Allyl isothiocyanate (AITC) activates nonselective cation currents in human cardiac fibroblasts: possible involvement of TRPA1

Gaku Oguri, Toshiaki Nakajima, Hironobu Kikuchi, Shotaro Obi, Fumitaka Nakamura, Issei Komuro

Department of Cardiovascular Medicine, University of Tokyo, Tokyo 113-8655, Japan
Department of Cardiovascular Medicine, Dokkyo Medical University, Tochigi, 321-0293, Japan
Teikyo University Chiba Medical Center, Ichihara, 299-0111, Chiba, Japan

ARTICLE INFO

Keywords:
Human cardiac fibroblast
Transient receptor potential ankyrin 1 (TRPA1) agonist
Allyl isothiocyanate
Methylglyoxal
Nonselective cation channels

ABSTRACT

The effects of allyl isothiocyanate (AITC), transient receptor potential ankyrin 1 (TRPA1) agonist, on cultured human cardiac fibroblasts were examined by measuring intracellular Ca²⁺ concentration [(Ca²⁺)i] and whole-cell voltage clamp techniques. AITC (200 μM) increased Ca²⁺ entry in the presence of [Ca²⁺]i. Ruthenium red (RR) (30 μM), and La³⁺ (0.5 mM), a general cation channel blocker, inhibited AITC-induced Ca²⁺ entry. Under the patch pipette filled with Cs⁺- and EGTA-solution, AITC induced the current of a reversal potential (Er) of approximately +0 mV. When extracellular Na⁺ ion was changed by NMDG⁺, the inward current activated by AITC was markedly reduced. La³⁺ and RR inhibited the AITC-induced current. The conventional RT-PCR analysis, Western blot, and immunocytochemical studies showed TRPA1 mRNA and protein expression. The present study shows the first evidence for functional Ca²⁺-permeable nonselective cation currents induced by AITC, possibly via TRPA1 in human cardiac fibroblast.

1. Introduction

Transient receptor potential ankyrin 1 (TRPA1) channel is a nonselective cation channel having ankyrin repeats on the N-terminus [1]. It is predominately expressed in sensory neurons [2], but there have been several reports that TRPA1 channel is identified in non-neuronal cells including lung fibroblasts [3] and arterial endothelial cells (ECs) [4]. And, TRPA1 channel plays an important role on cardiovascular diseases such as heart failure and myocardial fibrosis [5]. Some papers also illustrated that TRPA1 channel was identified in cardiomyocytes and activation of TRPA1 dose-dependently enhanced the contractile function and intracellular Ca²⁺ concentration ([Ca²⁺]), but not in cardiomyocytes prepared from Trpa1−/− mice [6, 7, 8]. Wang et al. [9, 10] have shown that TRPA1 expression was increased in mouse and human failure hearts, and the channel inhibition improved cardiac hypertrophy and function in the mouse pressure overload model due to transverse aortic constriction (TAC). Conklin et al. [8] also reported that the TRPA1 expression in cardiomyocytes was high in the intercalated disks and involved in acrolein-induced Ca²⁺ increase. According to these studies, TRPA1 appears to play a role in regulating cardiac function under various conditions including heart failure [5, 11].

Cardiac fibroblasts activated by various stimuli, such as myocardial injury and heart failure, mainly produce extracellular matrix (ECM), which play an essential role in cell signaling and fibrotic responses [12, 13, 14]. A number of evidences indicate that Ca²⁺-dependent pathways are involved in fibroblasts proliferation, differentiation and ECM production [15]. Among them, transient receptor potential (TRP) protein superfamily is composed of cation channels in various cells such as cardiac fibroblasts [16]. They play an integral role in pathophysiological and physiological conditions. Hatano et al. [17] showed the vanilloid type 4 transient receptor potential channel (TRPV4) in rat cardiac fibroblasts, by using 4 alpha-phorbol 12,13-didecanoate, a TRPV4 agonist. In recent years, we have reported that TRPA1 is identified in human cardiac fibroblasts (hCFs), and TRPA1 channel blocker inhibited Ca²⁺...
influx by methylglyoxal (MG), then inhibiting the proliferation [18]. Okada et al. [19] also reported that the TRPA1 channel inhibitor reduced fibrosis and inflammation by inhibiting transforming growth factor-β1 signaling cascades in ocular fibroblasts. Thus, these studies have shown that TRPA1 channel may become a potential therapeutic target for fibrotic reaction. However, Ca\(^{2+}\)-dependent pathways in hCFs are still not clearly known.

