Redox Biology 15 (2018) 418–434

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

# Blocking mitochondrial cyclophilin D ameliorates TSH-impaired defensive barrier of artery

Xiaojing Liu[a,b,c], Heng Du[d], Qiang Chai[e], Qing jia[e], Lu Liu[a,b,c], Meng Zhao[a,b,c], Jun Li[f], Hui Tang[f], Wenbin Chen[g], Lifang Zhao[a,b,c], Li Fang[a,b,c], Ling Gao[c,g,*], Jiajun Zhao[a,b,c,**]

[a] Deparment of Endocrinology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, China
[b] Shandong Key Laboratory of Endocrinology and Lipid Metabolism, Jinan, Shandong 250021, China
[c] Institute of Endocrinology and Metabolism, Shandong Academy of Clinical Medicine, Jinan, Shandong 250021, China
[d] Department of Biological Sciences, The University of Texas at Dallas, Richardson, TX 75080, United States
[e] Department of Cardiovascular Disease, Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan, Shandong 250001, China
[f] Department of Pharmacy, Shandong Provincial Hospital affiliated to Shandong University, Jinan, Shandong 250021, China
[g] Scientific Center, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, China

ARTICLE INFO

*Keywords:*
Mitochondria
Endothelial cell
Oxidative stress
Cyclophilin D
Thyroid stimulating hormone

ABSTRACT

*Aims:* Endothelial cells (ECs) constitute the defensive barrier of vasculature, which maintains the vascular homeostasis. Mitochondrial oxidative stress (mitoOS) in ECs significantly affects the initiation and progression of vascular diseases. The higher serum thyroid stimulating hormone (TSH) level is being recognized as a non-conventional risk factor responsible for the increased risk of cardiovascular diseases in subclinical hypothyroidism (SCH). However, effects and underlying mechanisms of elevated TSH on ECs are still ambiguous. We sought to investigate whether cyclophilin D (CypD), emerging as a crucial mediator in mitoOS, regulates effects of TSH on ECs.

*Methods and results:* SCH patients with TSH > = 10 mIU/L showed a positive correlation between serum TSH and endothelin-1 levels. When TSH levels declined to normal in these subjects after levothyroxine therapy, serum endothelin-1 levels were significantly reduced. Supplemented with exogenous thyroxine to keep normal thyroid hormones, thyroid-specific TSH receptor (TSHR)-knockout mice with injection of exogenous TSH exhibited elevated serum TSH levels, significant endothelial oxidative injuries and disturbed endothelium-dependent vasodilation. However, *Tshr*$^{-/-}$ mice resisted to TSH-impaired vasotonia. We further confirmed that elevated TSH triggered excessive mitochondrial permeability transition pore (mPTP) opening and mitochondrial oxidative damages in mouse aorta, as well as in cultured ECs. Genetic or pharmacological inhibition of CypD (the key regulator for mPTP opening) attenuated TSH-induced mitochondrial oxidative damages and further rescued endothelial functions. Finally, we confirmed that elevated TSH could activate CypD by enhancing CypD acetylation via inhibiting adenosine monophosphate-activated protein kinase/sirtuin-3 signaling pathway in ECs.

*Conclusions:* These findings reveal that elevated TSH triggers mitochondrial perturbations in ECs and provide insights that blocking mitochondrial CypD enhances the defensive ability of ECs under TSH exposure.

## 1. Introduction

Endothelial cells (ECs) are in a dynamic equilibrium with their environments and constitute a defensive barrier in the vasculature, controlling vascular permeability, smooth muscle tone, inflammatory and immune responses, angiogenesis, and thromboresistance. In many vascular diseases, the endothelium is thus both origin and victim [1]. The interactions within the redox reactions are richly elaborated in an oxygen-dependent life and contribute to spatiotemporal organization for differentiation, development, and adaptation to the environment

*Abbreviations:* Ach, Acetylcholine; AMPK, Adenosine monophosphate-activated protein kinase; CsA, Cyclosporine A; CVD, Cardiovascular diseases; ECs, Endothelial cells; eNOS, Endothelial nitric oxide synthase; ET-1, Endothelin-1; HA-VSMC, Human aortic smooth muscle cell; HUVEC, Human umbilical vein endothelial cell; L-NAME, N-nitro-L-arginine methyl ester; mitoOS, Mitochondrial oxidative stress; mPTP, Mitochondrial permeability transition pore; OCR, Oxygen consumption rate; ROS, Reactive oxygen species; SIRT3, Sirtuin-3; SNP, Sodium nitroprusside; TSH, Thyroid stimulating hormone; TSHR, Thyroid stimulating hormone receptor

