Table 1　CR と GH/IGF-1 抑制との比較

	CR による変化		GH/IGF-1 抑制による変化
	摂食後	摂食前	
血液生化学データ			
総脂質	↓	↓	↓
トリグリセリド	↓	↓	↓
総コレステロール	↓	↓	→
遊離コレステロール	↓	↓	↓
リン脂質	↓	↓	↓
遊離脂肪酸	↓	→	→
ケトン体	→	↑	→
グルコース負荷試験	↑		↑
インスリン負荷試験	↑		↑
肝臓の遺伝子発現			
β 酸化関連遺伝子	→	↑	→
脂肪酸合成関連遺伝子	↑	↓	→
ストレス応答遺伝子	→	↑	↑
白色脂肪組織の遺伝子発現			
脂肪酸合成関連遺伝子	↑		→
炎症関連遺伝子	↓		→

成長ホルモン（GH）/インスリン様成長因子 1（IGF-1）シグナル非依存的な CR のメカニズム

われわれは，同程度に寿命が延長した野生型（−/−）CR ラット（以下，CR ラット）および（tg/−）自由摂食ラット（以下，Tg ラット）のさまざまなパラメーターを野生型（−/−）自由摂食ラット（以下，AL ラット）と比較することで，GH/IGF-1 シグナル非依存的な CR の影響を明らかにしようと試みた．

血清総脂質，トリグリセリド，遊離コレステロール，リン脂質レベルは，AL ラットに比較して，摂食状態にかかわらず CR ラットおよび Tg ラットで，同様に有意に低値を示した．一方，CR による摂食後の遊離脂肪酸の低下および摂食後のケトン体の増加は，Tg ラットでは観察できなかった[14]．また，グルコース負荷試験およびインスリン負荷試験では，CR ラットでも Tg ラットでも，グルコース耐性およびインスリン高感受性を示した[15]．肝臓の網羅的遺伝子発現解析では，CR により摂食前ではミトコンドリア β 酸化関連遺伝子の発現が亢進した．また摂食後では脂肪酸合成関連遺伝子の発現が顕著に亢進した．しかしながら，これら遺伝子発現の変化は Tg ラット肝臓では観察できなかった．一方，MDR2 や OCT1A などストレス耐性遺伝子発現の亢進は CR ラットでも Tg ラットでも，同様に観察された（**Table 1**）．以上の結果から，CR により摂食前にはミトコンドリア β 酸化を介して脂質をエネルギー源とし，摂食後には脂質を貯蔵するシステムが活性化していること，この CR による代謝の変化は GH/IGF-1 非依存性に制御されていることが示唆された[14]．

前述したように，単一遺伝子の改変により長寿を示すと報告されているマウスやラットに，脂肪細胞特異的にもしくはアディポサイトカイン分泌を修飾したマウスが含まれる．具体的には脂肪特異的にインスリン受容体をノックアウトしたマウス，脂肪細胞の分化因子である c/EBPβ を c/EBPα locus にノックインした c/EBPβ/β マウス，さらに脂肪細胞特異的なサイトカインでありインスリン感受性を正に制御し，抗炎症性サイトカインでもあるアディポネクチンを肝臓で過剰発現させたトランスジェニックマウスが長寿命であることが報告されて

Presented by Medical*Online

Table 2　SREBP1c による影響およびカロリー制限における SREBP1c の影響

<table>
<tr><th rowspan="3"></th><th rowspan="3">SREBP1cKO
による影響</th><th colspan="4">CR による影響</th></tr>
<tr><th colspan="2">野生型マウス</th><th colspan="2">SREBP1cKO マウス</th></tr>
<tr><th>摂食後</th><th>摂食前</th><th>摂食後</th><th>摂食前</th></tr>
<tr><td>寿命</td><td>↓</td><td colspan="2">↑</td><td colspan="2">→</td></tr>
<tr><td>呼吸商</td><td>↑</td><td colspan="2">↓</td><td colspan="2">↓</td></tr>
<tr><td>グルコース負荷試験</td><td>→</td><td colspan="2">↑</td><td colspan="2">→</td></tr>
<tr><td>インスリン負荷試験</td><td>→</td><td colspan="2">↑</td><td colspan="2">→</td></tr>
<tr><td>腹部内臓脂肪組織量</td><td>↓</td><td colspan="2">↓↓</td><td colspan="2">↓</td></tr>
<tr><td>肩甲骨間褐色脂肪組織量</td><td>→</td><td colspan="2">↓</td><td colspan="2">→</td></tr>
<tr><td>血液生化学</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>　トリグリセリド</td><td>→</td><td>→</td><td>↓</td><td>→</td><td>→</td></tr>
<tr><td>　遊離脂肪酸</td><td>→</td><td>↓</td><td>↓</td><td>→</td><td>→</td></tr>
<tr><td>　ケトン体</td><td>↓</td><td>un</td><td>↓</td><td>un</td><td>→</td></tr>
<tr><td>肝臓含有脂質</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>　トリグリセリド</td><td>→</td><td>→</td><td>↓</td><td>→</td><td>↓</td></tr>
<tr><td>　遊離脂肪酸</td><td>↑</td><td>→</td><td>→</td><td>→</td><td>→</td></tr>
<tr><td>心臓含有脂質</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>　トリグリセリド</td><td>→</td><td>→</td><td>→</td><td>→</td><td>→</td></tr>
<tr><td>　遊離脂肪酸</td><td>→</td><td>→</td><td>↑</td><td>→</td><td>→</td></tr>
</table>

