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学 位 論 文 名	心不全予防における β カテニン転写阻害薬の薬効・薬理
論 文 審 査 委 員	(委員長) 新 保 昌 久 教 授 (委 員) 木 村 直 行 教 授 魚 崎 英 毅 准教授

論文内容の要旨

1 研究目的

Heart failure (HF) is a huge problem of health and causes high mortality. The increase of myocyte size and cardiac wall thickness is found when hypertrophic growth, caused by hypertension. Wnt/ β -catenin pathways are involved in organ development, injury repair, tissue remodeling, and inflammation. ICG001 is a low molecular weight compound that specifically impedes β -catenin-mediated gene transcription. The activation of ICG001 in the heart and kidney after transverse aortic constriction (TAC) by blocking the signal ameliorates tissue injury in both organs. However, the precise mechanisms of ICG001 to HF remain unclear. The precise roles and mechanisms of ICG001 should be unveiled in the pathophysiological processes in cardiac hypertrophy and fibrosis.

2 研究方法

1. Animals and pressure overload model

Male mice (C57BL/6), aged 8 weeks, were used in the present study. Pressure overload was produced by TAC. The mice were randomly assigned to either TAC or sham surgery groups.

2. Echocardiography

Echocardiography was performed before and at 4 weeks after TAC or sham surgery. The LV internal dimension at end-systole (LVIDs) and end-diastole (LVIDd), and ejection fraction (EF) were measured. EF was calculated for the measurement of wall thickness.

3. Treatments

After TAC, the mice were randomly assigned into two groups including vehicle group (negative control) and TAC treated with ICG001 group. The ICG001 (50 mg/kg/day) was intraperitoneally administered for 10 days (twice per day). Vehicle (DMSO) was intraperitoneally administered into sham and wild type groups.

4. Histology

Heart tissues were fixed by U-fix solution and embedded in paraffin. Five μ m thick sections were prepared and stained with Hematoxylin & Eosin (HE) or Masson & Trichrome (MT).

5. Immunohistochemistry

The sections were processed by using the Vectastain[®] Elite ABC-HRP Kit Peroxidase and ImmPACT[™] DAB peroxidase substrate following the manufacturer's protocol. The sections were incubated with anti-CD68 antibody for macrophage's detection and were incubated with anti-CD3 antibody for T cell's detection. The intensity of macrophages and T cells was analyzed by Image-J.

6. RNA extraction and real-time PCR

RNA was extracted from mouse LV tissues using RNeasy mini kit. cDNA was synthesized. Real-time PCR was conducted using an SYBR Premix Ex Taq II Kit. The relative expression levels of the target genes were determined after normalizing against the GAPDH gene.

7. Western blotting

Proteins were extracted from the cardiac tissue. Equal amounts of protein from each sample were fractionated by SDS-PAGE. The proteins were transferred from gel SDS-PAGE to membrane. Then, membrane was incubated with primary antibodies and secondary antibodies. The protein expression levels were normalized to the corresponding GAPDH levels.

8. Flow cytometry

Heart tissues were digested and blocked with the CD16/32 antibody. Next, cell was stained with anti-mouse antibodies including CD45, CD3 ϵ , Ly-6C, CD11b, F4/80, CCR2. Next, flow cytometry analysis was performed and analyzed with FlowJo.

9. Mass spectrometry

Heart samples were homogenized and filtered. Next, the supernatant was collected and dried. All samples were processed to Liquid Chromatography Mass Spectrometry (LCMS). LCMS was conducted using a LCMS 8030 and 8050.

3 研究成果

At 4 weeks after TAC, HW/BW was significantly decreased and EF was significantly increased in TAC mice treated with ICG001. The extent of fibrosis in LV wall was decreased in ICG001 group compared with vehicle group. The expression of markers for cardiac hypertrophy and fibrosis including *Bnp*, *Klf5*, fibronectin, CTGF, collagen I, and β -MHC genes were significantly decreased in ICG001-treated TAC mice. The protein expression of KLF5 and β -catenin were significantly decreased in mice treated with ICG001. In addition, ICG001 affected macrophages, but did not impact T cells. The macrophages detected by CD68 were significantly reduced in ICG001-treated TAC mice. Flow cytometry analysis revealed that the reductions in levels of macrophage infiltrating in the tissue were observed in ICG001-treated TAC mice. Next, treatment of mice with ICG001 led to a marked reduction in transcripts that encode inflammatory mediators including *Il4*, *Il10*, *Tnfa*, *Tgfb1*, and *Ccl2* in heart after TAC. Next, TAC mice showed the increase of amino acid including isoleucine, leucine, lysine, valine, phenylalanine, serine, tryptophan, and tyrosine, but this increase was significantly attenuated in ICG001-treated TAC mice. ICG001-treated TAC mice demonstrated the decrease of pyruvate,

alanine, and lactate in the glycolysis pathway. The oxidative stress, calculated by the ratio of GSH/GSSG, was decreased in ICG001-treated TAC mice. These results suggested that ICG001 prevents HF after pressure overload.

4 考察

This study revealed that ICG001 attenuates cardiac dysfunction and prevents HF after pressure overload. TAC caused cardiac dysfunction affected by raising the values of LVIDs and LVIDd while the EF was decreased. ICG001 increased survival rate of TAC mice and improved cardiac function by ameliorating EF and preventing increases of LVIDs, LVIDd, and LV mass. ICG001 then prevented raising of HW/BW ratio and reduced infiltration of fibrosis in cardiac interstitium after TAC. In addition, the expression of cardiac markers and fibrosis (*Bnp*, β -MHC, CTGF, fibronectin, and collagen I) was decreased after treatment with ICG001 in TAC mice. Thus, ICG001 attenuated pressure overload-induced cardiac fibrosis in TAC mice.

