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Inhibition of PERK Signaling Prevents Against Glucocorticoid-induced Endotheliocyte Apoptosis and Osteonecrosis of the Femoral Head

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Abstract

Vascular injury is considered an important pathological process during glucocorticoid (GC)-induced osteonecrosis of the femoral head (ONFH). In this study, we tried to investigate whether the endoplasmic reticulum (ER) stress is triggered in the GC-induced endotheliocyte (EC) apoptosis and ONFH. The results showed that a GC upregulated the expression of ER stress-related proteins, and PERK-CHOP signaling played an important role and induced EC apoptosis. The inhibition of PERK by GSK2656157 significantly decreased the GC-induced EC apoptosis in vitro and in vivo, thus protecting a rat model from vascular injury and significantly preventing GC-induced ONFH.

Keywords: glucocorticoid, osteonecrosis of femoral head, endoplasmic-reticulum stress, PERK signaling, endotheliocyte, apoptosis.

Introduction

Glucocorticoids (GCs) are widely used against a variety of diseases and are some of the most common causes of nontraumatic osteonecrosis of the femoral head (ONFH) (6, 15, 22, 26). It has been reported that osteonecrosis develops in 9–40% of patients receiving long-term GC therapy (35). Due to the lack of effective drugs for ONFH, patients often need surgical treatment. The long duration of this condition imposes persistent pain and an economic burden on the patients.

The adverse effects of GCs on bone are primarily due to direct actions, in particular, apoptosis of osteoblasts, osteoclasts, and endotheliocytes (ECs) (20, 42, 43). Many studies have suggested that the proapoptotic effect of GCs is cell type dependent (1, 13, 16, 34). Growing evidence shows that ECs are injured by a GC via induction of apoptosis and dysfunction during the pathological process of GC-induced ONFH (10, 20, 28, 46, 47). Thus, a better understanding of the mechanisms of action of GCs on ECs may lead to a better treatment option for ONFH.

The occurrence of endoplasmic reticulum (ER) stress-induced apoptosis has been proven in many diseases (7, 33). ER stress activates stress sensors, including protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1 (IRE1), involved in the regulation of cell homeostasis. On the other hand, prolonged and unmitigated ER stress may cause apoptosis (2, 14, 33). Although ER stress correlates with various diseases, the role of ER stress in the pathogenesis of GC-induced ONFH remains unclear. A few studies have indicated that treatment with GCs leads to ER stress and results in various changes, including dysfunction and apoptosis of osteoblasts, osteocytes, and trabecular-meshwork cells (34, 44, 45). Nevertheless, few studies have focused on the GC-induced EC apoptosis mediated by the ER stress signaling pathway.

We were thus prompted to investigate whether ER stress is triggered during GC-induced EC apoptosis and ONFH, and, if so, whether GC-induced EC apoptosis and ONFH could be prevented by inhibition of the GC-induced ER stress signaling pathway.

2. Materials and Methods

2.1 In Vitro Experiments

2.1.1 Cell culture, treatment, and small interfering RNA (siRNA) transfection

The human EC line EAhy926 and human alveolar-bone-derived osteoblasts (OBs) were obtained from KeyGENE BIOTECH (Nanjing, China). The cell lines were cultured (37°C, 5% CO_2) in DMEM supplemented with 10% of fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Human bone marrow stromal cells (BMSCs) were obtained from patients after amputation according to the method described by Kodama (21). Informed consent was obtained from all the patients. The BMSCs and OBs were also cultured (37°C, 5% CO_2) in DMEM supplemented with 10% of FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco).

The siRNA experiment was conducted via delivery of plasmids. To construct siRNA expression vectors, the sequences were purchased from GenePharma (Shanghai, China). The siRNA sequences are listed in Appendix Table 1. ECs were transfected with siRNA against PERK, IRE1α, or ATF6 or scrambled siRNA (GenePharma, Shanghai, China). At 8 h after transfection, the transfection medium was replaced with a fresh culture medium, and the cells were cultured for 40 h before treatment with dexamethasone (DEX; Selleck, Houston, TX, USA). The transfection efficiency was >80%.

