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ORIGINAL ARTICLE

Spexin protects cardiomyocytes from hypoxia-induced metabolic and mitochondrial dysfunction

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Abstract

Spexin (SPX) is a novel peptide with pleiotropic functions in adipose tissue including energy balance adjustment, fatty acid uptake, and glucose homeostasis. SPX level is closely associated with cardiovascular risk factors such as age, obesity, hypertension, and diabetes; however, its physiological significance in the cardiovascular system remains mostly undefined. We therefore here investigated the roles of SPX in regulating hypoxia-induced alterations in energy metabolism and mitochondrial function. We firstly confirmed that SPX is expressed in human and mouse cardiac tissue and documented that exposure to hypoxia in vitro reduces SPX level in rat H9C2 cardiomyocytes and primary neonatal rat ventricular myocytes (NRVMs). We then treated primary NRVMs with SPX before exposure to hypoxia, which (1) promoted fatty acid metabolism by enhancing expression of FAT/CD36, CPT1, ACADM, and PPAR-a and PGC1-a; (2) did not improve impaired glucose uptake; and (3) significantly prevented the downregulation of TFAM and mitochondrial electron transport chain complex and restrained UCP2 level and reactive oxygen species (ROS) production, thus enhancing ATP level in cardiomyocytes. In summary, SPX protects energy and mitochondrial homeostasis of cardiomyocytes during hypoxia, thereby highlighting the potential importance of SPX in the treatment of cardiovascular diseases.

Keywords Spexin · Hypoxia · Fatty acid metabolism · mitochondrial function

Introduction

Spexin (SPX), also known as neuropeptide Q, is a novel peptide identified by Markov modeling. The 14-amino acid long mature SPX peptide is encoded by the C12orf39 gene located in chromosome 12 of the human genome, which is evolutionarily conserved across vertebrate species as a member of the Spexin/Galanin/Kisspeptin gene family, which can activate galanin receptors type 2 (GALR2) and 3 (GALR3). SPX is widely expressed in central and peripheral tissues including brain, heart, lung, liver, kidney, thyroid, adrenal gland, testis, ovary, spleen, pancreas, skeletal muscle, adipose tissue, and stomach and different parts of the GI tract. SPX can be detected in systemic circulation and may serve as a neuroendocrine signal with pleiotropic functions. Walewski et al. reported that SPX decreased sharply in obese omental and subcutaneous adipose tissue as compared with that from healthy adults, and SPX level decreased in both obese adults and adolescents and was negatively associated with serum leptin level (Walewski et al. 2014; Kumar et al. 2018). Moreover, low SPX and high leptin levels positively correlated with higher serum hs-CRP level, which suggested a potential role for SPX level in risk stratification in cardiovascular diseases. Additionally, circulating SPX level was negatively correlated with levels of blood glucose, hemoglobin A1c, triglycerides, and LDL-C in type 2 diabetics; circulating levels of insulin and glucagon in obese women (Kolodziejski et al. 2018); and age, BMI, fasting glucose, and triglycerides in healthy women

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(Lin et al. 2018a), suggesting that SPX might regulate metabolic disorders. To this end, SPX has been shown to decrease fatty acid uptake in adipocytes and hepatocytes, increase pancreatic islets cell proliferation, and enhance bowel movement, thus restraining caloric intake and body weight (Wong et al. 2013; Walewski et al. 2014; Lin et al. 2015; Ma et al. 2017; Zheng et al. 2017; Kolodziejski et al. 2018; Sassek et al. 2018). SPX also mediates arterial blood pressure, salt and water balance, nociceptive responses, and reproductive functions (Toll et al. 2012; Liu et al. 2013). Therefore, a growing body of evidence indicates that SPX might regulate body energy metabolism and homeostasis. In addition, Porzionato et al. (Porzionato et al. 2012) suggested the possible role of SPX in the regulation of hyperoxia-induced plasticity of the carotid body. However, little is known about the role of SPX in cardiac energy metabolism.

Oxygen delivery reduction can lead to tissue hypoxia, and thus affect many cardiac pathophysiological processes, which could lead to cardiovascular disorders including ischemic disease, myocardial infarction, and heart failure (Abe et al. 2017). Chronic exposure to hypoxia may induce cardiac remodeling. Hypoxia can impair glucose and fatty acid utilization and might contribute to an increased risk of cardiac dysfunction (Azzouzi et al. 2015). Because SPX participates in cell fatty acid and glucose metabolism, the present study investigates the effects of SPX on metabolic and mitochondrial dysfunction of cardiomyocytes under hypoxic conditions.