Therefore, the present study is to clarify whether TRPA1 channels can function in hCFs, by using the \([\text{Ca}^{2+}]_i\) measurements and the patch-clamp techniques.

2. Methods

2.1. Cell culture of hCFs

Human adult ventricular cardiac fibroblasts (ACBRI 5118) were commercially purchased from DS PHARMA BIOMEDICAL Co., Ltd (Osaka, Japan) [20]. The cells were cultured in Cell System Corporation with defined cell boost (CSC Catalog 4ZO-50) and 10% serum (37° C under 5% CO\(_2\)). The cells at confluence were detached using 0.25% trypsin and cultured into the medium. The medium was changed twice a week. Cells at passage 3–6 were stripped from the culture dish with 0.25% trypsin and used for the experiments.

2.2. Drugs

The composition of control Tyrode solution was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 0.53, glucose 5.5, and N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid (HEPES)-NaOH buffer 5.5 (pH 7.4). The Ca\(^{2+}\)-free bath solution contained EGTA (0.5 mmol/L) in the Ca\(^{2+}\)-free Tyrode solution. In N-methyl-d-glucamine\(^{+}\) (NMDG\(^{+}\)) solutions, extracellular Na\(^{+}\) was changed with equimolar concentration of N-methyl-d-glucamine\(^{+}\) (NMDG\(^{+}\)). The patch pipette was filled with the following Cs\(^{+}\)-internal solution (in mM): CsCl 140, EGTA 5, MgCl\(_2\) 2, Na\(_2\)ATP 3, GTP 0.1, and HEPES-CsOH buffer 5 (pH 7.2). Allyl isothiocyanate (AITC), a selective TRPA1 agonist [21, 22], was purchased from Wako Pure Chemical Industries (Osaka, Japan). MG and ruthenium red (RR), a non-selective membrane-impermeable cation channel inhibitor including TRPA1 [1, 22, 23, 24, 25], were obtained from Nakarai Tesque (Kyoto, Japan). HC030031 (2-(1,3-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl) N-(4-isopropylphenyl) acetamide), a selective TRPA1 blocker [26], was obtained from Abcam Biochemicals (Cambridge, UK). Fura-2 acetoxymethyl ester (fura-2/AM, molecular probes) and lanthanum (La\(^{3+}\)) were obtained from Dojin Chemicals.

2.3. Measurement of \([\text{Ca}^{2+}]_i\)

\([\text{Ca}^{2+}]_i\) was measured with the fluorescence method as previously reported [27, 28]. The fibroblasts were trypsinized, washed, and adjusted to a cell density of 10\(^6\) cells/ml and loaded with fura-2 AM (2 μM) (37° C under 5% CO\(_2\)). Then the fura-2AM containing medium was removed, and fluorescent cells in suspensions were measured while stirred in a cuvette placed by a spectrofluorometer (CAF-100; Jasco, Tokyo, Japan). The excitation wavelengths were 340 and 380 nm, and the emission was 500 nm. The fluorescence intensity ratio of F340/F380 was used as an indicator of \([\text{Ca}^{2+}]_i\).

2.4. Recording techniques

Membrane currents were recorded under the whole-cell voltage clamp techniques with a patch-clamp amplifier (EPCC-7, List Electronics, Darmstadt, Germany) [29, 30]. The patch electrode had a tip resistance of 3–5 MΩ. All data were acquired, and analyzed on Power Macintosh.
7100/80 by using the PULSE + PULSEFIT software (HEKA Electronic) and Igor PRO (Wave Metrics, Lake Oswego, OR).