un：undetectable

いる[16)〜18)]．すなわち，脂肪細胞の代謝や分化，アディポカインは寿命制御に重要であることが示唆される．

一方，CR による個体のサイズや体重の減少は，脂肪組織量の減少を伴う．CR は体重に比べ脂肪組織量，さらには内臓脂肪量により強い影響を与えるといわれている[19)]．われわれは，CR 群および対照群の精巣上体周囲白色脂肪組織での網羅的遺伝子発現解析を行い，CR が多くの糖，アミノ酸，脂質およびミトコンドリアエネルギー代謝関連遺伝子の発現を増強すること，一方，多くの炎症，血管新生，細胞外器質および細胞骨格関連遺伝子の発現を抑制することを明らかにした[20)21)]．このような遺伝子発現の変化は，摂食パターンに影響を受けなかった．また，遺伝子発現の変化の一部は，*in vitro* での脂肪細胞の分化に伴う遺伝子発現の変化と同様であり，一方，すでに報告されている肥満動物の脂肪組織での遺伝子発現とは，逆相関する傾向を示した[20)21)]．さらに，CR ラットおよび Tg ラット，AL ラットの精巣上体周囲脂肪組織において，形態学的および網羅的遺伝子発現解析を行った．その結果，AL ラットに比較して，CR ラットの脂肪細胞のサイズは顕著に小型化したが，Tg ラットでの変化は乏しかった．また，CR ラットでは脂肪酸合成関連遺伝子の発現が亢進し，炎症関連遺伝子の発現は抑制されたが，このような遺伝子発現の変化は Tg ラットでは観察されなかった（**Table 1**）．さらに，CR ラットでのみ発現が亢進した脂肪酸合成関連遺伝子の多くは脂肪酸合成系のマスター転写調節因子である sterol regulatory element binding protein (SREBP) 1 により制御される遺伝子群であった（未発表データ）．

以上より，血中の遊離脂肪酸およびケトン体レベルの変化，肝臓におけるミトコンドリア β 酸化と脂肪酸合成関連遺伝子発現の変化，脂肪組織における *de novo* 脂肪酸合成と炎症関連遺伝子の発現の変化，すなわち脂質代謝の変化と脂肪組織のリモデリングが，GH/IGF-1 シグナル非依存的な CR による変化と考えられ，その一部は SREBP1 により制御されている可能性が示唆された．

Presented by Medical*Online

適応反応仮説からみたCRによる代謝の変化におけるSREBP1cの関与

さまざまなパラメーターにおいて，CRは絶食状態（fasting，starvation）とは異なるが，血中レプチン，インスリン，成長ホルモンおよびLHレベルはともに低値を示し，コルチコステロンレベルは高値を示すという変化は共通している[22)23)]．また絶食状態では，呼吸商（respiratory quotient）は低値を示し，エネルギー源が糖質から脂質へシフトし[24)25)]，白色脂肪組織由来の脂肪酸が，ミトコンドリアβ酸化の燃料として使われ，主に脳における燃料源であるケトン体が作られる[25)]．CRラットの呼吸商は，食餌摂取後では高値，摂取前では低値を示し，その日内変動は食餌摂取に依存し大きく変動する．一方，ALラットの呼吸商の日内変動は，CRラットに比べて小さい[26)]．そこで，われわれはSREBP1cノックアウト（KO）マウスと野生型マウスにおのおのCRを行い，寿命をはじめとするさまざまなパラメーターの解析を行った．その結果を**Table 2**に示す．SREBP1cKOマウス（以下，KOマウス）では，野生型マウスに比べて，平均および最大寿命とも有意に減少した．野生型マウスではCRに伴い平均および最大寿命とも延長したが，興味深いことにKOマウスではこのCRに伴う寿命延長効果がみられなかった．KOマウスでは野生型に比べて呼吸商は有意に高値を示し，日内変動も乏しかった．しかしながら，CR時の呼吸商は野生型，KOマウスとも有意な差はみられなかった．グルコース負荷試験およびインスリン負荷試験において，野生型ではCRによりグルコース耐性，インスリン感受性が亢進したが，KOマウスではこのようなCRの効果は失われていた．腹部内臓脂肪はKOマウスにおいて減少していた．CRにより野生型では腹部内臓脂肪量は顕著に減少したものの，KOマウスではその減少が乏しかった．また，肩甲骨間褐色脂肪組織量はCRにより野生型マウスでは顕著に減少するものの，KOマウスではこの減少がみられなかった．血液や肝臓および心臓含有トリグリセリドや遊離脂肪酸においても，野生型において観察されたCRの影響がKOマウスでは観察できないものがあった．摂食前の空腹時ケトン体レベルは野生型マウスに比べてKOマウスで減少した．野生型マウスではCRの空腹時ケトン体レベルは有意に増加したが，KOマウスではこのような変化は観察できなかった．以上の知見から，KOマウスではCRにより脂質をエネルギー源として有効に利用する能力が低下しているのではないかと考えられた（未発表データ）．

そこで，野生型およびKOマウスでの絶食に対する応答を比較した．するとKOマウスでは野生型に比べて，絶食に伴って観察される体重の減少，脂肪組織重量の減少，血糖値の低下および血清ケトン体の増加が抑制されていることが明らかとなった（未発表データ）．このことは，SREBP1cが絶食応答，さらにCRによる抗老化・寿命延長効果に重要な役割を担っていることを示唆する．

de novo 脂肪酸合成の重要性

われわれはCRおよび自由摂食ラットの白色脂肪組織においてプロテオーム解析を行った．その結果，CRによりATP-citrate lyase，NADP-dependent malic enzyme，pyruvate dehydrogenase E1 component subunit beta，pyruvate carboxylaseの発現が増加していた．さらに，citrate synthase活性が亢進していた．このことは，CRによりピルビン酸/リンゴ酸回路が活性化している可能性を示唆する（未発表データ）．また，前述したようにCRによりSREBP1cを介した脂肪酸合成が亢進していた．この2つのCR動物の白色脂肪組織の特徴を考え合わせると，白色脂肪組織はCR時エネルギー貯蔵装置としてではなく，グルコースをよりエネルギー効率の高い脂肪酸に変換する装置として機能している可

Presented by Medical*Online

能性を示唆する．一方，がん悪液質の白色脂肪組織では，CR と同じように摂食量が減少した状態にもかかわらず，SREBP1c を介した脂肪酸合成は抑制されているようである[27]．この違いは，白色脂肪組織での *de novo* 脂肪酸合成が亢進されるか抑制されるかが，生理的なやせか病的なやせかの違いの 1 つになる可能性を示唆する．