Materials and methods

Drugs and reagents

Spexin and M871 (catalog numbers 6090 and 2698) were obtained from Tocris Cookson Inc., USA. Antibodies: **a**nti-GALR2 (ab203072, 1:1000), anti-CD36 (ab64014, 1:1000), anti-PGC1α (ab54481, 1:1000), anti-PPARα (ab215270, 1:500), anti-ACADM (ab92461, 1:1000), anti-GLUT4 (ab654, 1:1000), anti-TFAM (ab131607, 1:1000), anti-UCP2 (ab203224, 1:500), and total OXPHOS Rodent WB Antibody Cocktail (ab110413, 1:1000) were purchased from Abcam, USA. Anti-CPT1 (15184-1-AP, 1:1000), anti-PDK4 (12949–1-AP, 1:500), and anti-PDHE1α (18068-1-AP, 1:500) were from Proteintech, China. Anti-GAPDH (bs-0755R, 1:1000), anti-β-actin (bsm-33139M, 1:1000), and horseradish peroxidase-conjugated goat anti-rabbit or mouse anti-rabbit IgG (bs-0295G and bs-0296G, 1:2000) were from Bioss, China. MitoSOX™ Red Mitochondrial Superoxide Indicator (M36008) was from Invitrogen, USA. RIPA (P0013B) and BCA (P0012S) protein assay kit were purchased from Beyotime Biotechnology, China.

Human atrial tissues

All patients recruited into the study signed written informed consent and the study complied with the principles that govern the use of human tissues outlined in the Declaration of Helsinki. Left atrial appendages were obtained as surgical specimens from patients undergoing cardiac surgery for mitral valve replacement, following established procedures approved by Ethic Committee of the Harbin Medical University. The specimens were stored in liquid nitrogen immediately and transported to laboratory.

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded cardiac tissue sections. Slices were incubated with anti-Spexin (H-023-81, Phoenix Pharmaceuticals, Germany, 1:400) overnight at 4 °C. The slides were incubated with peroxidase-conjugated goat anti-rabbit IgG (ZDR-5306, Zhongshan, China, 1:100) at 37 °C for 20 min.

Isolation and culture of cardiomyocytes

Primary neonatal rat ventricular myocytes (NRVMs) were prepared as previously described (Yuan et al. 2017). Briefly, neonatal Sprague-Dawley rats (postnatal days 1 to 3) were euthanized and the hearts were excised and cut into small pieces. The tissue was digested with collagenase type II (17101–015, Gibco, USA) at 37 °C, and the supernatant fluid was collected. All suspensions then were pelleted by centrifugation at 1000 rpm for 5 min and resuspended to dishes at 37 °C in a 5% CO_2 incubator. After allowing for adherence to cardiac fibroblasts for 1.5 h, non-adherent cardiac myocytes were removed by changing the culture medium. Cardiac myocytes were re-seeded on 6-well plates and cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. After 48 h, the culture medium was replaced with complete medium and subsequently subjected to different treatments.

Rat cardiomyocyte line H9C2 cells were purchased from the American Type Culture Collection (ATCC, USA) and were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Plasmid construction and cell transfection

The SPX overexpression plasmid and negative control vector were synthesized by GeneChem (Shanghai, China). The plasmid preparation, purification, and quality control analyses from transformed *Escherichia coli* cultures were performed according to standard protocol.

The H9C2 cell line was transfected with the plasmid using Lipofectamine 2000 reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The transfection efficiency was assessed by detecting green fluorescent protein (GFP) and polymerase chain reaction analysis. After 48 h transfection, cardiomyocytes were used in subsequent experiments.

Hypoxic treatment

Cells were pretreated with SPX (1000 nM) and M871 (100 nM) respectively for 2 h. For hypoxia stimulation, NRVMs and H9C2 cells were incubated in hypoxic incubator containing 94% N_2, 5% CO_2, and 1% O_2 for 24 h. The control cells were exposed to 24 h of normoxia.

Mitochondrial ROS detection

MitoSOX-red is a fluorogenic indicator of superoxide generated specifically from mitochondria. Mitochondrial reactive oxygen species (ROS) level in cardiomyocytes was determined according to the manufacturer's standard procedure. Briefly, cells were washed with warm PBS and stained with 5 μM MitoSOX reagent working solution for 10 min at 37 °C, protected from light. After washing gently with warm PBS, cells were visualized using a Zeiss fluorescence microscope (ZEISS, Germany).

