Original Research

Increases in energy intake, insulin resistance and stress in rats before Wenchuan earthquake far from the epicenter

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Abstract

The study of pre-earthquake (PE) behavior in animals has always been shrouded by controversy. There is very little scientific evidence showing that animals can sense the coming of an earthquake and that their organisms undergo physiological changes during the PE period. On the day of the Wenchuan earthquake, prior to the time of its actual occurrence, we were coincidentally able to measure the insulin sensitivity and stress level in rats that were originally part of another study. We detected defects in insulin signaling and a decrease in glucose uptake in skeletal muscle (SkM) and adipose tissue (AT), indicating impaired insulin sensitivity. These changes were associated with significantly increased plasma corticosterone concentration and elevated HSD11B1 mRNA expression in SkM and AT. The increase in insulin resistance (IR) could be attributed to elevated local (SkM and AT) and systemic stress. Interestingly, we also noticed that the food intake in rats showed a sudden increase two days before the earthquake and reached a peak on the day of the earthquake itself. Our observations suggest the possibility that the rats underwent PE physiological changes consisting of an increase in the stress level and consequently leading to an increase in food intake and IR.

Keywords: insulin resistance, stress, energy intake, pre-earthquake, rats

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Introduction

Throughout history man has always been fascinated by earthquakes and their sometimes terrifying power. Although earthquakes are mostly unpredictable forces of nature, we have always sought means to foresee their occurrence through pre-earthquake (PE) behavior in animals. There have been numerous naturalistic observations and anecdotal reports of abnormal behavior in animals days or hours before earthquakes occurred.[1] While some reports suggest that PE behavior is primarily a consequence of psychological focusing effect, other studies have ascribed this phenomenon to minor environmental changes and the emission of small waves that occur before an earthquake, detected only by animals but not by humans.[2] However, very few studies have been conducted on the physiological changes that occur in the PE period.[1] Since PE behavior is rather controversial and subjective, the measurement of physiological changes would provide us with objective evidence that animals undergo PE changes. Due to the unpredictable nature of earthquakes and the inability to reproduce the event in controlled settings, being able to measure the physiological changes involves mostly luck, and to be present at the right place and at the right time.

The Wenchuan earthquake of magnitude 8 on the Richter scale occurred on 12 May 2008, hitting the south-western part of China at 14:28 (local time). The epicenter of the earthquake was located in the Longmenshan fault zone, on the eastern edge of the Tibet Plateau.[3] Although our city Wuhan is situated 1000 km east of the epicenter which is relatively far away, we still felt the effects of the earthquake through the shaking of the ground and buildings, though no major structural damage or loss of lives were reported. Coincidentally and fortunately for us, on the morning of that same day we were measuring the insulin and cortisone levels in rats as part of our ongoing experiment on nutrition promotion. We had therefore already planned to measure the insulin sensitivity, plasma glucocorticoid levels and energy intake. We should point out that a similar experiment had been conducted in April on another group of rats, and we were performing a repeat of the experiment on the day of the earthquake. Surprisingly we noticed differences between the two sets

of results, even though we proceeded with the study using the same strict parameters and without any modifications in both groups. Our repeat experiment revealed a drastic insulin resistance (IR) and an increase in plasma glucocorticoid levels. Moreover, we also observed a significant increase in the food intake that started two days before the occurrence of the earthquake until the day of the earthquake itself. Since the only factor that varied between the control experiment and the repeat one was the earthquake, we surmised the hypothesis that the changes in stress level, IR and food intake could be attributed to PE physiological changes. Additionally, there were newspaper reports of unusual behavior in some animals of the Wuhan Zoo before the Wenchuan earthquake took place, and it is therefore possible that the changes seen in the rats of our study were due to the earthquake.

The stress response is essential in the survival of organisms, involving mainly glucocorticoid hormone. Glucocorticoid (corticosterone in rat, cortisol in human) is the common pathway of the sympatho-adrenomedullary and hypothalamo–pituitary–adrenocortical systems, which interact in a complex manner to maintain the internal environment during exposure of the organism to a wide variety of stressors.[4] The action of glucocorticoid on target tissues is determined by intracellular active glucocorticoid concentrations that is regulated not only by circulating steroid concentrations but also by intracellular 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1).[5] The role of HSD11B1 is to catalyze the *in vivo* conversion of inactive to active glucocorticoids[5] and the expression level of HSD11B1 positively correlates with its activity.[6] Studies have also shown that increased HSD11B1 activity in skeletal muscle (SkM) and adipose tissue (AT), which are the main sites of insulin-mediated glucose uptake, contributes to the pathogenesis of IR.[7] The molecular mechanisms involved include a defect in inhibition of Ser^{473} phosphorylation of PKB (protein kinase B) in SkM and AT.[8] We therefore further assessed the insulin signaling and HSD11B1 as a marker of local glucocorticoid activity.