おわりに

CR に関する研究は，主として老化生物学を専門とする研究者により研究されてきており，現在，この分野での CR に関する研究は，“その作用メカニズムの解明”に加え，“ヒトにおける CR の有効性”という 2 つの主要テーマに集約されている．そのうち前者に関して，白色脂肪組織リモデリングと SREBP1c を介した脂質代謝活性化の重要性を述べた．一方，同じく摂食量が減少した病態として，がん悪液質や神経性食欲不振症などの摂食障害があり，近年，モデル動物を用いた研究が広く行われるようになってきた．一般にがん悪液質における脂肪組織の萎縮は lipolysis の亢進によると考えられており，脂肪酸合成に関する知見は乏しい[27]．今まで異なった分野で研究されてきた CR モデルおよび摂食障害モデルから得られる知見を比較，検討することで，両分野に新たな切り口での研究の進展が期待される．

謝辞：第 52 回日本心身医学会総会のシンポジウムにおける講演の機会，さらにこの総説を執筆する機会を与えてくださった乾　明夫先生ならびに須藤信行先生に感謝いたします．

文献

1) Yu BP：Modulation of Aging Processes by Dietary Restriction. CRC Press, Boca Raton, 1994
2) Weindruch R, Walford RL：The Retardation of Aging and Disease by Dietary Restriction. Charles C Thomas, Springfield, 1988
3) Holliday R：Food reproduction and longevity：Is the extended lifespan of calorie-restricted animals an evolutionary adaptation? *Bioessays* **10**：125-127, 1989
4) Higami Y, Yamaza H, Shimokawa I：Laboratory findings of caloric restriction in rodents and primates. *Adv Clin Chem* **39**：211-237, 2005
5) Masoro EJ：Overview of caloric restriction and ageing. *Mech Ageing Dev* **126**：913-922, 2005
6) Sinclair DA：Toward a unified theory of caloric restriction and longevity regulation. *Mech Ageing Dev* **126**：987-1002, 2005
7) Colman RJ, Anderson RM, Johnson SC, et al：Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* **325**：201-204, 2009
8) Kemnitz JW：Calorie restriction and aging in nonhuman primates. *ILAR J* **52**：66-77, 2011
9) Roth GS, Lane MA, Ingram DK, et al：Biomarkers of caloric restriction may predict longevity in humans. *Science* **297**：811, 2002
10) Brown-Borg HM, Borg KE, Meliska CJ, et al：Dwarf mice and the ageing process. *Nature* **384**：33, 1996
11) Shimokawa I, Chiba T, Yamaza H, et al：Longevity genes：insights from calorie restriction and genetic longevity models. *Mol Cells* **26**：427-435, 2008
12) Shimokawa I, Higami Y, Utsuyama M, et al：Life span extension by reduction in growth hormone-insulin-like growth factor-1 axis in a transgenic rat model. *Am J Pathol* **160**：2259-2265, 2002
13) Shimokawa I, Higami Y, Tsuchiya T, et al：Life span extension by reduction of the growth hormone-insulin-like growth factor-1 axis：relation to caloric restriction. *FASEB J* **17**：1108-1109, 2003
14) Higami Y, Tsuchiya T, Chiba T, et al：Hepatic gene expression profile of lipid metabolism in rats：Impact of caloric restriction and growth hormone/insulin-like growth factor-1 suppression. *J Gerontol A Biol Sci Med Sci* **61**：1099-1110, 2006
15) Yamaza H, Komatsu T, Chiba T, et al：A transgenic dwarf rat model as a tool for the study of calorie restriction and aging. *Exp Gerontol* **39**：269-272, 2004
16) Blüher M, Kahn BB, Kahn CR：Extended longevity in mice lacking the insulin receptor in adipose tissue. *Science* **299**：572-574, 2003
17) Chiu CH, Lin WD, Huang SY, et al：Effect of a C/EBP gene replacement on mitochondrial biogenesis in fat cells. *Genes Dev* **18**：1970-1975, 2004
18) Otabe S, Yuan X, Fukutani T, et al：Overexpression of human adiponectin in transgenic mice results in suppression of fat accumulation and

Presented by Medical*Online

prevention of premature death by high-calorie diet. *Am J Physiol Endocrinol Metab* **293** : E210-218, 2007

19) Barzilai N, Banerjee S, Hawkins M, et al : Caloric restriction reverses hepatic insulin resistance in aging rats by decreasing visceral fat. *J Clin Invest* **101** : 1353-1361, 1998
20) Higami Y, Pugh TD, Page GP, et al : Adipose tissue energy metabolism : altered gene expression profile of mice subjected to long-term caloric restriction. *FASEB J* **18** : 415-417, 2004
21) Higami Y, Barger JL, Page GP, et al : Energy restriction lowers the expression of genes linked to inflammation, the cytoskeleton, the extracellular matrix, and angiogenesis in mouse adipose tissue. *J Nutr* **136** : 343-352, 2006
22) Nelson JF : Neuroendocrine involvement in the retardation of aging by dietary restriction : A hypothesis. In : Yu BP (ed) : Modulation of Aging Processes by Dietary Restriction. Boca CRC Press, Raton, Florida, pp37-55, 1994
23) Ahima RS, Prabakaran D, Mantzoros C, et al : Role of leptin in the neuroendocrine response to fasting. *Nature* **382** : 250-252, 1996
24) Overton JM, Williams TD, Chambers JB, et al : Central leptin infusion attenuates the cardiovascular and metabolic effects of fasting in rats. *Hypertension* **37** : 663-669, 2001
25) Salway JG : Metabolism at a Glance. Blackwell Science, Oxford, UK, 1999
26) McCarter RJ, Palmer J : Energy metabolism and aging : a lifelong study of Fischer 344 rats. *Am J Physiol* **263** : E448-452, 1992
27) Bing C, Trayhurn P : New insights into adipose tissue atrophy in cancer cachexia. *Proc Nutr Soc* **68** : 385-392, 2009

Abstract

Altered Energy Metabolism in Anti-aging and Pro-longevity Effects of Caloric Restriction