Determination of adenine triphosphate content

The NRVMs adenosine triphosphate (ATP) concentration was measured using an ATP assay kit according to the manufacturer's instructions (A095-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Sun et al. 2017). To correct for any variability in the number of cells, measurements were normalized to the total protein amount detected by a bicinchoninic acid (BCA) assay.

Western blot

Total protein was extracted from cardiomyocytes for western blot analysis using RIPA buffer containing 1% protease inhibitor as described previously (Yuan et al. 2018). Briefly, the samples were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by 5% nonfat dry milk in PBS and incubated overnight at 4 °C with different primary antibodies. After washing on the following day, the membranes were incubated with secondary antibody for 1 h. The images were captured on a ChemiDoc XRS gel documentation system (Bio-Rad, CA). Band intensity was quantified by Image Lab software.

Quantitative reverse-transcription polymerase chain reaction

Total RNAs were isolated with reagent (Axygen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed with the kit (TOYOBO, Japan). qRT-PCR assay (SYBR Green Assay, Roche, Switzerland) was performed on Applied Biosystem. The relative expression levels of mRNAs were calculated and quantified using the $2^{-\Delta\Delta CT}$ method after normalization to GAPDH levels. The primers used were listed: Spx-forward (5′ to 3′) CTGGTGCTGTCTGTTCTG; Spx-reverse (5′ to 3′) TTGGGTTTCGTCTTTCTGG.

Statistical analysis

All data are presented as mean ± SD and were analyzed using GraphPad Prism 5.0 software. Comparison between two groups was conducted by Students' *t* test. Multiple-group comparisons were carried out using one-way ANOVA followed by Tukey's tests. $P < 0.05$ was considered to be statistically significant.

Results

Hypoxia decreased SPX expression in cardiomyocytes

To evaluate whether SPX was expressed in cardiomyocytes, we detected cytoplasmic immunostaining in human and mouse cardiac tissue (Fig. 1), which had been documented by Porzionato et al. in rat heart tissue (Porzionato et al. 2010). To explore the role of SPX in cardiomyocytes, first, we examined whether the expression of SPX was affected by hypoxia treatment. H9C2 cells were transfected with plasmids overexpressing SPX, and decreased fluorescence intensity was observed in hypoxia conditioned cells (Fig. 2a). which indicted SPX was consumed under hypoxia. On this basis, we detected mRNA expression of SPX in NRVMs by qRT-PCR. Similarly, reduced SPX expression was confirmed in hypoxia-conditioned NRVMs compared with control group (Fig. 2b). These results come to the conclusion that hypoxic stimulus could deplete intracellular SPX content. Meanwhile, the protein level of HIF1-a in NRVMs was detected to ensure the reliability of hypoxia stimulation (Fig. 2c). In addition, surface expression of GALR2 in hypoxia cells was less than that in normal condition (Fig. 2d), while that of GALR3 was unchanged (data not shown).

Effects of SPX on cardiomyocytes fatty acid and glucose metabolism

SPX had been shown to stimulate lipolysis and to inhibit lipogenesis in adipocytes in vitro. In our study, we firstly

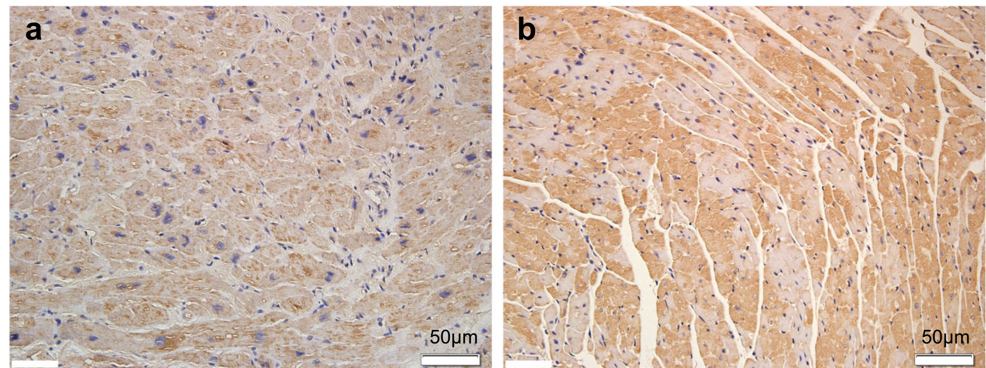

Fig. 1 SPX is expressed in cardiomyocytes. **a**, **b** Immunohistochemical staining of SPX in human and mouse cardiac tissue. Magnification: × 400, scale bar 50 μm