### 2.5. Immunoﬂuorescence staining

The immunoﬂuorescence staining was performed on hCFs with anti-TRPA1 (extracellular) antibody (ACC-037, Alomone Labs, Jerusalem, Israel). The hCFs were cultured on collagen I-coated chamber slide (177402, Nunc, Rochester, NY), ﬁxed with 2% paraformaldehyde in phosphate-buffer saline (PBS). The cells were blocked with PBS containing 1% bovine serum albumin. They were incubated for overnight with primary antibody in the presence or absence of blocking peptide (BLP-CC037, Alomone Labs) diluted with 1% bovine serum albumin in PBS into 1:400. Alexa Fluor 555 labeled Donkey anti-rabbit IgG antibody (A31572, ThermoFisher SCIENTIFIC, Waltham, MA) diluted 1:1000 was used to visualize the channel expression. The hCFs were mounted by using Fluoroshield with DAPI (ImmunoBioScience Corp, Mukilteo, WA) to visualize nuclei. A ﬂuorescence microscope system (BZ-X700, KEYENCE Corp, Osaka, JAPAN) was used for observations.

### 2.6. Western blotting

The hCFs were washed in PBS, and lysed in RIPA buffer with 1% Nonidet P-40, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, and 1% protease inhibitor cocktail (25955, Nacalai Tesque, Kyoto, Japan). Samples were centrifuged at 14,000 rpm (4 °C, 25 min), and total proteins in the supernatant were separated. The ProteoExtract™ Native Membrane Protein Extraction Kit (Merck KGaA, Darmstadt, Germany) was used to isolate native membrane protein. The isolated proteins were kept at -80 °C before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated on a 7% polyacrylamide gel at 250 V and transferred onto a PVDF membrane (Amersham Hypond-P, Cytiva, Tokyo, Japan) at 130 mA with semi-dry method for 60 min. After that, the membrane was blocked with Blocking One (Nacalai Tesque, Tokyo, Japan) for 1 h at room temperature. It was then exposed to anti-TRPA1 antibody (NB110-40763, Novus Biologicals, Littleton, CO, USA) diluted to 1:500 with Signal Enhancer HIKARI Solution A (Nacalai Tesque, Kyoto, Japan) at 4 °C overnight. Subsequently, the probed membrane was washed three times by using 0.1% tween20 in Tris-buffered saline (TBS-T) and incubated with anti-rabbit IgG linked to peroxidase (7074s, Cell Signaling Technology, Danvers, MA, USA) diluted to 1:2000 with Signal Enhancer HIKARI Solution B (Nacalai Tesque, Kyoto, Japan). After washes, the bound antibodies were revealed via Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan) and detected by a LuminoGraph I (ATTO, Tokyo, Japan).

### 2.7. RNA extraction, and reverse transcription-PCR (RT-PCR)

All cellular RNAs were extracted from hCFs using ISOGEN II (NIPPON GENE CO. LTD. Tokyo, Japan). For RT-PCR, complementary DNA (cDNA) was synthesized from 40ng of total RNA for 10μl of each reverse transcription reaction with a ReverTra Dash (TOYOBO CO., LTD. Osaka, Japan). The reaction mixture was subjected to PCR ampliﬁcation with speciﬁc forward and reverse oligonucleotide primers for 40 cycles with heat denaturation, annealing, and extension. The PCR products were size-fractionated on 2% agarose gels and controlled under the blue LED light. The primers were determined based on the sequence of human TRPA1. The forward primer sequence was 5’-TGTTGACAAATA-GACCCAGT-3’ and the reverse primer sequence was 5’-TGGGCACCCCTTAGAGTAGC-3’.

Figure 2. TRPA1 mRNA and protein expression. A: Expression of a subunit gene of TRPA1 channel mRNA in hCFs. Left, marker. B: Western blotting of TRPA1 protein isolated from total cells. C: Western blotting of TRPA1 protein isolated from membrane fraction. Two representative data (C1 and C2) are shown.
2.8. Data analysis

All values are expressed as means ± S.D.

3. Results

3.1. TRPA1 mRNA and protein in hCFs

The expression of TRPA1 channel was confirmed by immunocytochemistry (Figure 1A). The hCFs were counterstained with DAPI to visualize nuclei, and double staining of nucleus and TRPA1 protein. TRPA1 staining was blocked by coincubation of TRPA1 antibody with a blocking peptide, which decreased TRPA1 staining (Figure 1B). The channel expression was not observed in negative controls with normal rabbit IgG, when primary antibody was absent (Figure 1C).