2.1.2 Annexin V/Propidium Iodide (PI) Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells were analyzed for phosphatidylserine exposure by the annexin-V fluorescein isothiocyanate (FITC)/PI double-staining method according to the manufacturer's instructions (Dojindo Molecular Technologies, Inc. Gaithersburg, MD). Briefly, ECs were harvested by gentle trypsinization and then washed with PBS twice and resuspended in annexin-coupling buffer at a concentration of $\sim 10^6$ cells/mL. A total of 100 μL of the cell suspension was incubated with 5 μL of the annexin V-FITC conjugate and 5 μL of a PI solution at room temperature for 15 min. A FACS machine was employed to evaluate the rate of apoptosis. Approximately 5,000 events were analyzed for apoptotic, necrotic, and live cells. All the experiments were repeated three times, and the results are expressed as a percentage of all the events in each experiment.

2.1.3 Western Blot Analysis

ECs were treated with DEX under different conditions. The cells were harvested and lysed with cell lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO) on ice for 15 min. Protein samples were diluted 1:5 with protein loading buffer (Transgen Biotech, Beijing, China). A total of 30 μg of protein was subjected to SDS-PAGE after denaturation at 95°C for 5 min. The cell lysates were analyzed on a 10% gel (based on Tris-HCl buffer) under reducing conditions. After electrophoresis, the proteins were electrophoretically transferred to 0.22 μm polyvinylidene difluoride membranes (Merck, Darmstadt, Hesse, Germany) and blocked with 5% nonfat dry milk at 4°C overnight. The membranes were then incubated for 3 h at 37°C with anti-ATF6 (Thermo Fisher Scientific, Waltham, MA), anti-phosphorylated-IRE1α, (p-IRE1α; Abcam, Cambridge, MA), anti-PERK, anti-phosphorylated-PERK (p-PERK), anti-IRE1α, anti-CHOP, anti-BIP, anti-XBP1-s, anti-Caspase-3 (Casp3), anti-cleaved Caspase-

114 3(cCasp3), anti-β-Tubulin, or anti-GAPDH (Cell Signaling Technology, Danvers, MA) antibodies.
115 The membranes were next immersed in a solution of a secondary antibody: an anti-rabbit or anti-
116 mouse IgG antibody (Cell Signaling Technology) for 1 h at 37°C.
117 After three washes with Tris-buffered saline containing 0.1% of Tween 20, the membranes were
118 added to an ECL substrate in a dark room for imaging on a FluorChem M Gel Documentation
119 System (ProteinSimple, San Jose, CA, USA). The results were analyzed in densitometric analysis
120 software Quantity One (Bio-Rad Laboratories, Inc., Hercules, CA, USA) β-Tubulin or GAPDH
121 served as an internal reference.
122
123
124 **2.2 In Vivo Experiments**
125
126 2.2.1 Establishment of the Osteonecrosis Model and Treatment
127
128 A short-term GC treatment model was set up as follows. The Sprague-Dawley（SD） rats were
129 randomly and equally divided into the following three groups: (1) Control group (n = 10); (2)
130 methylprednisolone (MPS) group (rats treated with MPS, n = 10); and (3) treatment group
131 (osteonecrotic rats treated with MPS and PERK inhibitor GSK2656157, n = 10). In parallel, 0.2 mL
132 of normal saline was intramuscularly injected into the rats in the control group. MPS (20 mg/[kg·d],
133 Pfizer, New York NY) was intramuscularly injected once a day for 3 days.
134
135 The in vivo animal model of ONFH was constructed according to the description by Guo (11). The
136 rats were randomly and equally subdivided into the control group (n = 10), MPS group (n = 10),
137 and treatment group (n = 10).
138
139 According to a manufacturer's protocol, PERK inhibitor GSK2656157 (Selleck) was
140 intragastrically administered (25 mg/kg) 8 h before every MPS injection in the treatment group.
141
142 None of the rats died before the scheduled euthanasia under 4% chloral hydrate anesthesia. Next,
143 the femoral heads were collected for micro-computed tomography (CT) scanning, immunostaining,
144 and immunohistochemical staining. All the experimental and animal care procedures were approved
145 by the Animal Research Ethics Committee of Shanghai Sixth People's Hospital and were in
146 compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory
147 Animals.
148
149 2.2.2 Angiography and Micro-CT Scanning
150 Before euthanasia, the rats were anesthetized and successively perfused with 4% heparinized saline
151 and Microfil (MV-112, Flow Tech, Carver, MA) according to the manufacturer's protocol. The
152 bodies of the rats were stored at 4°C overnight, and then the bilateral femoral heads were collected
153 for further experiments. The femoral heads were subjected to micro-CT scanning before and after
154 decalcification in a 10% EDTA solution for 4 weeks. The micro-CD scanner was set to a resolution
155 of 9 μm per pixel. The trabecular bone was segmented from the bone marrow and analyzed to
156 determine trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular bone pattern factor
157 (Tb.pf), bone volume per tissue volume (BV/TV), and trabecular number (Tb.N). Three planes