detected expression of fatty acid translocase, FAT/CD36, which was decreased during hypoxia and ameliorated by treatment with SPX (Fig. 3a, b). Furthermore, SPX reversed downregulation of CPT1, ACADM, PPAR-a, and PGC1-a induced by hypoxia (Fig. 3a, b). To validate the role of GALR2 in conferring the effects of SPX on fatty acid metabolism, we used the specific antagonist M871 for GALR2 inhibition and showed that it partially neutralized the effect of SPX on fatty acid metabolism during hypoxia but displayed no significant effect in normal conditions (Fig. 3a, b). As the major glucose transporter in the myocardium, GLUT4, consequently decreased significantly under hypoxia leading to a decrease in glucose uptake. However, SPX had little influence on glucose uptake or on the key regulators of glucose oxidation PDK4

Fig. 2 Hypoxia exposure decreased expression of SPX. **a** Fluorescence intensity detection in H9C2 cells transfected with SPX overexpressed plasmid and negative control vector. Magnification: × 100, scale bar: 200 μm and magnification: × 200, scale bar: 200 μm. **b** Relative mRNA expression of SPX in NRVMs detected by qRT-PCR. **c**, **d** Western blot detection of HIF1-a and GALR2 in NRVMs. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control group, $n = 5$ per group

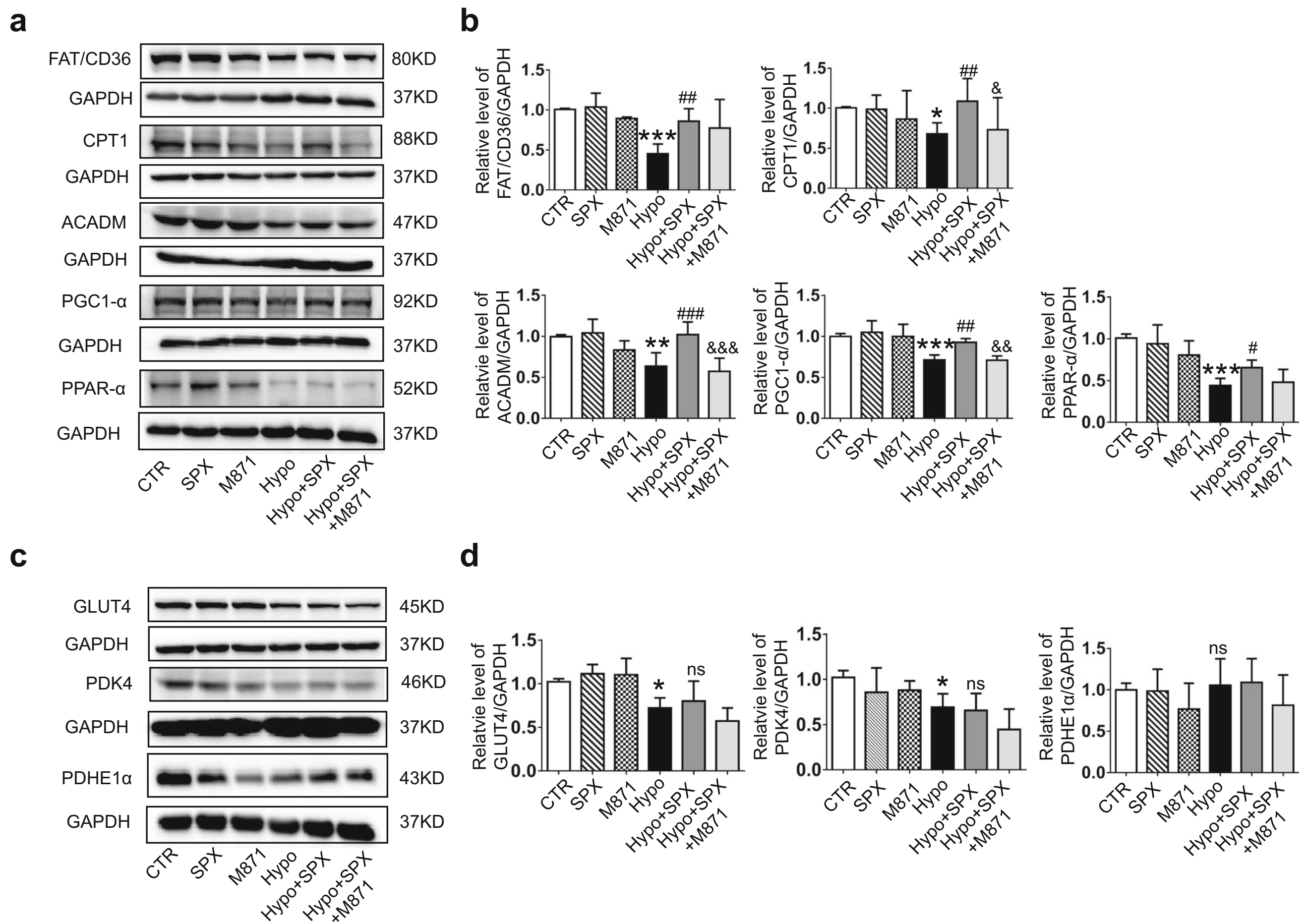

Fig. 3 Effects of SPX on fatty acid and glucose metabolism in hypoxic NRVMs. **a**, **b** Representative bands and statistical results for the protein expression of FAT/CD36, CPT1, ACADM, PPAR-a, and PGC1-a. **c**, **d** Representative immunoblots and results for the protein expression of GLUT4, PDK4, PDH. The expression levels of these proteins were normalized to that of GAPDH. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. CTR group, #$P < 0.05$; ##$P < 0.01$; ###$P < 0.001$ vs. hypoxia group, &$P < 0.05$; &&$P < 0.01$; &&&$P < 0.001$ vs. hypoxia+SPX group, $n = 5$ per group