Next, we examined TRPA1 mRNA expression in hCFs (Figure 2A). TRPA1 mRNA was detected in hCFs. The amplitude of cDNA fragments was of predicted molecular size (317 bp), identical to cDNA fragments amplified from reverse transcribed mRNA (Figure 2A). In addition, to identify the channel protein expression, Western blot analysis of total cell protein was performed. A specific antibody for TRPA1 channel protein showed a strong band at approximately 100 kDa (Figure 2B). Similarly, Western blot analysis of membrane protein showed a band (Figure 2C). These immunocytochemistry and western blotting studies revealed the TRPA1 protein expression in hCFs.

3.2. Effects of allyl isothiocyanate (AITC) on \([\text{Ca}^{2+}]_i\)

To examine whether TRPA1 can function in hCFs, \([\text{Ca}^{2+}]_i\) was measured. Figure 3 shows the effects of AITC on \([\text{Ca}^{2+}]_i\). In the presence of extracellular \(\text{Ca}^{2+}\), the application of AITC (200 μM) induced a rapid increase of \([\text{Ca}^{2+}]_i\), and subsequently decreased to a steady-state level (Figs. 3Aa & Ca). In contrast, AITC did not affect \([\text{Ca}^{2+}]_i\) in the absence of extracellular \(\text{Ca}^{2+}\) (Figure 3B). Furthermore, we examined the effects of various blockers on AITC-induced \([\text{Ca}^{2+}]_i\) rise. First, the effects of RR, a non-selective TRP blocker including TRPA1 [1, 22, 23, 24, 25], were investigated (Figure 3A). The additional of RR (30 μM, Figure 3Ab) with AITC markedly inhibited AITC-induced \([\text{Ca}^{2+}]_i\), compared with control cells (Figure 3Aa). RR (30 μM) inhibited it by 95 ± 4% (Figure 3Da, n = 8). La\(^{3+}\) (0.5 mM), an agent known to block various subtypes of TRP channels [30, 31], also inhibited AITC-induced \([\text{Ca}^{2+}]_i\) rise (Figure 3Cb), compared with control cells (Figure 3Ca). La\(^{3+}\) inhibited it by 90 ± 12% (Figure 3Db, n = 5).

Figure 3. Effects of allyl isothiocyanate (AITC) on intracellular \(\text{Ca}^{2+}\) concentration \([\text{Ca}^{2+}]_i\). A: Effects of AITC and ruthenium red (RR) on \([\text{Ca}^{2+}]_i\). AITC (200 μM; Aa) increased \([\text{Ca}^{2+}]_i\). However, the addition of RR (30 μM; Ab) with AITC reduced the AITC-induced \([\text{Ca}^{2+}]_i\), compared with AITC only (Aa). B: Effects of AITC on \([\text{Ca}^{2+}]_i\), in the absence of extracellular \(\text{Ca}^{2+}\). C: Effects of La\(^{3+}\) (0.5 mM) on \([\text{Ca}^{2+}]_i\). The additional application of La\(^{3+}\) with AITC (Bb) abolished the AITC-induced \([\text{Ca}^{2+}]_i\), compared with AITC only (Ba). D: Inhibitory effects of RR (30 μM; Da) and La\(^{3+}\) (0.5 mM; Db) on AITC-induced \([\text{Ca}^{2+}]_i\) increase. The increased value in F340/F380 induced by AITC was obtained in the bath solution with and without drugs. The increased value in the absence of drugs is considered as 100%. The percent inhibition of these agents is illustrated in D.
3.3. AITC-induced nonselective cation currents in hCFs

We examined the effects of AITC (200 μM) on membrane currents in hCFs. The cells were held at -40 mV. The patch pipette was filled with the Cs⁺-internal solution. AITC (200 μM) increased the inward current at a holding potential of -40 mV (Figure 4A). The current-voltage (I-V) relationships were obtained by ramp pulses from -60 to +60 mV (200 ms duration). The I-V relationships were shown in control, in the presence of AITC (200 μM). The I-V relationships of the AITC-induced current was obtained by subtracting the control current from the current in the presence of AITC. It was almost linear with the reversal potential (Er) of approximately -3 ± 2 mV (n = 5). La³⁺ markedly inhibited the AITC-induced current (Figure 4C). Furthermore, replacement of extracellular Na⁺ to NMDG⁺, an impermeable cation, decreased the AITC-induced current (Figure 4D). Effects of RR (30 μM) on AITC-induced currents were investigated by using ramp pulses (Figure 5A). The I-V relationships are shown in control, in the presence of AITC (200 μM), and AITC plus RR (30 μM). RR markedly inhibited the AITC-induced currents at any potential with Er of approximately +0 mV. However, RR did not significantly affect the control (background) I-V relationships in the absence of AITC (data not shown).