* Corresponding author at: Institute of Endocrinology and Metabolism, Shandong Academy of Clinical Medicine, 544, Jing 4 Rd., Jinan, Shandong 250021, China.
** Correspondence to: Deparment of Endocrinology, Shandong Provincial Hospital Affiliated to Shandong University, 324, Jing 5 Rd., Jinan, Shandong 250021, China.
*E-mail addresses:* gaoling1@medmail.com.cn (L. Gao), jjzhao@medmail.com.cn (J. Zhao).

https://doi.org/10.1016/j.redox.2018.01.004
Received 5 December 2017; Received in revised form 5 January 2018; Accepted 7 January 2018
Available online 09 January 2018

[2]. Disruption of the balance between oxidants and anti- oxidants in favor of the oxidants leads to the occurrence of oxidative stress [3], in which the damaging effects of reactive oxygen species (ROS) exceed the ability of biological systems to neutralize the oxidizing agents and to repair cellular damage [4]. Studies in human subjects and animals have demonstrated that oxidative stress and the associated endothelial dysfunction significantly correlate to the classic cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus and chronic smoking, and also play important roles in the development of vasculopathies, including atherosclerosis, hypertension, restenosis after angioplasty, angiogenesis, cardiac injury associated with post-ischemic reperfusion and heart failure [4–8].

Subclinical hypothyroidism (SCH) is characterized by elevated serum thyroid stimulating hormone (TSH) levels with normal serum thyroid hormone concentrations [9,10]. It is a common thyroid disease with a prevalence ranging from 4% to 20% in adults, and the prevalence is progressively increasing [11]. Although defined as an asymptomatic state, SCH is proposed to lead adverse consequences including systemic hypothyroid symptoms, neuromuscular dysfunction, progression to overt hypothyroidism, hypercholesterolemia and cardiovascular dysfunction [12–14]. The higher serum TSH level has been recognized as a nonconventional risk factor responsible for the increased risk of cardiovascular diseases (CVD) in SCH [15]. Although with strong positive relationship to increased markers of oxidative stress [16] and endothelial dysfunction [17] in serum, the effects and underlying mechanisms of elevated TSH on ECs still remain to be established.

Although with lower content, mitochondria are crucial in maintaining redox homeostasis and cellular functions of ECs [18]. Imbalance of redox state in mitochondria leads to excessive generation of mitochondrial ROS, which serve as kindling radicals to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, xanthine oxido-reductase and uncoupled endothelial nitric oxide synthase (eNOS) [19], with endothelial dysfunction as a result [20].

The mitochondrial permeability transition pore (mPTP) is a key regulator of mitochondrial homeostasis. Transient opening of the pore is important for release of ions and metabolites to maintain mitochondrial health [21]. However, under extreme stress, excessive activation of mPTP can be triggered to aggravate mitochondrial degeneration, leading to matrix expansion and mitochondrial membrane rupture, which dissipates the mitochondrial membrane potential, deregulates $Ca^{2+}$ homeostasis, generates and releases pathological mitochondrial ROS [22]. Cyclophilin D (CypD), a protein with peptidyl-prolyl cis-trans isomerase (PPIase) activity, is a critical regulator of the mPTP opening. It resides in the mitochondrial matrix in resting state. Once activated, CypD translocates to the mitochondrial inner membrane and binds to the mPTP constitutes, for instance ANT [23] or $F_1F_0$ ATP synthase [24–26], to facilitate mPTP opening. Although Marcu et al. [27] confirmed the effect of CypD on endothelial proliferation and angiogenesis, it remains to determine whether CypD-mediated mPTP opening and altered mitochondrial redox state are involved in the effects of TSH on ECs.

Here we report for the first time that elevated TSH triggers mitochondrial oxidative stress in ECs and shed light onto the crucial role of CypD in modulating TSH-induced mitochondrial and endothelial perturbations.

## 2. Materials and methods

### 2.1. Human subjects and data collection

Human subjects were recruited from Ningyang County, Shandong Province, China. Subjects under pregnancy or breast-feeding, taking medicines that affect thyroid status, and without good compliance were excluded.