(coronal section, sagittal section, and transverse section) of the representative samples from each group were generated in the DataViewer software (Bruker Micro-CT). The total vessel volume was calculated by CTAn.

2.2.3 Immunofluorescent Staining

The femoral heads were sectioned at 5μm thickness in the coronal plane. The deparaffinized sections were processed by 0.25% trypsin antigen retrieval and were blocked with 10% FBS for 1 h at 37°C. The sections were incubated with anti-CD31 (1:200, Sigma-Aldrich) and anti-cleaved caspase 3 (cCasp3) (1:600, Cell Signaling Technology) antibodies at 4°C overnight (primary antibodies), and then the appropriate secondary antibodies (Servicebio, Wuhan, China) were applied for incubation at room temperature for 1 h. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Servicebio) for 5 min. Immunofluorescence photomicrographs were captured by means of a fluorescence microscope (Leica DMI6000B, Germany).

2.2.4 Immunohistochemical Staining

After 1 h 0.25% trypsin antigen retrieval and 1 h 10% FBS incubation at room temperature, the sections were incubated with the anti-cCasp3 (1:200, Cell Signaling Technology) primary antibody overnight at 4°C. After that, the sections were incubated with a biotinylated secondary antibody (Servicebio, Wuhan, China) according to the manufacturer's instructions. The sections were stained with a 3,3-diaminobenzidine precipitate and counterstained with hematoxylin. Photomicrographs were acquired using a LEICA DM 4000 (Leica Microsystems, Germany).

2.3 Statistical Methods

Means of multiple groups were compared by one-way analysis of variance (ANOVA). Fisher's exact test was conducted to compare the incidence of the disease between two groups. The independent-sample *t* test was performed to compare means between two different groups. Statistical analysis was conducted in SPSS 20.0 software (IBM Corp., Armonk, NY, USA). Data with P values <0.05 were considered statistically significant.

3. Results

3.1 DEX Induces EC Apoptosis

The results of the flow cytometric analysis revealed DEX-induced apoptosis in ECs, BMSCs, and OBs. Compared with OBs and BMSCs, the ECs showed a stronger apoptotic tendency (Figure 1A, B). The flow cytometric analysis also revealed that the apoptosis of ECs increased with the increase of DEX stimulation time and concentration (Figure 1C–F). cCasp3, a representative activated Caspase involved in several types of cell death, was also detected here by western blotting, which supports the conclusions from the flow cytometric analysis (Figure 1G, H). To observe apoptosis in vivo, we performed cCasp3 and CD31 double-label immunofluorescence staining on rat femoral head sections. The results meant that short-term GC treatment induced apoptosis in the inner wall of blood vessels, whereas no apoptosis was found in other tissues of a femoral head (Figure 1I, J). We concluded that apoptosis occurs in the ECs in response to GC treatment, which revealed that the apoptosis was stronger in ECs than in other cells in bone tissue.