and PDHE1α (Fig. 3c, d). Taken together, SPX was involved in fatty acid but not glucose metabolism during hypoxia.

SPX protected mitochondrial function during hypoxia

Previous studies have implicated mitochondrial dysfunction in heart diseases as reflected by decreased ATP levels. As illustrated in Fig. 4a, normoxia groups presented regular mitochondrial structure under transmission electron microscopy visualization, whereas many swollen mitochondria were detected in cardiac myocytes under hypoxia. SPX treatment protected NRVMs from severe mitochondrial injury induced by hypoxia. Hence, SPX prevented the decrease of ATP caused by hypoxia exposure (Fig. 4b), which was contrary to the expression of mitochondrial uncoupling proteins 2 (UCP2, Fig. 4c). Mitochondrial transcription factor A (TFAM) and mitochondrial electron transport chain (mtETC) complexes were examined, and this study confirmed that SPX restored the protein expression level of TFAM, complex I (C I, NDUFB8), complex II (C II, SDHB), complex III (C III, UQCRC2), and complex V (C V, ATP5A) that had been reduced by hypoxia, which was attenuated by adding M871 to the culture medium (Fig. 4d, e). Taken together, hypoxia stimulation caused energy deficiency and mitochondrial biogenesis dysfunction in cardiomyocytes, and SPX recovered energy storage.

SPX reduced mitochondrial ROS caused by hypoxia

Mitochondrial ROS production was measured by MitoSOX-red staining. The fluorescence intensity in NRVMs from hypoxia group was obviously enhanced, and SPX therapy reduced ROS generation (Fig. 5). Furthermore, adding M871 to the condition medium neutralized the aforementioned effect of SPX, indicating that SPX prevented hypoxia-induced damage to the respiratory chain in cardiomyocytes.