The main objective of this present study is to report the detailed changes in stress level, IR and energy intake in the PE period. We also tentatively postulate that the increase in IR and energy intake were due to the stress response, which itself could be ascribed to PE physiological changes.

Materials and methods

Animals and diets

Normal male Sprague–Dawley rats (*Rattus norvegicus*; aged 6 weeks, weighing 140–180 g) were obtained from the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All of them were housed with free access to water and subjected to controlled temperature ($22 \pm 3^{\circ}$C), lighting (lights on 06:00–18:00) and relative humidity ($50 \pm 10\%$). All the rats, which were originally intended to be used as normal controls in another experiment, were fed with a standard rodent chow (13.68%, 64.44% and 21.88% of calories derived, respectively, from fat, carbohydrate and protein) for four weeks to assess insulin sensitivity. Blood and tissue samples were collected at the end of the experiment. The chow was provided by the Laboratory Animal Centre. All the experimental procedures performed were approved by the Animal Ethics Committee of our university and in accordance with Hubei Province Laboratory Animal Care Guidelines for the use of animals in research.

Study site and time

The study was conducted in the city of Wuhan, which is located in the Jianghan Plain 1000 km away from the epicenter of the Wenchuan earthquake (Figure 1). Insulin sensitivity in PE animals ($n = 4$) was coincidentally measured on the morning of May 12, 2008, while that of their controls (NC, $n = 4$) was tested at the end of April 2008. Plasma and tissue samples were harvested in each case on the day of the experiments.

Energy intake

Food intake of animals was determined on alternate days in the evening as planned originally. In order to exclude the impact of body weight on food intake, the energy intake was standardized to body weight in this study. The energy intake was calculated by the following equation:

$$Y = aX/Z \quad (1)$$

where Y is the energy intake (kJ/d/g), X is the food intake (g/d), a is the energy density (kcal/g) and Z is the body weight (g).[9] The energy intake corresponds to the mean value for two days.

Body weight, Lee index and body fat percentage

All the rats were weighed before the start of the hyperinsulinemic–euglycemic clamp. The nasoanal lengths were measured prior to their sacrifice. Lee index was calculated from the formula:[9]

$$\text{Lee index} = 1000\, a^{1/3}/b \quad (2)$$

where a is the weight (g) and b is the nasoanal length (cm), respectively.

Subcutaneous (groin), perirenal and epididymal white ATs were carefully dissected out and weighed. Body fat percentage of various AT compartments was determined by dividing the weight of the calculated fat per animal by the carcass weight. The sum of perirenal and epididymal fat was considered as visceral fat.[9]

Animal preparation and hyperinsulinemic–euglycemic clamp in conscious rats based on tail artery and vein catheterization technique

After a 10- to 12-h overnight fast, a 120-min hyperinsulinemic–euglycemic clamp was conducted in conscious rats according to a modified form of the procedure described by Shang *et al.*[10] In short, under local anesthesia with 5% lidocaine around the tail root of rats, tail artery and vein

