after pre-incubated with BAPTA/AM and LPZ treatment by CCK8.

Figure 5

Relative calcium fluorescence change curves. A Relative [Ca²⁺]_i response in MC3T3-E1 cells pre-incubated with 2-APB in Ca²⁺-free medium. B Relative [Ca²⁺]_i response in MC3T3-E1 cells pre-incubated with Ryanodine in Ca²⁺-free medium. C Relative [Ca²⁺]_i response in MC3T3-E1 cells pre-incubated with Verapamil in Ca²⁺-containing medium. D Relative [Ca²⁺]_i response in MC3T3-E1 cells pre-incubated with BTP-2 in Ca²⁺-containing medium (n = 3).

Figure 6

Representative confocal images of MC3T3-E1 cells. A MC3T3-E1 cells double-loaded with ER-tracker (red) and Mag-fluo4/AM (green) to determine ER Ca²⁺ levels. B MC3T3-E1 cells double-loaded with Mito-tracker green and Rhod-2AM (red) to determine mitochondrial Ca²⁺ levels. (a-h Representative time series, n = 3, 20x).

Figure 7

[Ca²⁺]_i level after long-term treatment of LPZ. A [Ca²⁺]_i levels of MC3T3-E1 cells pre-incubated with BTP-2 in calcium-contained medium, detected by flow cytometry. B [Ca²⁺]_i levels of MC3T3-E1 cells pre-incubated with TG in calcium-free medium, detected by flow cytometry. C Quantitative analysis of [Ca²⁺]_i levels (n = 3).

Figure 8

[Ca^{2+}]_i level after long-term treatment of LPZ and TG. A [Ca^{2+}]_i levels of MC3T3-E1 cells pre-incubated with TG in calcium-contained medium, detected by flow cytometry. B Quantitative analysis of [Ca^{2+}]_i levels after treatment with LPZ or TG for 24 h.

Figure 9

Patch clamp recording of currents. A The recorded current was determined to be NCX. B Initial induced current of NCX, effect of different lansoprazole concentration and Ni²⁺ on INCX; gray: 5 mM NiCl₂; brown: 0 μM lansoprazole; blue: 10 μM lansoprazole; black: 50 μM lansoprazole; red: 100 μM lansoprazole. C NCX current peak statistics of MC3T3-E1 cells at different lansoprazole concentrations; forward current density peak statistics, #: P<0.05 vs the normal group; reverse current density peak statistics, *: P < 0.05 vs the normal group, **: P<0.01 vs normal group. (x + s, n = 6).

Figure 10

The expression of OB functional gene and ER stress apoptosis pathway related mRNA expression levels (ALP, OCN, Runx2, COL1α, Caspase-12, ATF4). TG was used as a positive reference group (n = 3).

A

B

C

Figure 11

The expression of OB functional gene and ER stress apoptosis pathway related protein levels. A WB images of LPZ, 2-APB, TG treatment. B Semi-quantitative analysis of protein levels in MC3T3-31 cells. C WB images of different concentrations of LPZ treatment (n = 3).

Figure 12

Effects of LPZ on Ca^{2+} -ATPase of MC3T3-E1 cells. A LPZ reduce Ca^{2+} -ATPase activity. B LPZ inhibits plasma membrane Ca^{2+} -ATPase gene expression (n = 3).

Figure 13

HE staining of femurs and expression of CHOP after intragastric administration of LPZ for 6 months (n = 3, Scale = 2 mm). A HE staining of mice femur. B Mouse femur immunohistochemistry of CHOP (Scale = 2 mm). C Semi quantitative calculation of CHOP with ImageJ.

Figure 14

Possible mechanisms underlying changes of $[Ca^{2+}]_i$ LPZ could enter MC3T3-E1 cells through the cell membrane, inhibit the SERCA and PMCA, Ca^{2+} was released from ER, and sustained at a higher level, causing calcium toxicity, and triggering the apoptosis pathways of ER and mitochondrial pathways. causing decreased osteoblast viability and function ultimately.