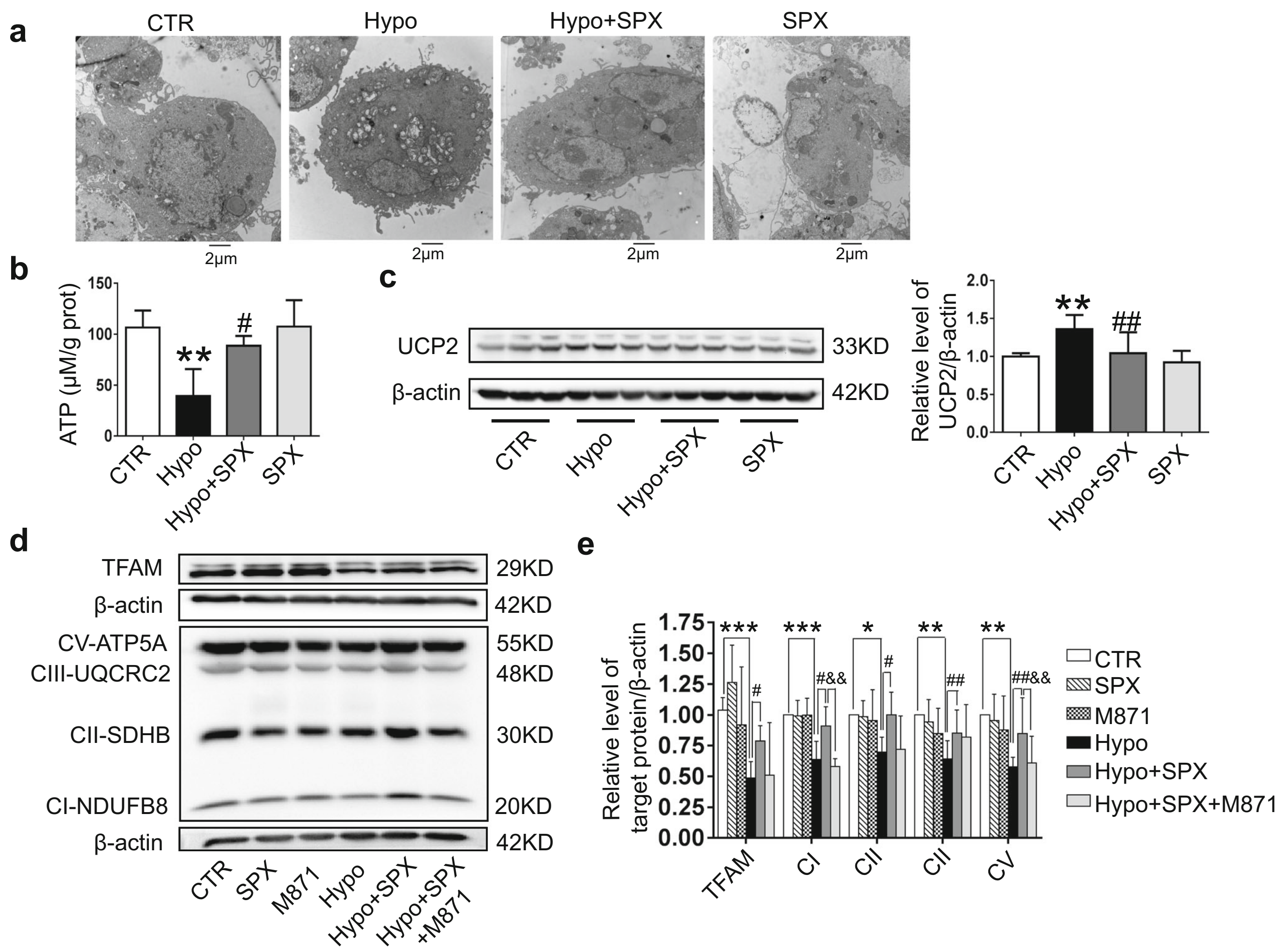

Fig. 4 SPX protected mitochondrial function against hypoxia exposure in NRVMs. **a** Transmission electron microscope images of control and hypoxia groups with or without SPX. Magnification: × 8000× (scale bar 2 μm). **b** The concentration of ATP was measured using an ATP assay. The data are presented as the mean ± SD, $^{**}P < 0.01$ vs. CTR group, $^{\#}P < 0.05$ vs. hypo group, $n = 4$ per group. **c** Western blot results for the expression of UCP2 in control and hypoxia groups with or without SPX. **d**, **e** Representative bands and statistical results for the protein expression of TFAM and mitochondrial respiratory chain complexes (NDUFB8, SDHB, UQCRC2, and ATP5A) in control and hypoxia groups with or without SPX or M871. The expression levels of these proteins were normalized to that of β-actin. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ vs. CTR group, $\#P < 0.05$; $\#\#P < 0.01$, vs. hypoxia group, $\&\&P < 0.01$ vs. hypoxia + SPX group, $n = 5$ per group

Discussion

The most frequent cardiovascular diseases undoubtedly include hypoxic states. While there has been extensive research on the effect of hypoxia on the myocardium, much less is known about the management of energy metabolism disorders caused by hypoxia. The present study is the first to our knowledge to demonstrate that SPX ameliorated hypoxia-induced energy metabolism and mitochondrial dysfunction in cardiomyocytes by the following potential mechanisms which might be partially mediated by activating GALR2: (1) facilitating fatty acid uptake, transport, and oxidation by increasing content of CD36, CPT1, ACADM, PPAR-a, and PGC1-a; (2) upregulating protein expression of TFAM, mitochondrial electron transport chain complexes, and downregulating UCP2 level thereby enhancing ATP production; and (3) attenuating mitochondrial ROS generation.