Yoshikazu Higami*

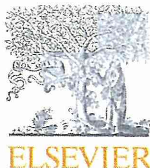
*Molecular Pathology & Metabolic Disease, Faculty of Pharmaceutical Sciences, Tokyo University of Science
(*Mailing Address* : Yoshikazu Higami, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan)

Caloric restriction (CR) has been applied as a powerful tool in aging research. CR is accepted as a robust, reproducible and simple experimental manipulation known to extend both median and maximum lifespans, and to retard and suppress a broad spectrum of pathophysiological changes in a variety of mammals. In general, CR delays skeletal and sexual maturation, reduces body size with less adiposity, lowers body temperature, modulates hyperglycemia and insulinemia, alters lipid and energy metabolisms, protects against internal oxidative and environmental stresses, and activates mitochondrial biogenesis and sirtuins. Based on the adaptive response hypothesis against food shortage, I propose that CR promotes adipose tissue remodeling and modulates energy metabolism via sterol regulatory element binding protein (SREBP) 1c, a master transcriptional factor of fatty acid biosynthesis. Activation of *de novo* fatty acid biosynthesis regulated by SREBP1c might play an important role in the anti-aging and lifespan extension by caloric restriction.

Key words : caloric restriction, aging, lipid metabolism, adipose tissue remodeling, SREBP1c

Presented by Medical*Online

Biochemical and Biophysical Research Communications 421 (2012) 15–19



Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Reversible induction of PARP1 degradation by p53-inducible cis-imidazoline compounds

Wataru Nagai [1], Naoyuki Okita [*,1], Hiroshi Matsumoto, Hitoshi Okado, Misako Oku, Yoshikazu Higami [*]

Department of Molecular Pathology and Metabolic Disease, Faculty of Pharmaceutical Sciences, Tokyo University of Science, Yamazaki 2641, Noda, Chiba 278-0022, Japan

ARTICLE INFO

Article history:
Received 16 March 2012
Available online 23 March 2012

Keywords:
Nutlin3a
Nutlin3b
Caylin2
Poly(ADP-ribose) polymerase1
p53
MDM2
Proteasome

ABSTRACT

PARP1 is an important enzyme involved in various patho-physiological phenomena such as ischemia/reperfusion (I/R) injury, which occurs when blood flow is restored after cerebral infarction, myocardial infarction and transplantation of various organs. I/R-induced PARP1 over-activation is mediated by production of reactive oxygen species and is involved in NF-κB transactivation. For these reasons, PARP1 is an attractive target for strategies to protect against I/R injury. We previously reported that an MDM2 inhibitor Nutlin3a, a cis-imidazoline compound, induces PARP1 degradation in a p53 and proteasome-dependent manner. In this study, we evaluated the effect of Nutlin3a analogs, Nutlin3b and Caylin2, on PARP1 degradation. Like Nutlin3a, Caylin2, but not Nutlin3b, induced PARP1 degradation in both 3T3-L1 and 3T3-F442A. This result occurred almost in parallel with p53 accumulation. Furthermore Caylin2-induced PARP1 degradation was not observed in p53 deficient mouse embryonic fibroblasts or in the presence of the proteasome inhibitor MG132. These results suggest that Caylin2 induces PARP1 degradation by the same mechanism as Nutlin3a. Finally, we showed that Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response. For protection against I/R injury, our results support the usability of the p53 inducible cis-imidazoline compounds, Nutlin3a and its analogs, as PARP1 inhibitors.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

Poly(ADP-ribosyl)ation, which is the post-translational protein modification, is involved in cell replication, DNA repair, cell death, and inflammation [1,2]. PARP1 is the most abundant PARP family member in cells, and is dramatically activated by DNA breaks. Therefore, massive DNA damage induces over-activation of PARP1, and then decreases ATP levels via over-consumption of cellular NAD^+, which is required for the ATP production in glycolysis and TCA cycle pathways. It has been also reported that PARP1 over-activation is involved in ischemia/reperfusion (I/R) injury, which occurs during the restoration of blood flow after cerebral infarction, myocardial infarction and organ transplantation [3–5]. For these reasons, PARP1 is an attractive target for protection against I/R injury [6].

We previously reported that Nutlin3a, an MDM2 ubiquitin ligase antagonist, induces p53 and proteasome-dependent PARP1 protein degradation [7]. It has been thought that Nutlin3a is a candidate for anti-tumor drugs, because MDM2 inhibition by Nutlin3a induces p53 stabilization, followed by p53-dependent apoptosis in tumor cells [8]. The discovery of Nutlin3a-induced PARP1 degradation prompted the use of Nutlin3a as a PARP1 inhibitor. Furthermore, considering that p53 has the potential to up-regulate anti-oxidant and anti-inflammatory genes [9–11], Nutlin3a may be a potent anti-I/R drug that has multiple points of action. However, the Nutlin3a pharmacophore that induce PARP1 protein degradation has not been identified. In the present study, to clarify whether Nutlin3a analogs were also able to induce PARP1 protein degradation in a manner similar to Nutlin3a, we examined the effect on PARP1 degradation by the commercially available Nutlin3a enantiomer, Nutlin3b [12,13], and by the Nutlin3a derivative, Caylin2 [14]. Furthermore, by using compounds possessing PARP1 degradation activity, we evaluated the reversibility of PARP1 degradation and the effect on anti-inflammatory IL6 gene expression.

2. Materials and methods

2.1. Cell culture and drugs

Mouse fibroblast 3T3-L1 and 3T3-F442A cell lines were purchased from the RIKEN Bioresource Center (Japan) and the European Collection of Animal Cell Cultures (UK), respectively. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, low glucose) (WAKO, Japan) with 10% fetal calf serum

* Corresponding authors. Fax: +81 4 7124 3676.
E-mail addresses: nokita7@rs.noda.tus.ac.jp (N. Okita), higami@rs.noda.tus.ac.jp (Y. Higami).