Previous studies have already shown that upregulated β -catenin and KLF5 expression can contribute to fibrosis. In this study, treatment with ICG001 in TAC mice showed downregulated β -catenin and KLF5 expression and reduced fibrosis. Since *Klf5* haploinsufficiency has been reported to decrease M1 macrophage accumulation, this study also investigated whether ICG001-treated TAC mice can deactivate the accumulation of inflammatory cells in cardiac tissues. The result exhibited that ICG001 suppressed the infiltration of macrophages but ICG001 did not influence T cells. In addition, a reduction in transcripts encoded inflammatory mediators (*Il10*, *Tgfb1*, and *Ccl2*) also supported previous result. The reduction of CCL2 by ICG001 may have minimized the recruitment of monocytes, resulting in decreasing macrophage in the heart. Due to the fact that the response of inflammatory and fibrotic to cardiac injury is necessary for compensation and cardiac repair, ICG001 was injected in the early period after TAC and reduced the accumulation of macrophages resulting in preventing HF. Thus, the timing of ICG001 injection may be crucial in achieving to prevent HF. This finding suggested that ICG001 may prevent further macrophage proliferation in the early stage or later remodeling phase and has a potential effect to prevent the macrophage accumulation in TAC mice.

Next, the evaluation of metabolic changes in hypertrophic and HF in ICG001-treated TAC mice remain unrevealed. ICG001 elevated the expression of PPAR- α after TAC. Activating PPAR- α transcription reportedly improves the function and energetics of the heart, hence, I evaluated the effect of ICG001 on metabolite concentration in the heart. Metabolic profiling demonstrated that amino acids including isoleucine, leucine, lysine, valine, phenylalanine, serine, tryptophan, and tyrosine were increased in TAC mice but significantly decreased in ICG001-treated TAC mice. Reduction of the cardiac intratissue concentration of BCAA in TAC mice by increasing the BCAA catabolism preserves cardiac function and structure, thus, stimulating BCAA catabolism via PPAR- α upregulation may be one of the mechanisms that ICG001 protects the heart from HF. Furthermore, ICG001 also reduced the pyruvate, alanine,

and lactate levels. These alanine and lactate levels were increased in TAC mice but not in sham, suggesting to decrease the flux of pyruvate into the TCA cycle with diversion to lactate. This finding indicated that ICG001 might control the metabolism in TAC mice by retarding the accumulation of lactate, alanine, and aspartate, resulting in prevention of cardiac hypertrophy and HF. Moreover, the GSH/GSSG ratio also displayed that treatment by ICG001 in TAC mice can reduce the oxidative stress, suggesting that ICG001 may mediate the distinct alteration of oxidative stress during myocardial hypertrophy and HF. These studies provide the knowledge of ICG001 to improve cardiac function after pressure overload.

5 結論

In this study, ICG001 can prevent HF from cardiac hypertrophy and fibrosis due to pressure overload by inhibition of Wnt/ β -catenin signaling involved in reducing KLF5 expression and macrophage recruitment. In addition, this study reveals the novel mechanism associated with the reduction of oxidative stress via metabolic alteration.

論文審査の結果の要旨

申請者は、心不全をもたらす病態として重要性が高い高血圧による心筋肥大・線維化に着目し、臓器障害や組織修復、リモデリングに関与することが知られている Wnt/ β -catenin 経路に対する転写阻害薬の薬効・薬理を解析した。ICG001 は、これまで高血圧性心疾患の病態モデルである大動脈縮窄（TAC）モデルを用いた研究で、心臓、腎臓に対して保護的に作用することが報告されているが、本研究ではこれまで明らかにされていなかった心肥大や線維化を抑制（予防）する機序について詳細な検討がなされた。すなわち、ICG001 が Wnt/ β -catenin シグナル経路を抑制することにより、KLF5 の発現低下、マクロファージの動員抑制を介して炎症の惹起を抑制すること、また PPAR α を介する代謝変化が心肥大・線維化を抑制することを明らかにした。

本研究は、臨床的、社会的に重要性が高まっている心不全の予防・治療という課題について、新たな薬物治療の可能性を探索する意義のある研究で、詳細かつ新たな作用機序を見いだすために多くの検討がなされており、新規性、独創性があると評価した。

審査会では、学位論文としては研究の背景・目的の提示が不十分であること、薬剤の標的細胞の考察が不十分であること、審査会のプレゼンと比較して機序の図示が不十分であり追加が望ましいこと、まだ不明な機序について今後の方向性を示すこと、文章や英語表記の多くの誤りなど、数多くの指摘があった。これに対し、申請者は指摘内容を理解し、限られた時間の中で誠実に改訂を行った。

以上より、本論文は学位論文に相応しいと審査員全員で判断した。

最終試験の結果の要旨

最終審査会に際しては、論文の内容について明快にプレゼンテーションがなされ、審査委員からの質問にも適切に答えていた。関連する過去の知見にも精通し、研究者として十分な資質・能

力を有していると思われた。

以上の観点から、本論文は学位論文として相応しく、申請者は学位に値する学識が備わっていると審査委員全員が判断し、最終試験に合格とした。