SPX is widely distributed in different tissues of fish and mammals (Porzionato et al. 2010; Wong et al. 2013), with pleiotropic functions via various receptors. SPX gene expression has been detected in the human systemic circulation, endocrine, and epithelial tissues, and it is modified by physiological status or hormonal signals such as obesity and insulin (Walewski et al. 2014; Gu et al. 2015). Hypoxia is a potential trigger for cardiac diseases such as ischemic heart disease, myocardial infarction, and heart failure. In our study, we detected decreased SPX expression in H9C2 and NRVM cardiomyocytes induced by pathological stimulus of hypoxia (Fig. 2a, b). The level of intracellular SPX decreased sharply under hypoxia, which indicated SPX was consumed under

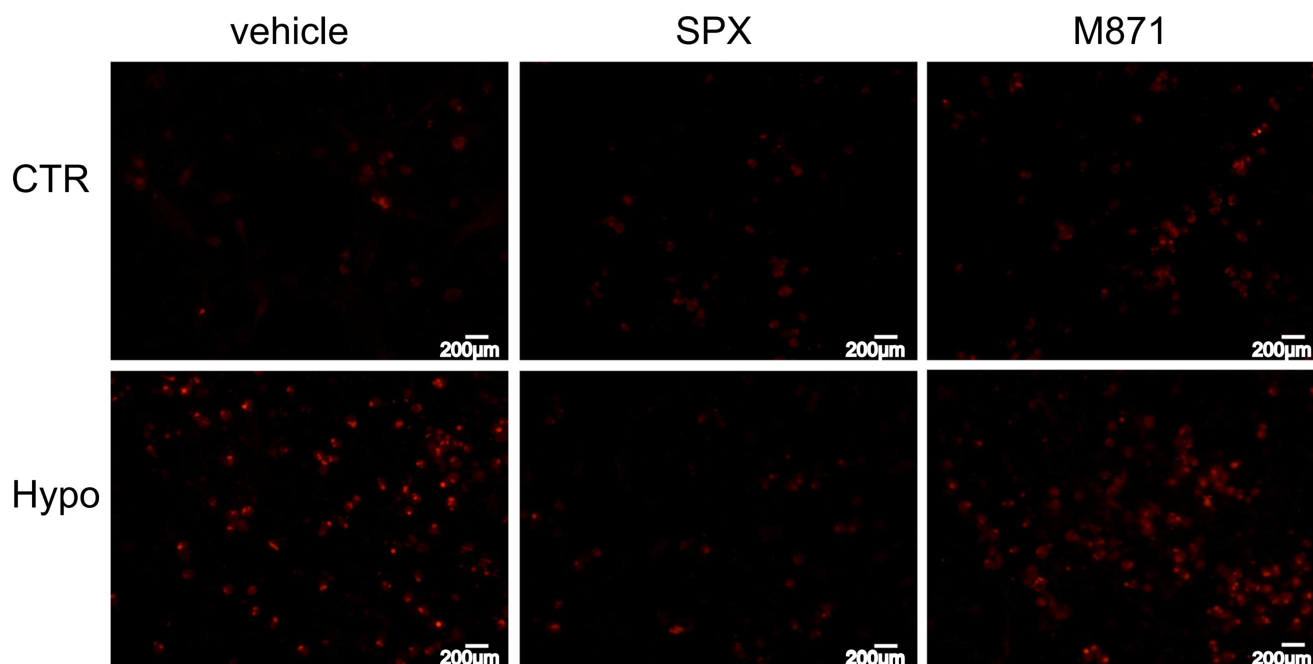

Fig. 5 SPX reduced mitochondrial ROS generation induced by hypoxia. Representative images show level of mitochondrial ROS in NRVMs from control and hypoxia groups with or without SPX or M871. Magnification × 200, scale bar 200 μm

hypoxia. Interestingly, Porzionato et al. found that after exposure to hyperoxia, SPX expression was increased in peripheral chemoreception suggesting SPX was sensitive to oxygen concentration (Porzionato et al. 2012). Given that both glucose and fatty acid metabolic disorders contribute to cardiac injury in hypoxia models (Tao et al. 2019), it will be essential to detect the regulatory effects of SPX on glucose and lipid metabolism. In our study, supplement with exogenous SPX protects cardiomyocytes from imbalance of energy homeostasis and mitochondrial dysfunction during hypoxia, forecasting that metabolic disturbance may partly be due to SPX consumption. Thereby, it highlights the potential importance of SPX as a diagnostic and therapeutic target in cardiovascular diseases especially under ischemia/hypoxia condition.