[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.03.091

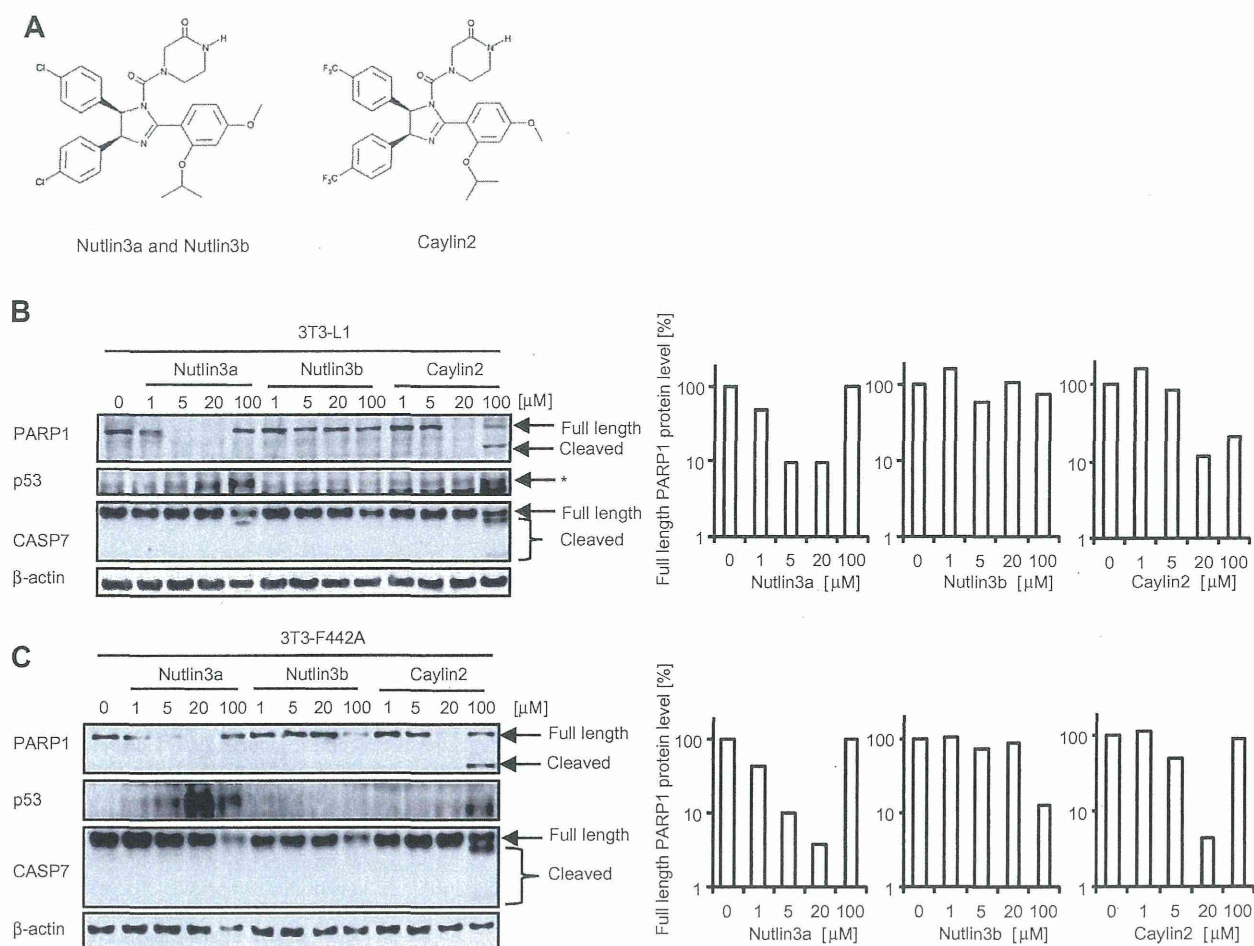


Fig. 1. Caylin2 but not Nutlin3b decreases in PARP1 protein levels in mouse fibroblasts. (A) Structures of Nutlin3a, Nutlin3b, and Caylin2. Mouse fibroblast 3T3-L1 (B) or 3T3-F442A (C) were treated with the indicated concentrations of Nutlin3a, Nutlin3b or Caylin2 for 8 h. The cell lysates were analyzed by Western blotting using the indicated antibodies (left pannel). Quantitative data are shown (right panel). In the p53 panel, the arrow and asterisk show the p53 and nonspecific bands, respectively. All experiments were performed at least three times, and representative data is shown.

and 1% penicillin/streptomycin (Sigma). p53+/+ or −/− MEFs were prepared as described previously [7]. The established MEFs were maintained in DMEM (high glucose) with 10% FCS, 0.1 mM 2-mercaptoethanol, and 1% penicillin/streptomycin. The proteasome inhibitor MG132 was purchased from WAKO (Japan). Nutlin3a, Nutlin3b, and Caylin2 were supplied by Cayman (USA).

2.2. Western blotting

Cell preparation and Western blotting were performed as described previously [7]. As primary antibodies, anti-PARP1 (clone C-2-10, WAKO, Japan), anti-p53 (clone Ab-1, Calbiochem, USA), anti-β actin (clone AC-15, SIGMA, USA), or anti-CASP7 (clone 1F3, MBL, Japan) antibodies were used. For secondary antibodies, horseradish peroxidase-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG or anti-rabbit IgG (Jackson Immunoresearch, USA) were used. The specific proteins were visualized with ImmunoStar LD reagent (WAKO, Japan) and LAS3000 (Fuji Film, Japan), and the data were analyzed using MultiGauge software (Fuji Film, Japan).

2.3. RNA purification and RT-PCR

RNA purification and RT-PCR were performed using RNAiso PLUS, FastPure RNA kit, PrimeScript Reverse Transcriptase and random hexamers (all from TaKaRa, Japan) as described previously [7]. The PCR was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, USA) and primers for *TNFα* (forward, 5′-CCCTCACACTCAGATCATCTTCTC-3′; reverse, 5′-GCCTTGTCCCTTGAA GAGAACC-3′) *IL6* (forward, 5′-GCCTTCCCTACTTCACAAGTCC-3′; reverse, 5′-CAGAATTGCCATTGCACAAC-3′), or *TBP* (forward, 5′-CAG TACAGCAATCAACATCTCAGC-3′; reverse, 5′-CAAGTTTACAGCCAAG-ATTCACG-3′) as follows: initiation step, at 94 °C for 1 min; amplification step, at 94 °C for 1 min, at 60 °C for 15 s, at 68 °C for 15 s; termination step, 68 °C 15 s. PCR products were subjected to 1.8% agarose gel electrophoresis, stained with ethidium bromide, and visualized with LAS3000. The data was analyzed using MultiGauge software (Fuji Film, Japan).