We have previously showed that MG increased [Ca²⁺], in hCFs [17]. Figure 5B shows the I-V relationships of MG-induced currents. Similarly, in cases with AITC (Figure 5A), MG (100 μM) induced nonselective cation currents with Er of approximately +0 mV (Figure 5B). La³⁺ (0.5 mM) completely abolished the MG-induced currents. However, La³⁺ further decreased the background current with Er of approximately +0 mV. HC030031 (10 μM) significantly decreased MG-induced nonselective cation currents (Figure 5C).

4. Discussion

The major findings of the present study are as follows. 1) AITC increased [Ca²⁺]; and activated nonselective cation currents with Er of approximately +0 mV. 2) La³⁺ and RR, a general nonselective TRP channel blocker, inhibited the AITC-induced current and calcium responses. 3) Existence of TRPA1 channel mRNA and proteins were shown by the RT-PCR analysis, immunocytochemistry and western blotting studies. These results provide the evidence for functional existence of nonselective cation channels, possibly via TRPA1 channels, activated by AITC in hCFs, by using patch clamp techniques.

TRPA1 was first isolated by a screen of transformation-sensitive protein in cultured fibroblasts [32]. We have recently showed the evidence for TRPA1 channel mRNA, and protein expression in hCFs, and MG increases [Ca²⁺], that is blocked by HC030031 or by siRNA-induced knockdown of TRPA1 [18]. The present study also confirmed it by using the conventional RT-PCR analysis, western blotting and immunocytochemical studies. The western blotting analysis of membrane protein also revealed a single band of TRPA1 protein, suggesting membrane expression of TRPA1 channel in hCFs. Similarly, Conklin et al. [9] showed TRPA1 expression in cardiomyocytes by the immunocytochemistry and western blotting analysis using the same antibody of TRPA1. TRPA1 responds to pungent plant compounds such as AITC and acrolein [33]. AITC is known as a selective and most potent agonist of TRPA1 channels [21, 22]. Activation of TRPA1 by AITC occurs through the covalent modification of cysteine residues located on intracellular amino (N)-terminus [34, 35]. And a lysine residue also contributes to AITC-induced activation of TRPA1 [34]. The present study showed the direct evidence for TRPA1 channel currents elicited by AITC and MG in hCFs. Replacement of extracellular Na⁺ to NMDG⁺ markedly reduced the AITC-induced current. Furthermore, La³⁺ and RR, a general TRP channel blocker, inhibited the AITC-induced currents. The I-V relation of the AITC-induced current was almost linear with the reversal potential (Er) of approximately +0 mV. Outward rectification has been reported to be commonly observed when TRPA1 channels are activated by chemical agonists, intracellular Ca²⁺, and low temperature [1, 35, 36, 37]. However, it is also not uncommon that the linear I-V relationships are...
observed between −100 and +100 mV when the channel is strongly activated [38].