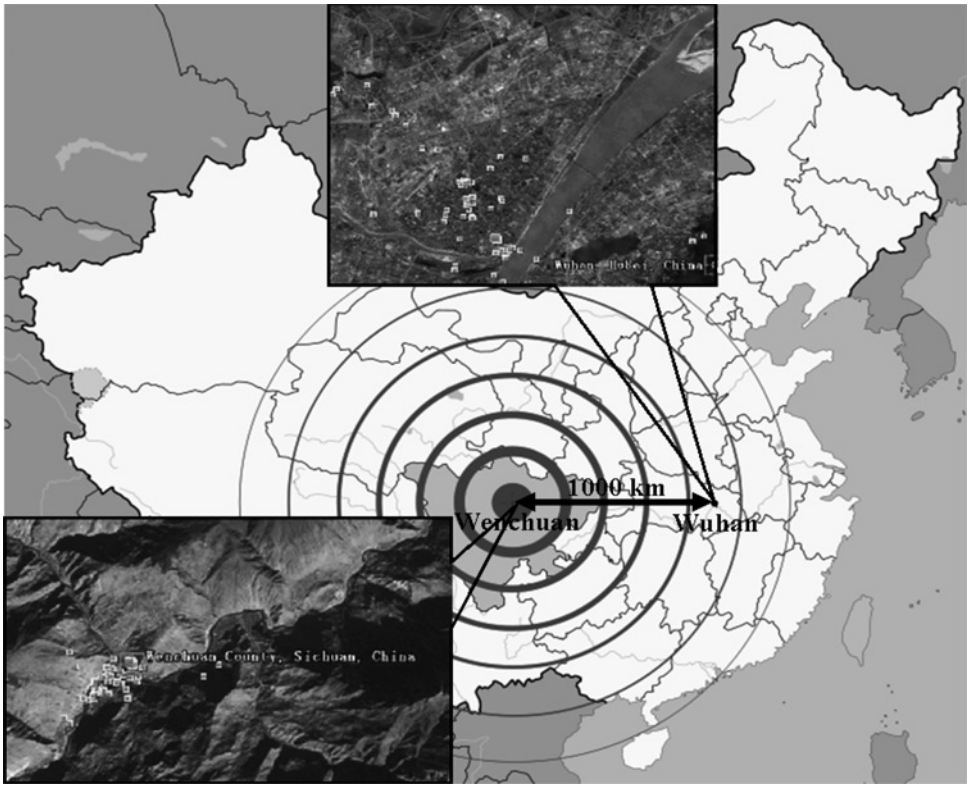

Figure 1 The study was performed in Wuhan, a city located in Jianghan Plain 1000 km away from the epicenter of Wenchuan earthquake

catheterizations were performed with intravenous integrated catheters (24G × 19 mm, Weihai Jierui Medical Products Co. Ltd, Weihai, China) filled with heparin-saline solution (50 IU heparin/mL 0.9% saline). The arterial catheter was used for blood sampling and the venous catheter for intravenous infusion of insulin and glucose. Fasting plasma glucose was measured using a glucose oxidase kit (Beijing Chemical Industry, Beijing, China).[11] Plasma insulin level was estimated using a rat insulin ELISA kit (Linco Research, St Charles, MO, USA). A prime-continuous infusion of human insulin (Novolin R, Novo Nordisk, Denmark) was maintained at a rate of 0.25 U/kg/h to raise plasma insulin for 120 min, and 25% glucose solution was infused at variable rates and periodically adjusted to clamp the plasma glucose levels at approximately 4.5 mmol/L. Blood samples (20 μL) were collected every five minutes for plasma glucose determination using a glucometer (One Touch® Ultra, Lifescan, Milpitas, CA, USA). The average glucose infusion rate between the 60th and 120th min (GIR_{60-120}) was used to evaluate insulin sensitivity. To estimate insulin-mediated glucose transport activity in individual tissues, 2-deoxy-D-glucose (2-DG; Sigma-Aldrich, St Louis, MO, USA) was administered through the venous cannula as a bolus (2 mmol/kg in saline)[12] 45 min before the end of the clamps.

Measurement of *in vivo* 2-deoxyglucose uptake in SkM during hyperinsulinemic-euglycemic clamp

At the end of hyperinsulinemic–euglycemic clamp, the rats were killed with a lethal dose (150 mg/kg) of intraperitoneally injected pentobarbitone sodium. Anterior tibial muscle was carefully dissected, removed and quickly frozen in liquid nitrogen after exsanguination. The rate of 2-deoxyglucose uptake by selected SkM samples was determined using a non-radioisotope enzymatic assay described by Yamamoto *et al.*[12] In brief, two different reaction mixtures were prepared in the following manner. Assay cocktail A contained all reagents (50 mmol/L triethanolamine hydrochloride [pH 8.1], 50 mmol/L KCl, 0.02% bovine serum albumin, 0.1 mmol/L nicotineamide adenine dinucleotide phosphate [$NADP^+$], 2–10 μmol/L resazurin sodium salt, 20 U/mL *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase [G6PDH] and 0.2 U/mL diaphorase). Cocktail B was similar in composition but contained 0.1 U/mL of *Candida utilis* (tolura yeast) G6PDH, in place of *L. mesenteroides* G6PDH (all reagents were purchased from Sigma-Aldrich). Usually G6PDH from *C. utilis* reacts only to G6P (glucose-6-phosphate) while G6PDH from *L. mesenteroides* reacts with both G6P and DG6P (2-deoxy-D-glucose-6-phosphate). Then frozen muscle (50–100 mg) was homogenized on ice in a nine-fold volume of ice-cold deionized water. An aliquot (100 μL) of the homogenate was transferred to a 1.5-mL Eppendorf tube and 200 μL of 0.1 N NaOH was added, followed by heat treatment at 85°C for 45 min to destroy endogenous NAD(P)H, NAD(P) and enzymes. After the tubes were cooled, 200 μL of 0.1 N HCl was added to neutralize the alkali, and the sample was centrifuged at 10,000*g* for five minutes. The supernatant (160 μL) was transferred and incubated with 800 μL assay cocktail A or B, and compared against mixtures of standard solutions of glucose and 2-DG (160 μL) and assay cocktails A or B (800 μL). The reaction time was set to 60 min for cocktail A and 30 min for cocktail