3. Results

3.1. Caylin2. but not Nutlin3b induces a decrease in PARP1 protein levels in mouse fibroblast cell lines

Although we previously reported that Nutlin3a induces PARP1 protein degradation, we did not address whether Nutlin3a analogs also have the potential to induce PARP1 degradation [7]. Here, we investigated the inducibility of PARP1 degradation by two such analogs, Nutlin3b and Caylin2 in mouse fibroblast cell lines

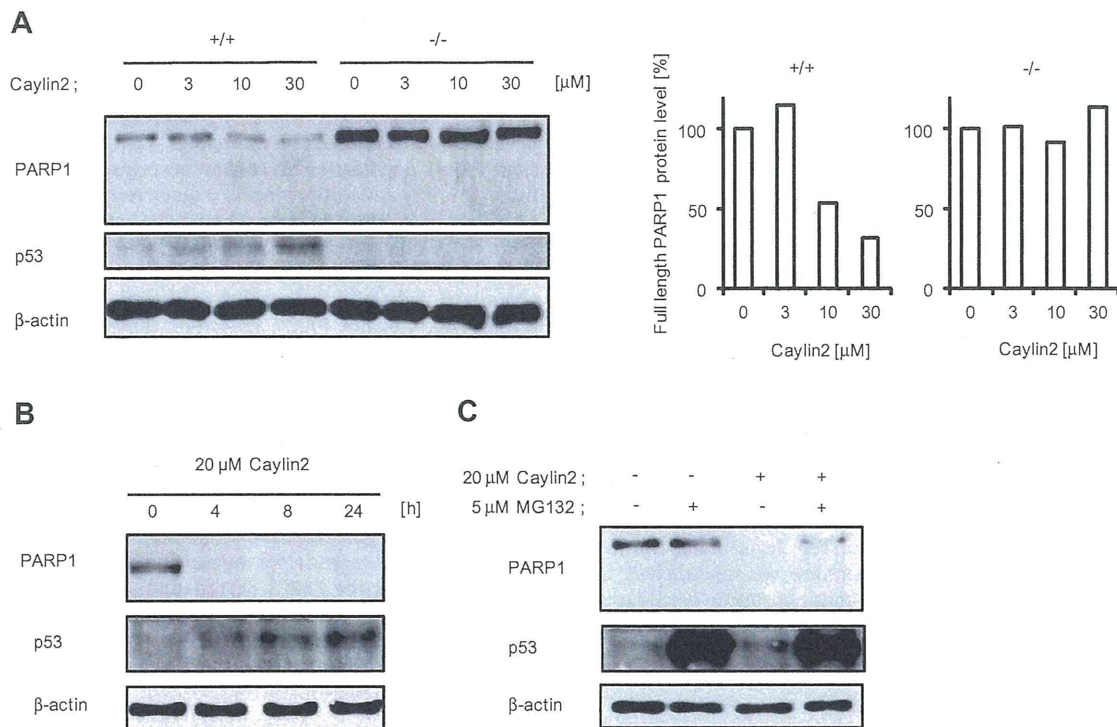


Fig. 2. Caylin2-induced PARP1 degradation is p53 status and proteasome-dependent. (A) p53+/+ and p53−/− MEFs were treated with the indicated concentrations of Caylin2 for 8 h. Cell lysates were analyzed by Western blotting using the indicated antibodies (left panel). Quantitative data are shown (right panel). Each 2 to 3 clones of p53+/+ and p53−/− MEFs were analyzed and representative data are shown. (B) 3T3-L1 cells were treated with 20 μM Caylin2 for the indicated times. The proteins were subjected to Western blotting. (C) 3T3-L1 cells were treated with 20 μM Caylin2 in the presence or absence of 5 μM MG132 proteasome inhibitor (MG) for 8 h, and cell lysates were then subjected to Western blotting using the indicated antibodies.

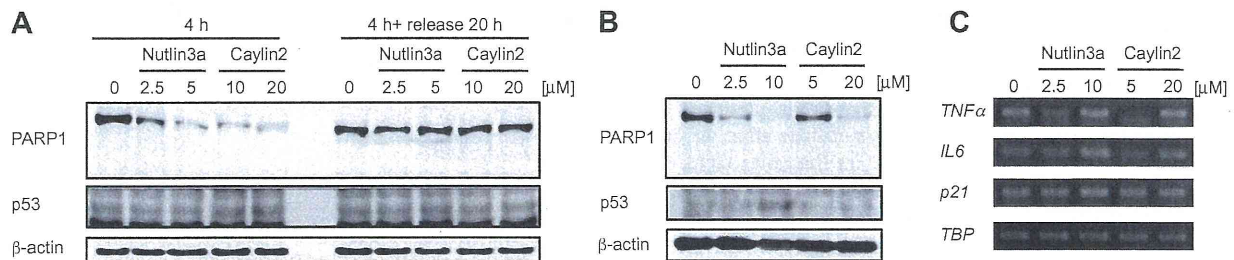


Fig. 3. Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response. (A) 3T3-L1 cells were treated with Nutlin3a (2.5 or 5 μM) or Caylin2 (10 or 20 μM) for 4 h. After these treatments, cells were also cultured in normal growth medium without treatment for a further 20 h. The cell lysates were analyzed by Western blotting using the indicated antibodies. (B, C) 3T3-L1 cells were treated with 2.5 μM Nutlin3a or 5 μM Caylin2 for 4 h. The protein and RNA expression were analyzed by Western blotting (B) or RT-PCR (C).