Under normal conditions, the healthy heart relies predominantly (60–90%) on fatty acid oxidation for energy production. Although uptake of fatty acids into cardiomyocytes can occur passively across cell membranes, it is primarily mediated by transporter mechanisms involving FAT/CD36 (Kuang et al. 2004). ACADM and CPT1 mediate the key step of fatty acid oxidation in mitochondria. Hypoxia-induced cardiac fatty acid metabolism remodeling is tightly regulated by PPAR-α, which plays a vital role in upregulating genes correlated with fatty acid uptake and oxidation including ACADM and CPT1. PGC1-α co-activated with PPAR-α, thus promoting fatty acid oxidation (Bertero and Maack 2018). Previous research demonstrated that SPX inhibited long-chain fatty acid uptake in adipocytes and hepatocytes thereafter reducing food intake and body weight (Walewski et al. 2014; Ge et al. 2016). Our study confirmed that SPX treatment significantly attenuated the hypoxia-induced downregulation of FAT/CD36, CPT1, ACADM, PPAR-α, and PGC1-α caused thus attenuating impaired fatty acid metabolism (Fig. 3). Furthermore, SPX stimulates glucose uptake in human adipocytes and murine 3T3-L1 cells (Kolodziejski et al. 2018). However, in the present study, SPX showed little effect on glucose uptake and utilization in cardiomyocytes, which might be explained by differences in the function of SPX on fatty acid and glucose metabolism in different tissues and physiological states.

Expression of HIF-1α, the major transcription factor that decreases mitochondria oxidative phosphorylation in adaptation to hypoxia, is significantly upregulated during hypoxia. As a part of repressed mitochondrial respiration, decreased mitochondrial biogenesis and electron transport chain complex remodeling is a well-established cellular adaptation to hypoxia (Das et al. 2012; Wenz 2013). As a major mitochondrial gene-regulator and a transcription factor, TFAM modifies gene expression and protects mitochondrial DNA from mutation (Kunkel et al. 2016; Kunkel et al. 2018). UCP2, as a member of UCPs, is strongly expressed in the heart, which protects against mitochondrial oxidative damage by reducing the production of ROS (Cadenas 2018). In the present study, hypoxia was accompanied by derangements in mitochondrial function, including reduced expression of TFAM and mitochondrial respiratory chain proteins and increased UCP2 expression leading to impaired ATP production and increased generation of ROS. However, SPX prevented mitochondrial dysfunction and excessive ROS production.

SPX activates GALR2 and GALR3 but not GALR1 (Walewski et al. 2014). Several studies demonstrated that GALR2 is linked to several metabolism pathways including

insulin, lipid, and bile acid synthesis and metabolism (Fang et al. 2018; Kolodziejski et al. 2018; Lin et al. 2018b). However, the impact of GALR2 on energy metabolism in cardiomyocytes has not been investigated. After hypoxia exposure, protein expression of GALR2 but not GALR3 was decreased (Fig. 2c). Furthermore, inhibition of GALR2, to some extent, blocked the SPX-mediated improvement of hypoxia-induced impaired fatty acid and mitochondrial metabolism in cardiomyocytes.

Conclusions

The present study documented that expression of SPX was decreased by hypoxia, and that pretreatment with SPX alleviated hypoxia-induced dysfunction of fatty acid metabolism and mitochondria possibly via GALR2 activation. These findings provide insight into cardiovascular disease pathophysiology and render SPX a potential therapeutic target.

Authors' contributions YL and LS conducted the experiments, analyzed the data, and supervised and wrote the manuscript; LQZ participated equally in the first authorship and conducted experiments, drafted the figure, and analyzed the data and co-wrote the manuscript; MQS, HL, YW, and YKW conducted the experiments and analyzed the data; DL, CGD and CYZ performed the experimental approaches and data to the work; YTG co-wrote the manuscript and revised it for critically important intellectual content; YL* helped to identify and formulate the research and interpreted the experiments. All the authors approved the final version of the manuscript.

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Compliance with ethical standards

All patients recruited into the study signed written informed consent and the study complied with the principles that govern the use of human tissues outlined in the Declaration of Helsinki. Left atrial appendages were obtained as surgical specimens from patients undergoing cardiac surgery for mitral valve replacement, following established procedures approved by Ethic Committee of the Harbin Medical University. The specimens were stored in liquid nitrogen immediately and transported to laboratory

Conflict of interest The authors declare that they have no conflicts of interest.

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