B as described by Yamamoto *et al.* [G6P + DG6P] was measured using assay cocktail A and [G6P] was measured using assay cocktail B, followed by detection on an F-4500 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan) at emission 615 nm with excitation at 530 nm. The values for [G6P + DG6P] and [G6P] were then determined from their respective standard curves. [DG6P] value was calculated from the difference between [G6P + DG6P] and [G6P], and the uptake of 2-DG was clearly evaluated.

Immunoblot analysis

Protein concentrations were analyzed using the BCA reagent (Pierce, Rockford, IL, USA), and the volume required for 80 μg of protein was determined. Samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose. Immunoblotting was done, followed by detection with enhanced chemiluminescence and quantified by densitometry.[10] Rabbit polyclonal antibodies recognizing anti-PKB/Akt and anti-phospho-PKB/Akt (Ser^{473}) were obtained from Cell Signaling Technology (Beverly, MA, USA).

Plasma corticosterone levels

In both the control and study groups, blood for plasma corticosterone was drawn at 07:00–09:00, through the vena caudalis of the rats on the day of the hyperinsulinemia–euglycemia clamp, before the start of the experiment. The corticosterone concentrations were measured by commercially available kits (Assaypro, St Charles, MO, USA).

Reverse transcriptase polymerase chain reaction

Total RNA was extracted with 1 mL of TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol, and RNA concentration was determined by spectrophotometry. Three micrograms of total RNA sample was used to prepare cDNA. The polymerase chain reaction (PCR) amplification cycles were carried out using primer sequences (Sangon, Shanghai, China) as follows: HSD11B1 (HSD11B1; NM_017080), sense primer 5′-GCAGAGCGATTTGTTGTT-3′ and antisense primer 5′-TGTCTATGAAGCCGAGGA-3′ (364-bp product); β-actin (Actb; NM_031144), sense primer 5′-CTATCGGCAATGAGCGGTTC-3′ and antisense primer 5′-CTTAGGAGTTGGGGGTGGCT-3′ (762-bp product). The PCR conditions used for all the reactions were as follows: 96°C, 3 min, for initial denaturation; 95°C, 10 s; 55°C, 30 s; and 72°C 1 min for cycle amplification (30 cycles); and 72°C, 6 min for final extension. Aliquots of the PCR products were separated by agarose gel electrophoresis and visualized under ultraviolet. The signal intensity was analyzed by computerized densitometry and assessed after normalization using β-actin as an internal standard.

Statistical analysis

Results are presented as means ± SEM. Statistical analyses were performed to assess the differences among the groups at the same sampling points using two-tailed unpaired Student's *t*-test with SPSS 11.5 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Energy intake

The energy intake standardized to body weight in PE rats was significantly higher three days before and one day after the earthquake compared with NC rats (Figure 2).

Overall changes in body weight, Lee index and body fat percentage

Since fat accumulation affects insulin sensitivity adversely, the body weight, Lee index and body fat percentage of these animals were examined in our study. However, we found that there was no significant difference ($P > 0.05$) between the groups of PE and NC for body weight (Figure 3a), Lee index (Figure 3b), and visceral and subcutaneous body fat percentage (Figures 3c and 3d).

Systemic and peripheral (SkM and AT) insulin sensitivity

The fasting blood glucose and fasting plasma insulin were notably increased ($P < 0.05$) and the average GIR_{60-120} at hyperinsulinemic–euglycemic clamp was decreased ($P < 0.05$) in the PE group compared with NC (Figure 4a–c). We further found that the insulin-mediated 2-DG uptake (Figure 4d and 4e) and Ser^{473} phosphorylation of PKB (Figure 4f–h) in SkM and AT in PE rats were significantly lower than that in NC ($P < 0.05$).