(Fig. 1). Nutlin3b is an inactive enantiomer of Nutlin3a, whereas Caylin2 is a Nutlin3a derivative in which trifluoromethyl groups are substituted for chlorine on the 2 phenyl rings (Fig. 1A) [8,12]. As shown in Fig. 1B and C, for both cell lines, 1–20 μM Nutlin3a treatment markedly decreased PARP1 protein levels in a dose dependent manner, whereas 100 μM Nutlin3a treatment had no effect, as per our previous report. p53 accumulation was dose dependent, increasing with the concentration range. Additionally, after 100 μM Nutlin3a-treated, both cell lines were detached from the culture dish and appeared to die without significant CASP7 activation. This observation was consistent with our previous data [7]. Nutlin3b treatment did not markedly alter p53 protein levels in either cell line. In 3T3-L1 cells, Nutlin3b treatment did not affect PARP1 protein level (Fig. 1B). On the other hand, in 3T3-F442A cells, only 100 μM Nutlin3b treatment decreased the PARP1 protein level (Fig. 1C). Similar to the Nutlin3a treatment, 100 μM Nutlin3b-treated cells seemed to die without significant CASP7 activation. Interestingly, Caylin2 treatment showed a signature profile of PARP1 protein in both cell lines. 20 μM Caylin2 treatment induced a significant decrease in PARP1 protein and 100 μM Caylin2 treatment induced PARP1 cleavage, which is considered as an apoptotic hallmark as well as activation of apoptotic caspases such as CASP2, 3, 6, 7, 9, and 10 [15–17]. Indeed, a trypan blue exclusion assay showed that Caylin2-treated cells were viable at 20 μM and dead at 100 μM (Supplemental Fig. 1).

3.2. PARP1 down-regulation by Caylin2 treatment is p53 and proteasome-dependent

Since we previously reported that Nutlin3a-induced PARP1 degradation occurs in a p53 and proteasome dependent manner, we sought to confirm using the same methods as our previous report whether Caylin2-induced PARP1 degradation is inhibited by p53 depletion or proteasome inhibition. As shown in Fig. 2A, p53 WT MEFs, but not p53 KO MEFs, displayed decreasing PARP1 protein levels in a Caylin2 dose dependent manner. Furthermore, as shown in Fig. 2B, Caylin2-induced PARP1 degradation was inhibited by co-treatment with the proteasome inhibitor MG132. These results indicate that Caylin2, like Nutlin3a, induces PARP1 degradation in a p53 and proteasome-dependent manner.

3.3. Nutlin3a or Caylin2 treatment induces reversible PARP1 down-regulation without an inflammatory response

Since PARP1 plays roles in the maintenance of cellular homeostasis through various signal transduction pathways [1,2], reversible down-regulation of the PARP1 protein level is important to protect tissues from I/R injury. Therefore we investigated the reversibility of Nutlin3a- or Caylin2-induced PARP1 degradation. 3T3-L1 cells were treated with Nutlin3a (2.5 or 5 μM) or Caylin2 (10 or 20 μM) for 4 h, and then cultured for 20 h. After 4 h of Nutlin3a or Caylin2 treatment (transient treatment), PARP1 protein levels decreased, although p53 protein levels were not markedly altered (Fig. 3A). After release from those treatments (+20 h), PARP1 protein levels were recovered (Fig. 3A). These results show that Nutlin3a or Caylin2-induced PARP1 degradation is reversible. As it has been reported that Nutlin3a-induced p53 activation leads to up-regulation of inflammatory cytokines [18], we also investigated the influence on inflammation by the transient Nutlin3a or Caylin2 treatment (Fig. 3B). 3T3-L1 cells were treated with the indicated doses of Nutlin3a or Caylin2 for 4 h, and then analyzed the TNFα and IL6 inflammatory genes by RT-PCR. Under these conditions, Nutlin3a or Caylin2 treatment induced PARP1 degradation in a dose dependent manner. Interestingly, we observed different inflammatory responses under these condition (Fig. 3C). The higher dose treatments of Nutlin3a or Caylin2 significantly induced IL6 mRNA expression. However, these doses had little effect or only slightly induced TNFα mRNA expression. On the other hand, the lower dose treatments of Nutlin3a or Caylin2, which were capable of inducing PARP1 degradation, inhibited TNFα mRNA expression and did not affect or only slightly inhibited IL6 mRNA expression. Taken together with Fig. 3B and C, these results indicate that the lower dose treatment of Nutlin3a or Caylin2 has the potential to induce PARP1 degradation without inducing an inflammatory response.

4. Discussion

In this study, we examined the effect of treatment by Nutlin3a analogs on PARP1 protein levels. We demonstrated that Caylin2 induces PARP1 degradation in a similar manner to Nutlin3a. Taken together with our previous study, these results indicate that p53-inducible cis-imidazoline compounds have the potential to induce PARP1 degradation. In the context of using Nutlin3a, Caylin2 and related derivatives as "PARP1 degradation inducers" for I/R injury therapy, a major advantage of this study is that it has demonstrated that Nutlin3a- or Caylin2-induced PARP1 degradation is reversible (Fig. 3A). I/R injury is the tissue damage that occurs during the ischemic and reperfusion period, and as such commonly occurs as a result of ischemic infarction and its treatment or during organ transplantation. In the injured tissues, PARP1 is over-activated by reactive oxygen-mediated DNA damage, resulting in decreases in ATP levels via over-consumption of cellular NAD^+ [1,2]. Therefore, PARP1 inhibition has protective effects on I/R injury. Furthermore, PARP1 itself plays roles in the maintenance of cellular homeostasis through its involvement in the regulation of various signal transduction pathways [1,2]. Taken together, transient PARP1 degradation is valuable in regard to both protection from I/R injury and to allowing for a quick recovery from the harmful effects of PARP1 inhibition. There have been some previous reports of IL6 regulation by p53 or PARP1. p53 has been reported to repress not only IL6 but also the promoter activity of NF-κB, a transcriptional factor of various inflammatory genes including IL6 [10,11]. Additionally, PARP1 activation inhibits the DNA-binding activity of NF-κB [19]. In this study, we showed that Nutlin3a or Caylin2 causes differential effects on inflammatory responses depending on the magnitude of the doses used (Fig. 3C). Our results suggest that the choice of appropriate doses and timing of treatments would be critical to obtain only the beneficial effects on PARP1 degradation when using Nutlin3a or Caylin2 for protection from I/R injury.