201 **3.2 DEX Induces ER Stress in ECs**

202 To find out the cause of apoptosis and to confirm the presence of ER stress, we quantified ER stress-
203 related proteins including PERK, p-PERK, IRE1α, p-IRE1α, ATF6, CHOP, XBP-1s, and BIP. The
204 western blot revealed that ER stress-related proteins were upregulated with the increase of
205 stimulation time and concentration, and three classical signaling pathways were found to be
206 activated simultaneously after DEX treatment (Figure 2A, B). The CHOP and CD31 double-label
207 immunofluorescence staining indicated that CHOP expression in ECs increased after short-tern GC
208 treatment (Figure 2C, D). These findings confirmed GC-induced ER stress in ECs.

209

210 **3.3 PERK Inhibition Prevents EC Apoptosis In Vitro**

211 Given that we observed the activation of all three ER stress-related downstream signaling pathways,
212 further experiments were necessary to determine the specific pathway associated with the apoptosis.
213 We blocked the three signaling pathways respectively by transfection of short interfering RNAs
214 against PERK, IRE1α, or ATF6. The siRNA-treated ECs were then stimulated with DEX and
215 assessed by flow cytometry. Compared with the control group, neither the IRE1α-deficient nor
216 ATF6-deficient ECs showed a decrease of apoptosis, whereas the inhibition of PERK significantly
217 decreased the apoptosis after DEX treatment (Figure 3A, B).

218 After that, we identified a PERK inhibitor, GSK2656157, and pretreated the cells with the
219 inhibitor 8 h before the DEX treatment. downstream of PERK signaling pathway. We quantified
220 GADD34 and CHOP, which are downstream of the PERK signaling pathway and participate in
221 apoptosis. The western blot showed that the expression of GADD34 and CHOP diminished after
222 GSK2656157 treatment. Besides, the decreased expression of cCasp3 indicated the antiapoptotic
223 effect of PERK inhibition (Figure 3C). The flow cytometric analysis also showed the decrease of
224 apoptosis in the GSK2656157-pretreated ECs, indicating that the PERK inhibitor exerted a
225 protective action against GC-induced apoptosis (Figure 3D, E).

226

227 **3.4 PERK Inhibition Prevents the GC-induced Vascular Damage In Vivo**

228 The immunofluorescent staining of CD31 and cCasp3 revealed that PERK inhibitor GSK26556157
229 reduced the EC apoptosis after short-term MPS treatment. The PERK inhibitor provided protection
230 against GC-induced apoptosis, as verified by our findings in vitro (Figure 4A, B) The micro-CT
231 analysis of the perfused vessels in the femoral heads also suggested that the long-term GC treatment
232 injured the vessels in the femoral head. The PERK inhibitor GSK2656157 had a protective effect
233 against GC-induced vascular injury (Figure 4C–E).

234

235 **3.5 PERK inhibitor GSK2656157 Prevents GC-induced ONFH In Vivo**

236 The results of an in vitro experiment revealed that 13 in 20 femoral heads (10 Sprague-Dawley rats)
237 in the MPS group showed visible signs of osteonecrosis, while only one osteonecrotic femoral head
238 was found in the treatment group ($p < 0.001$). Hematoxylin and eosin (H&E) staining revealed
239 visible subchondral necrosis with fatty-tissue invasion in the subchondral bone trabecular area,
240 whereas PERK inhibitor GSK2656157 successfully prevented ONFH in the treatment group (Figure
241 5A). The trabecular changes in the subchondral region of the femoral heads were detected by micro-
242 CT (Figure 5B). Besides, the micro-CT analysis yielded results on bone parameters, including Tb.Th,
243 Tb.Sp, Tb.pf, and BV/TV. We noticed a significant preventive effect of GSK2656157 (Figure 5C–
244 G). The bone mineral density (BMD) of the rats in the model group was 0.076 g/cm^3, which was