Plasma corticosterone level

The plasma corticosterone level was used as a marker of systemic stress. Our data indicate that the plasma corticosterone concentration was markedly elevated ($P < 0.05$) in PE (Figure 5a) compared with the NC group.

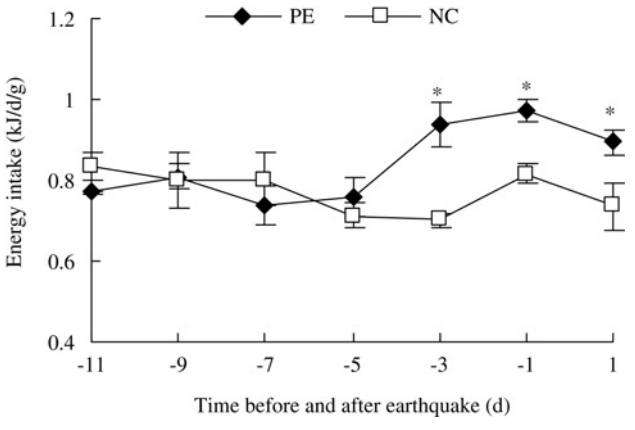

Figure 2 Increased energy intake (standardized to body weight) in pre-earthquake (PE) rats. Significant difference was observed between PE and control (NC) rats three days before and one day after the Wenchuan earthquake, but not 4–11 days before. *$P < 0.05$ versus NC group

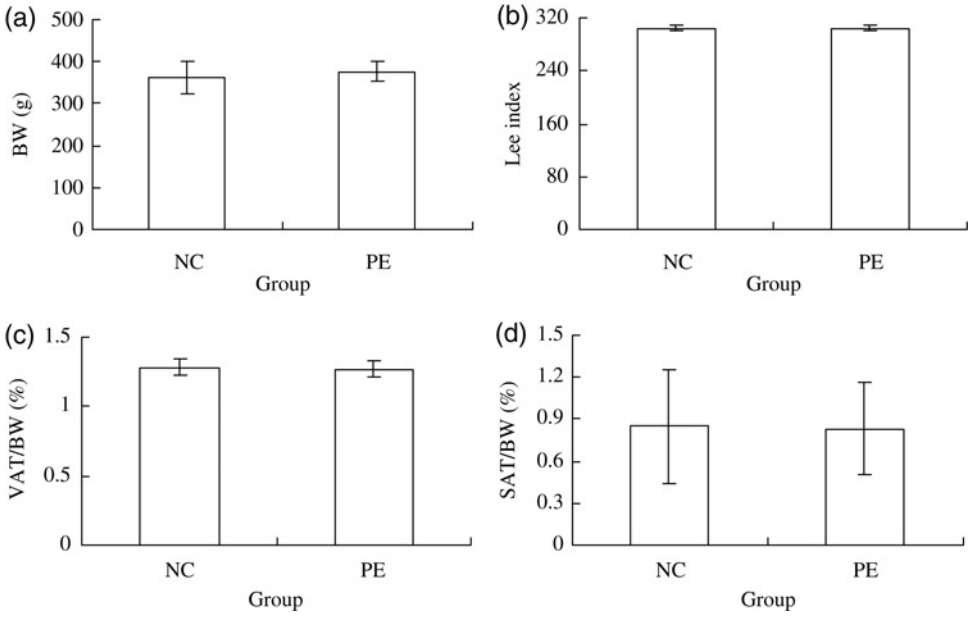

Figure 3 No significant differences were observed in body weight (a); Lee index (b); or body fat percentage (c, d) between pre-earthquake (PE) rats and their controls. VAT/BW, body fat percentage of visceral adipose tissues; SAT/BW, body fat percentage of subcutaneous adipose tissues

Alteration of HSD11B1 in mRNA expression level in SkM and AT

The mRNA expression level of HSD11B1 in SkM and AT was dramatically up-regulated ($P < 0.05$) in the PE group compared with that in NC (Figure 5b–d).

Discussion

In this study we observed that in the rats from the experiment carried out at the end of April 2008 (2 weeks before the earthquake), which served as our control, the energy intake remained constant and there was no IR or increase in stress level noted. On the other hand, the rats in the PE period displayed increased energy intake, severe systemic IR, defects in insulin signaling and glucose uptake in SkM and AT. These metabolic changes were associated with increased plasma corticosterone, and elevated HSD11B1 expression level in muscle and fat.