Recently, it was reported that inflammasome activation of cardiac fibroblasts is essential for myocardial I/R [20]. So far, our work has revealed that the PARP1 degradation pathway functions efficiently in fibroblast cell lines [7]. These findings support the possibility of practical use of this PARP1 degradation pathway. Further research will require several lines of investigation. Firstly, it will be interesting to identifiy the stereocenter that specifically induces PARP1 degradation. The chiral separation of Nutlin3 (Nutlin3a and Nutlin3b) has been achieved, although the absolute stereocenter has not been known [12,13]. In Caylin2 the chiral separation has not been achieved. We predict that Caylin2a (Caylin2 of Nutlin3a type), but not Caylin2b (Caylin2 of Nutlin3b type), may be the potential to induce PARP1 degradation and are performing further analyses now. Secondly, it will be important to explore PARP1 degradation inducers that different structures than the cis-imidazoline compounds such as Nutlin3a or Caylin2. Thus, elucidation of the mechanism of reversible PARP1 degradation induction is important for the optimization of compounds which induce this phenomenon, resulting in the establishment of selective chemotherapeutic strategies against I/R injury.

Acknowledgments

The authors thank Dr. Susumu Kobayashi (Tokyo University of Science, Japan) and Dr. Takahiro Suzuki (Tokyo University of Science, Japan) for their valuable expertise and all members of Higami laboratory for their cooperation. This work was supported by Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology (23790201) (N.O.) and partially by Challenging Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology (23659207) (Y.H.). RIDAI SCITEC holds a patent (PCT/JP2012/052565) on the method of treating ischemia/reperfusion injury, and N.O., and Y.H. are the inventors of the patent.

References

[1] M. Masutani, H. Nakagama, T. Sugimura, Poly(ADP-ribosyl)ation in relation to cancer and autoimmune disease, Cell. Mol. Life Sci. 62 (2005) 769–783.
[2] M. Miwa, M. Masutani, PolyADP-ribosylation and cancer, Cancer Sci. 98 (2007) 1528–1535.
[3] H.K. Eltzschig, T. Eckle, Ischemia and reperfusion – from mechanism to translation, Nat. Med. 17 (2011) 1391–1401.
[4] S.J. van Wijk, G.J. Hageman, Poly(ADP-ribose) polymerase-1 mediated caspase-independent cell death after ischemia/reperfusion, Free Radic. Biol. Med. 39 (2005) 81–90.
[5] P. Pacher, C. Szabo, Role of the peroxynitrite-poly(ADP-ribose) polymerase pathway in human disease, Am. J. Pathol. 173 (2008) 2–13.

[6] D.V. Ferraris, Evolution of poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors, from concept to clinic, J. Med. Chem. 53 (2010) 4561–4584.
[7] S. Matsushima, N. Okita, M. Oku, W. Nagai, M. Kobayashi, Y. Higami, An Mdm2 antagonist, Nutlin-3a, induces p53-dependent and proteasome-mediated poly(ADP-ribose) polymerase1 degradation in mouse fibroblasts, Biochem. Biophys. Res. Commun. 407 (2011) 557–561.
[8] L.T. Vassilev, B.T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, E.A. Liu, In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, Science 303 (2004) 844–848.
[9] A.A. Sablina, A.V. Budanov, G.V. Ilyinskaya, L.S. Agapova, J.E. Kravchenko, P.M. Chumakov, The antioxidant function of the p53 tumor suppressor, Nat. Med. 11 (2005) 1306–1313.
[10] U. Santhanam, A. Ray, P.B. Sehgal, Repression of the interleukin 6 gene promoter by p53 and the retinoblastoma susceptibility gene product, Proc. Natl. Acad. Sci. USA 88 (1991) 7605–7609.
[11] E.A. Komarova, V. Krivokrysenko, K. Wang, N. Neznanov, M.V. Chernov, P.G. Komarov, M.L. Brennan, T.V. Golovkina, O.W. Rokhlin, D.V. Kuprash, S.A. Nedospasov, S.L. Hazen, E. Feinstein, A.V. Gudkov, p53 is a suppressor of inflammatory response in mice, FASEB J. 19 (2005) 1030–1032.
[12] Z. Wang, M. Jonca, T. Lambros, S. Ferguson, R. Goodnow, Exploration of liquid and supercritical fluid chromatographic chiral separation and purification of Nutlin-3 – a small molecule antagonist of MDM2, J. Pharm. Biomed. Anal. 45 (2007) 720–729.
[13] Cayman Chemical, http://www.caymanchem.com/app/template/Product.vm/catalog/10009816.
[14] Cayman Chemical, http://www.caymanchem.com/app/template/Product.vm/catalog/10005002.
[15] S.H. Kaufmann, S. Desnoyers, Y. Ottaviano, N.E. Davidson, G.G. Poirier, Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis, Cancer Res. 53 (1993) 3976–3985.
[16] Y.A. Lazebnik, S.H. Kaufmann, S. Desnoyers, G.G. Poirier, W.C. Earnshaw, Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE, Nature 371 (1994) 346–347.
[17] C. Pop, G.S. Salvesen, Human caspases: activation, specificity, and regulation, J. Biol. Chem. 284 (2009) 21777–21781.
[18] B. Huang, D. Deo, M. Xia, L.T. Vassilev, Pharmacologic p53 activation blocks cell cycle progression but fails to induce senescence in epithelial cancer cells, Mol. Cancer Res. 7 (2009) 1497–1509.
[19] W.J. Chang, R. Alvarez-Gonzalez, The sequence-specific DNA binding of NF-kappa B is reversibly regulated by the automodification reaction of poly(ADP-ribose) polymerase 1, J. Biol. Chem. 276 (2001) 47664–47670.
[20] M. Kawaguchi, M. Takahashi, T. Hata, Y. Kashima, F. Usui, H. Morimoto, A. Izawa, Y. Takahashi, J. Masumoto, J. Koyama, M. Hongo, T. Noda, J. Nakayama, J. Sagara, S. Taniguchi, U. Ikeda, Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury, Circulation 123 (2011) 594–604.