Clinical assessments of the enrollments were performed at baseline and end-of-study. Venous blood samples were drawn between 8:00 a.m. and 10:00 a.m. after a minimum 10-h fasting, followed by the measurement of weight (kilograms) and standing height (meters). Body mass index (BMI) was calculated by dividing weight by the square of the height. The methods for determining waist circumference (WC) and blood pressure were according to the previous described [28].

The following serum variables were all completed at the clinical laboratory of the Shandong Provincial Hospital. Lipid profiles and fasting plasma glucose (FPG) were quantified using a BECKMAN Chemistry Analyzer AU5800 System (Beckman Coulter, Tokyo, Japan). Non HDL cholesterol (non HDL-C) was calculated by subtracting HDL-C from TC. Serum free triiodothyronine ($FT_3$), free thyroxine ($FT_4$), and TSH levels were measured by chemiluminescence methods (Cobas E601; Roche, Basel, Switzerland).

Euthyroidism was defined as serum TSH level between 0.27 and 4.2 mIU/L with normal serum $FT_4$ levels. Subclinical hypothyroidism (SCH) was TSH $\geq$ 4.2 mIU/L with normal serum $FT_4$ confirmed on the basis of at least two hormonal assays with a three-month interval [29]. All SCH patients underwent levothyroxine ($LT_4$, Euthyrox, 50 μg per tablet, Merck Serono, Darmstadt, Germany) replacement therapy with the initial dosage 25 μg/day. The dosage of $LT_4$ was adjusted according to serum TSH and $FT_4$ levels. The dosage with which SCH patients achieved euthyroidism was subsequently maintained. All participants were followed up for 15 months.

SCH patients who didn't achieve euthyroidism after $LT_4$ therapy were also excluded in the present study. After being matched by age, sex, BMI, TC and LDL-C, 33 euthyroid subjects, 33 mild SCH patients (TSH of 4.2–10 mIU/L) and 33 significant SCH patients (TSH > = 10 mIU/L) were finally enrolled in the study. Serum endothelin-1 (ET-1) levels of these humans were measured using Elisa Kits (abcam, USA). Measurement processes followed strictly to the manufacture's instructions.

The human study was performed according to the Declaration of Helsinki, approved by the Ethics Committee of Shandong Provincial Hospital, and was registered at ClinicalTrials.gov (NCT01848171). All participants signed an informed consent.

### 2.2. Animals and treatment

#### 2.2.1. Generation of TT-KO mice

We utilized a Cre/LoxP strategy to yield thyroid-specific TSH receptor (TSHR)-knockout (TT-KO) mice. *Tshr*$^{flox/+}$ mice with C57BL/6J background were obtained from Cyagen Biosciences (Guangzhou, China). For the generation of TT-KO mice, *Tshr*$^{flox/flox}$ mice were crossed with the heterozygous mice expressing TPO-driven Cre recombinase (kindly donated by Shioko Kimura, National Institutes of Health, Bethesda). Mice homozygous for the floxed gene and heterozygous for TPO-Cre (TT-KO) were used as experimental animals.

The TT-KO mice were genotyped by polymerase chain reaction. Primer pairs of 5-GAGGATTTCTGTTGGTGGCTGG-3/5-CACCCTTGATC CCCTTGACC-3 and 5-GTAAACTGCTGGAGTACATGA-3/5-AAAATTTA GCCTATGTGTAGCTT-3 were used to identify floxed allele. The primers of 5-TGC CACGACCAAGTGACAGCA ATG -3/5- AGAGACGGA AATCC ATCGCTCG -3 were used to identify mice with TPO-Cre.

#### 2.2.2. Treatments to TT-KO mice

After discontinuing breast feeding at 4 weeks old, all male TT-KO mice were supplemented with exogenous $T_4$ (Sigma) to keep normal thyroid hormone levels. Mice aged 9–10 weeks were subcutaneously injected with freshly prepared TSH (TT-KO + TSH, 7 mIU/g·d, Sigma,) or solvent (TT-KO + solvent, same volume as TSH) for additional 2 weeks before sacrificed. To evaluate the consequences of pharmacological blocking of CypD in vivo, we injected cyclosporin A (CsA, 15 mg/kg·d, Sandimmune, Novartis) or PBS (same volume as CsA) to TT-KO mice for 4 weeks prior to TSH or solvent co-injection for another 2 weeks (TT-KO + solvent, TT-KO + TSH, TT-KO + CsA, TT-KO