Studies have shown that a relationship exists between glucocorticoids and calorie intake. It is reported that glucocorticoids increase food intake in humans and animal models.[13] Hence, the increased energy intake in PE rats could be attributed to elevated plasma corticosterone. Nevertheless, the short-term increase in energy intake by itself would not result in drastic IR, especially since the same normal chow was used in both experiments as evidenced by the absence of any difference in body fat and weight. Peripheral IR in SkM and AT plays an important role in the development of systemic IR[8] and is characterized by reduced glucose uptake and inhibition of insulin-mediated Ser^{473} phosphorylation of PKB in peripheral tissues.[8] Our study has shown that GIR_{60-120}, as well as glucose uptake and insulin-mediated Ser^{473} phosphorylation of PKB, all decreased significantly in PE rats, confirming the presence of peripheral and systemic IR. IR can be caused by factors such as obesity, starvation, sepsis, cancer cachexia, burn trauma, pregnancy, acromegaly, etc.[14] However, none of these factors was involved in our experiment. Our data did not indicate any significant differences between PE and NC groups in the degree of obesity (Figure 3). Thus, obesity never seems to play an important role in the formation of IR in PE rats. In contrast, stress, another important causative factor in the etiology of IR,[15] has been observed in organisms after an earthquake.[16] Glucocorticoid hormone elevates HSD11B1 activity[17] in SkM and HSD11B1 activity increases in SkM during abdominal surgery as part of the physiological stressor response.[18] In this study, we showed that the plasma corticosterone level significantly increased in PE rats, which is associated with up-regulation of HSD11B1 expression in SkM and AT. Since HSD11B1 mRNA expression closely and positively correlates with its activity,[6] it is highly probable that the HSD11B1 activity is elevated in SkM and AT in the PE rats, although actual levels of the enzyme were not measured. In addition, HSD11B1 is elevated in AT in insulin-resistant humans and rodents, and transgenic mice with overexpression of HSD11B1 in AT develop obvious IR.[19] Therefore, from the findings of our experiment we can postulate that an increase in systemic and local glucocorticoid levels results in drastic IR.

What is the probable mechanism of increased systemic and local stress in these PE animals? A number of studies have illustrated an increase in the cortisol level in earthquake survivors and most of these data are obtained from human beings diagnosed with post-traumatic stress disorder due to the experience of trauma in the disaster.[20] We found that all rats in the PE period never underwent any visible trauma. Thus, the mechanism of the elevated stress level in this present study might be different from that in the post-earthquake organisms. Many reports on possible seismic precursors suggest that environmental factors such as tilt, humidity, electric and magnetic signals