245 significantly lower than that of the control group, whereas the PERK inhibitor significantly
246 prevented the reduction in BMD (Figure 5H). The results above led to the conclusion that PERK
247 signaling blockage (by GSK2656157) may prevent ONFH.
248

249 Discussion

250 The most important findings of this study are that ER stress is strongly involved in GC-induced EC
251 apoptosis and ONFH. ECs are more sensitive to apoptosis than other cells in bone tissue when
252 stimulated by GCs. PERK-CHOP signaling plays a critical part in this process. The PERK inhibitor
253 GSK2656157 was demonstrated to be effective in preventing GC-induced vessel injury and ONFH
254 in vivo, thereby further proving the decisive participation of PERK signaling in GC-induced ONFH.
255 This study provides new insight into the inherent relation among EC apoptosis, vascular injury, and
256 GC-induced ONFH. Namely, GCs cause ER stress in ECs and induce apoptosis, which leads to
257 microvascular damage and eventually causes ONFH.
258

259 Apoptosis in bone is thought to be the key determinant of GC-induced ONFH (17, 18, 43). Some
260 studies have mainly focused on the direct adverse effects of administered GCs on BMSCs,
261 osteoblasts, and osteoclasts; GCs decrease the formation of both osteoblasts and osteoclasts and
262 increase the apoptosis of osteoblasts while prolonging the lifespan of osteoclasts (12, 34, 36, 37, 43,
263 48). However, recent studies pointed out that apoptosis of ECs in ONFH is essential but ignored.
264 One of the possible reasons is that the apoptotic effect of a GC is cell-type dependent (9, 46).
265 Compared to other cells, ECs show a stronger and earlier apoptotic tendency in response to GC
266 treatment. Furthermore, there is an even higher GC concentration in blood vessels than in other
267 tissues in vivo (30, 38). It has been reported that during high-dose GC treatment (blood
268 concentration of 15–100 μM, according to a GC treatment guide) (39), the vessels are more affected
269 than any other tissues in the femoral head. This observation is consistent with earlier studies, which
270 suggest that vessel injury is an initiating factor for the pathological processes of osteonecrosis (19,
271 25, 47), and that osteonecrosis is a series of secondary pathological changes in response to ischemia
272 (4).
273

274 ER stress is known to activate three major signaling pathways, such as the PERK-ATF4 axis, ATF6
275 signal transduction, and IRE1a cascade (24, 33, 41). ER stress may act as a two-edged sword in
276 such diseases as ONFH. The ER responds to stress by activating the unfolded protein response
277 (UPR). If various UPR-induced mechanisms fail to alleviate ER stress, both the intrinsic and
278 extrinsic pathways of apoptosis can get activated. When activated upon sensing ER stress, PERK
279 oligomerizes and phosphorylates itself and the ubiquitous translation initiation factor eIF2α, thereby
280 indirectly inactivating eIF2α and inhibiting mRNA translation. In this way, PERK helps reduce the
281 flux of protein entering the ER to alleviate ER stress (40). It has been demonstrated that a
282 dephosphorylation inhibitor of eIF2α can reduce ER stress (3, 25, 34). Nevertheless, strong or
283 prolonged ER stress may break the homeostatic balance of UPR (32, 40). Subsequently, the PERK-
284 CHOP signaling pathway is activated, inducing apoptosis (5, 8, 24, 31).
285

286 PERK is the major protein responsible for attenuation of mRNA translation under ER stress,
287 preventing the influx of newly synthesized protein molecules into the already stressed ER

compartment. The activation of eIF2α phosphorylation has been demonstrated to reduce the flux of protein into the ER and thus alleviate ER stress. Nonetheless, different levels of stimulation have been found to cause various ER stress responses. Prolonged or extra strong stimulation is thought to break the PERK-eIF2α regulatory mechanism, mediated by the activation of ATF4-CHOP signaling, which has a proapoptotic activity and is crucial for triggering apoptosis in response to ER stress (29, 31, 33, 40). The experiment on the relation between GC-induced ER stress and stimulus intensity (time/concentration) was under consideration. Because apoptosis in patients with steroid-induced ONFH has been widely reported, it is believed that long-term or large-dose GC treatment may directly cause this apoptosis (27).

More recently, a few studies described the participation of ER stress in ONFH. Sato AY et al. have reported that salubrinal or guanabenz, specific inhibitors of eIF2α dephosphorylation, block apoptosis of osteocytic MLO-Y4 and osteoblastic OB-6 cells, when this apoptosis is induced by either GC or ER stress inducers (34). Liu et al. have reported that ER stress is an important pathological outcome in the surgery-induced ONFH model, and salubrinal alleviates ONFH symptoms by enhancing angiogenesis and bone healing via suppression of ER stress (23). These two studies show that inhibition of eIF2α dephosphorylation reduces ER stress and is proven to be effective at launching the ER stress-related response (3). Furthermore, they focused on the direct effects of ONFH on osteoblasts and osteoclasts. In this study, we proved that a GC activates PERK-CHOP signaling, indicating that GC-induced ER stress breaks ER homeostasis and leads to apoptosis. Furthermore, we confirmed that ER stress is strongly involved in GC-induced EC apoptosis and ONFH. GSK2656157, an inhibitor of PERK phosphorylation, has been proven to be effective against ER stress-induced apoptosis (23). In our study, GSK2656157 blocked the whole downstream signaling of PERK and attenuated GC-induced cell apoptosis. GSK2656157 was demonstrated to be effective in the protection from steroid-induced vessel injury and from ONFH in vivo. These findings confirmed the decisive role of PERK signaling in ONFH.

Our study has several limitations. First, a CHOP knockout transgenic model was not constructed due to the lack of osteonecrotic murine models. Second, although the therapeutic effect is clear-cut, PERK inhibitor GSK2656157 is still not an approved drug and further clinical studies are required.

This work should improve the understanding of the relation between intraosseous microvascular injuries and steroid-induced ONFH. GC-induced ER stress was found to induce EC apoptosis, which triggers other secondary changes and finally causes osteonecrosis. Our experimental results also suggest a promising drug for the prevention of ONFH. PERK signaling, as a brand-new research direction for ONFH prevention, requires much lucubration in the future. Furthermore, a more in-depth study on ONFH may change the traditional paradigm of several bone diseases and could contribute to the discovery of a novel therapeutic method.

Abbreviations

ATF6：activating transcription factor 6; BIP：immunoglobulin heavy-chain-binding protein
BMSCs: bone marrow stromal cells; Casp3: caspase 3; CHOP: C/EBP-homologous protein;
cCasp3: cleaved caspase 3; DEX: dexamethasone; EC: endotheliocyte; ER: endoplasmic reticulum;
FACS：fluorescence-activated cell sorting; GC: glucocorticoid; IRE1：inositol-requiring kinase 1;

OBs: osteoblasts; ONFH: osteonecrosis of the femoral head; PERK: protein kinase-like ER kinase; p-IRE1α: phosphorylated IRE1α; p-PERK: phosphorylated-PERK; XBP1s: X-box-binding protein 1 sliced

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Authors' contributions

YC Gao and HY Zhu carried out the design and conception of the study and drafted the manuscript. Y Feng and CQ Zhang participated in the drafting of the manuscript and its critical revision. YC Gao and QY Wang conducted all data acquisition, analysis, and interpretation. All the authors read and approved the final manuscript. YC Gao and HY Zhu contributed equally to this work; Y Feng and CQ Zhang are co-senior authors and contributed equally to this work.

Competing Interests

The authors have declared that no competing interests exist.