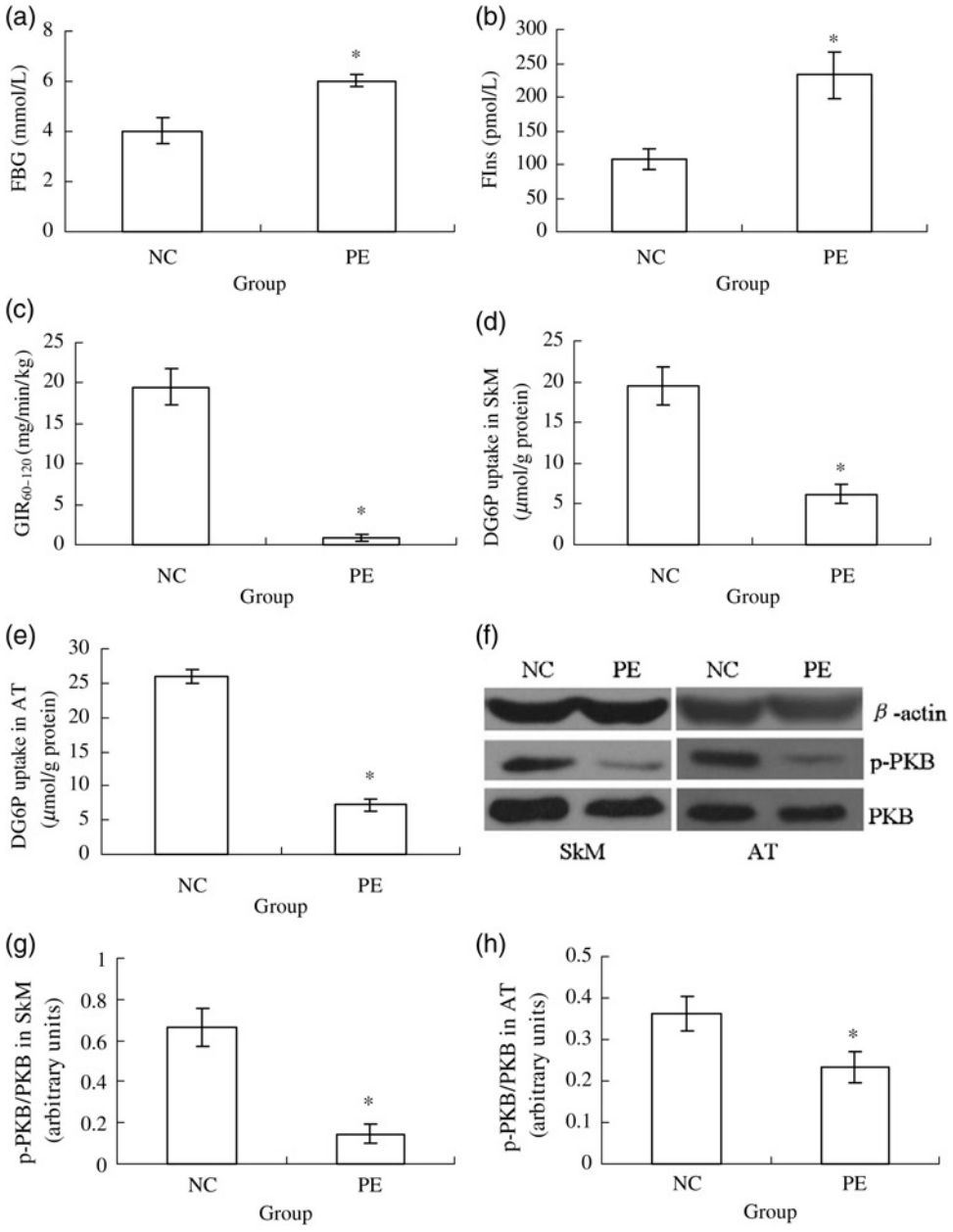

Figure 4 Altered systemic and peripheral (SkM and AT) insulin sensitivity in pre-earthquake (PE) animals. Compared with the NC group, the FIns (a) and FBG (b) were significantly elevated ($P < 0.05$), while the average GIR_{60-120} at hyperinsulinemic–euglycemic clamp (c), insulin-mediated 2-DG uptake (d, e) and Ser^{473} phosphorylation of PKB (f–h) in SkM and AT were notably decreased in PE rats. FIns, fasting plasma insulin; FBG, fasting blood glucose; SKM, skeletal muscle; AT, adipose tissue; 2-DG, 2-deoxy-D-glucose; PKB, protein kinase B; GIR_{60-120}, average glucose infusion rate between the 60th and 120th min. $^{*}P < 0.05$ versus NC group

could be linked to aberrant behavior in animals.[21] Indeed, anomalous electromagnetic signals have been repeatedly observed before big earthquakes and weak electromagnetic fields can produce biological effects.[22] Studies have observed that the excretion rates of cortisol significantly increased in operators working under radiofrequency electromagnetic waves.[23] Additionally, weak electromagnetic fields can elicit significant stress response in human cells.[24] Hence, electromagnetic signals could be an important causative factor in the increase in the glucocorticoid level in PE rats. Seismic electric signals can also be recorded at sensitive sites which are a hundred or more kilometers from the epicenter,[25] and the cortisol concentrations have been found to increase in cows exposed to electric shock.[26] Therefore, the results of our research to some extent might be attributed to the PE electric signals. Unfortunately, for reasons independent of our will, we have not been able to obtain an official record of the extent of the seismic electrical activity in Wuhan. As for tilt and humidity, all rats were housed in our Laboratory Animal Center without observable inclination change in the PE period and subjected to controlled relative humidity before the seism occurred. We can reasonably presume that tilt and humidity are not related to the increased stress level in our PE animals, although both of these factors can cause an elevation in the glucocorticoid level under certain conditions in organisms.[27,28]