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470 **Figure legends**
471 **Figure 1**
472 Apoptosis in the ECs in response to GC treatment. **A** ECs, BMSCs, and MC3T3 were treated with
473 DEX (10 μM) for 48 h. The FACS chart of Annexin V-FITC/PI staining is shown. **B** The histogram
474 of FACS results indicates the apoptotic status of different cells. **C** ECs were treated with DEX (10
475 μM) for different periods (0–48 h). The FACS chart of Annexin V-FITC/PI staining is shown. **D** The
476 histogram of FACS results indicates the apoptotic status of the cells. **E** Additional ECs were treated
477 with different concentrations of DEX (0–10 μM) for 48 h. The results are presented as a FACS chart.
478 **F** The histogram of FACS results indicates the apoptotic status of the cells. **G, H** ECs under the
479 same treatment conditions presented in A and B were harvested from 6-well plates. The expression
480 levels of Casp3 and β-tubulin are shown in a western blot. **I** A representative image of cCasp3
481 immunohistochemical staining in the femoral head from a 3-day steroid-treated rat shows a pattern
482 consistent with the shape of a microvessel. **J** The histogram of immunofluorescence results indicates
483 the percentage of apoptotic ECs in the femoral head. *$p < 0.05$.
484 **Figure 2**
485 **A** DEX Induces ER stress in ECs. ECs were treated with DEX at different concentrations (0–10 μm)
486 for 24 h. ER stress-related proteins (PERK, p-PERK, IRE1α, p-IRE1α, ATF6, CHOP, XBP-1s, and
487 BIP) were examined by western blotting. **B** ER stress-related proteins were also observed among
488 ECs treated with DEX for different periods (0–24 h, 10 μM). **C** A representative image of CHOP
489 immunohistochemical analysis in the femoral head from a 3-day steroid-treated rat shows a pattern
490 consistent with the shape of a microvessel. **D** The histogram of immunofluorescence results reveals
491 the CHOP-positive percentage of ECs in the femoral head. *$p < 0.05$.
492
493 **Figure 3**
494 PERK inhibition by siRNA or PERK inhibitor GSK2656157 decreased the apoptosis of ECs in vitro.
495 **A** ECs were transfected with siRNAs to knockdown PERK, IRE1α, or ATF6, followed by treatment
496 with vehicle or DEX (10 μM) for 48 h. The FACS charts of the Annexin V-FITC/PI staining are
497 presented. **B** The histogram indicates the apoptotic status of the cells. **C** The ECs were treated under
498 the same above-mentioned conditions, and the expression levels of Casp3 were examined by
499 western blotting. **D** The cells were pretreated with the PERK inhibitor GSK2656157 8 h before the
500 DEX treatment (10 μM; 48 h). The FACS charts are shown. **E** The histogram of the Annexin V-
501 FITC/PI staining is shown. *$p < 0.05$.

502
503 **Figure 4**
504 PERK inhibitor GSK2656157 prevented vascular damage in an ONFH model. **A** Representative
505 immunostaining of the femoral heads from the rats that received 3-day steroid injections shows the
506 colocalization of CD31 and cCasp3 in the GC group, whereas the immunostaining of the femoral
507 heads from both the control and treatment groups shows low expression levels of cCasp3. **B** The
508 histogram based on densitometric analysis of immunostaining from various groups ($n = 10$; * $p <$
509 0.05). **C** A representative 3D reconstructed microangiographic image shows the general form of the
510 intraosseous microvessels in the femoral head. **D, E** Quantification of the total vessel volume (mm^3)
511 and the ratio of total vessel volume to total tissue volume in the femoral heads from the different
512 groups are shown ($n = 10$). *$p < 0.05$
513
514 **Figure 5**
515 PERK inhibitor GSK2656157 protected the femoral head from osteonecrosis in vivo. **A** The H&E
516 staining of coronal sections of the representative femoral heads from each group. The arrows
517 indicate the empty lacunae and hypertrophic fat cells, which indicate osteonecrosis. **B**
518 Representative images from the micro-CT analyses in the sagittal, horizontal, and coronal planes.
519 The triangle indicates lower density changes in the subchondral area in the ONFH group. **C–H** The
520 histograms of the morphometric analysis show the BMD and bone parameters (BV/TV, Tb.Th, Tb.N,
521 Tb.Pf, and Tb.Sp) of the upper outer subchondral bones of the femoral heads ($n = 20$). *$p < 0.05$