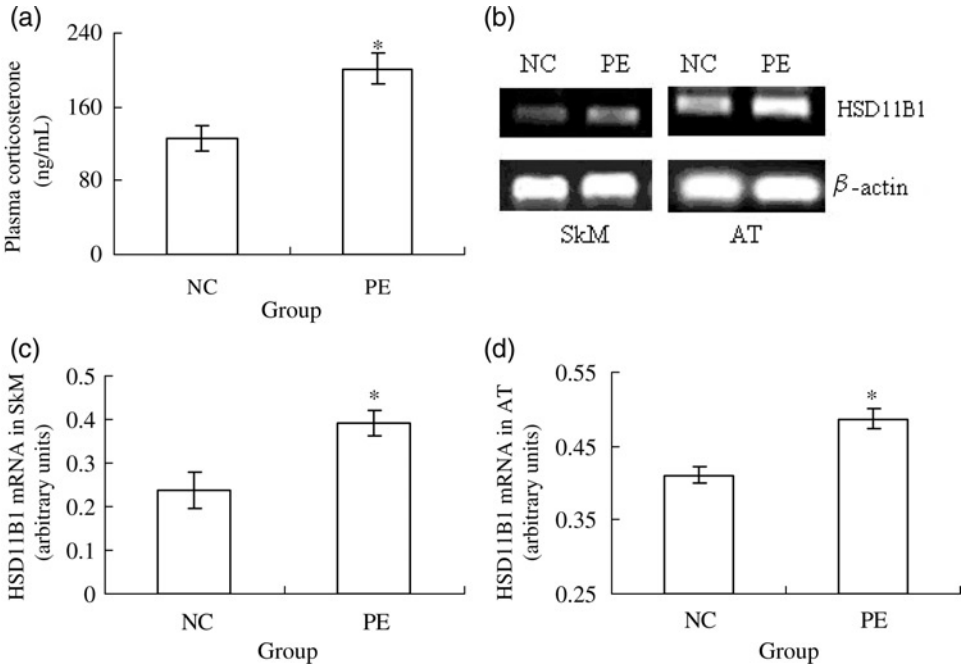

Figure 5 Changes in plasma corticosterone and HSD11B1 mRNA expression level in SkM and AT. The plasma corticosterone concentration (a) and HSD11B1 expression (b–d) in SkM and AT were significantly up-regulated in PE rats compared with that in NC. $^{*}P < 0.05$ versus NC group. SKM, skeletal muscle; AT, adipose tissue; HSD11B1, hydroxysteroid dehydrogenase type 1; PE, pre-earthquake

Yet, it should be noted that this study was not predesigned but was rather adjusted from an ongoing experiment after an unexpected discovery. We have examined only these hormonal and metabolic changes, and the proposed causal link could not be definitely tested in this investigation. Moreover, we were not able to determine the activity of HSD11B1 in time, unfortunately, for practical reasons involving the unavailability of radioisotope agents, although its mRNA expression closely and positively correlates with its activity. Although the earthquake was the only variable factor observed in our experiment, there could also have been other factors or events involved that precipitated the stress response in rats, which we were not able to observe. We also could not repeat the experiment using an earthquake model due to obvious practical limitations. We therefore could not determine whether similar results would be obtained in a controlled setting. Concerning earthquake prediction, one may be tempted from the results of our experiment to point out that it is possible to foresee earthquakes through a rise in stress level and IR. However, we should exercise utmost caution and restraint in that respect as an increase in insulin and corticosteroid levels occurs in a wide variety of settings, and therefore cannot be used to specifically predict earthquakes. As explained before, the epicenter of the earthquake was situated 1000 km west of the city of Wuhan, which is relatively far. Even then, the rats showed PE physiological changes, which is probably indicative of a high degree of sensitivity, although the strong intensity of the earthquake would also probably act as a contributing factor. However, the great distance also diminishes the specificity of the physiological changes observed, whereby factors other than the earthquake could have caused these changes. Nevertheless, the physiological changes may provide important clues as to the mechanisms involved in earthquake occurrence, so that in the future we may be able to predict earthquakes accurately.

Notwithstanding these limitations, we tentatively put forward the hypothesis that increased level of stress in the PE period could result in impaired systemic and peripheral (SkM and AT) insulin sensitivity and increase in food intake. We also presume that the stress could be due to PE physiological changes, implying that the rats might have been able to sense the coming of the earthquake through mechanisms that could not be fully explained by us. Therefore, further studies are still necessary to verify this hypothesis conclusively.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; XH, JZ, H-HZ, WK, W-HY, T-SZ, J-YZ, and LY conducted the experiments; L-LC, XH and JZ wrote the manuscript.

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