TRPA1 has been reported as a highly Ca-permeable nonselective cation channel [1, 39, 40] with an unitary conductance of 98 pS [22]. Recently, using the cell-attached patch techniques, Conklin et al. [8] showed single channel activity of the sarcolemmal patches enhanced by cinnamaldehyde, a known TRPA1 agonist. In the present study, AITC did not affect \([\text{Ca}^{2+}]_i\) in the absence of extracellular \([\text{Ca}^{2+}]_o\), but in the presence of extracellular \([\text{Ca}^{2+}]_o\), AITC increased \([\text{Ca}^{2+}]_i\). The AITC-induced \([\text{Ca}^{2+}]_i\) was blocked by RR or \(\text{La}^{3+}\) in a similar manner as AITC-induced nonselective cation currents. These results provide the direct evidence for functional existence of TRPA1 channels in hCFs, by using \([\text{Ca}^{2+}]_i\) measurements and the patch-clamp techniques. However, it remains unclarified whether basal activity of TRPA1 channels without agonists can contribute to basal \([\text{Ca}^{2+}]_i\). RR failed to affect basal \([\text{Ca}^{2+}]_i\) and background current, proposing that TRPA1 channels are less activated during basal conditions, compared with agonist stimulation. This is somewhat compatible with the following results. Andrei et al. [6, 7] reported that TRPA1 channel was present in cardiomyocytes and AITC increased the contractile function and \([\text{Ca}^{2+}]_i\) in cardiomyocytes, but not cardiomyocytes prepared from \(\text{Trpa1}^{-/-}\) mice. However, Bodkin et al. [40] showed that ventricular contraction under basal conditions did not change in \(\text{Trpa1}^{-/-}\) mice, suggesting that TRPA1 channels in basal state do not modify cardiac function. In the present study, \(\text{La}^{3+}\) further inhibited the background current with \(E_r\) of approximately +0 mV, suggesting that hCFs have background nonselective cation channels other than TRPA1 as previously reported in various types of cells [30, 41, 42]. The further detailed studies including single channel recordings are required to identify the molecular basis of the background currents in hCFs.

Several lines of evidence have shown that Ca\(^{2+}\) entry plays an essential role in fibroblasts' biological functions [15, 43]. Previous studies have also reported that TRPA1 is associated with fibrosis in various tissues such as cardiac tissue [19, 44, 45]. Furthermore, cardiac fibroblasts not only produce ECM, but also mechanically and electrically bind to cardiomyocytes, affecting the electrical activity [46, 47]. Because TRPA1 is a nonselective cation channel, Na\(^+\) and Ca\(^{2+}\) pass the TRPA1 channel, resulting in membrane depolarization and action potential generation [48]. Thus, the activation of TRPA1 in hCFs may contribute to affect electrical activity in cardiomyocytes under the pathophysiological conditions.
conditions. The TRPA1 channel has also been demonstrated to play an important role in mediating several pathophysiological conditions such as tissue injury and inflammation [2, 5, 9, 49]. Further, mounting evidence suggests that TRPA1 may be a key gatekeeper in regulating the inflammatory response [49] and oxidative stress [50], and play an important role in the pathophysiology of cardiac disease [9, 40]. Wang et al. [9] have reported that TRPA1 expression was increased in human failing heart. Conklin et al. [8] also showed that TRPA1 in cardiomyocytes is a target of lipid peroxidation-derived aldehydes, such as acrolein generated during ischemia-reperfusion, and TRPA1 activation may contribute to cardiac ischemia-reperfusion injury. Thus, TRPA1 appears to be involved in the regulation of cardiac function including fibroblasts under various conditions (myocardial infarction, heart failure, etc.) [5, 11]. The further research is required to clarify the pathological roles of TRPA1 in hCFs.

We should acknowledge several limitations. We had used the commercially cultured healthy hCFs in this experiment. Ion channels, receptor function and calcium regulation mechanisms in hCFs may be changed after the isolation and during the culture conditions. Furthermore, we used RR and La3+, a non-specific blocker, and the further studies using a more specific TRPA1 blocker and single channel recording techniques as well as freshly isolated hCFs are required to clarify the identification of TRPA1 channels in hCFs.

The present results provide the first evidence for functional Ca2+-permeable nonselective cation currents activated by AITC and MG, possibly via TRPA1 in hCFs.

Declarations

Author contribution statement

T. Nakajima: Performed the experiments; Wrote the paper.
G. Oguri, H. Kikuchi and S. Obi: Conceived and designed the experiments; Performed the experiments.
F. Nakamura and I. Komuro: Contributed reagents, materials, analysis tools or data.

Funding statement

S. Obi was supported by JSPS KAKENHI (18K08084) and T. Nakajima was supported by JSPS KAKENHI (19H03981).

Data availability statement

Data included in article supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05816.

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T. Nakajima: Performed the experiments; Wrote the paper.
G. Oguri, H. Kikuchi and S. Obi: Conceived and designed the experiments; Performed the experiments.
F. Nakamura and I. Komuro: Contributed reagents, materials, analysis tools or data.

Funding statement

S. Obi was supported by JSPS KAKENHI (18K08084) and T. Nakajima was supported by JSPS KAKENHI (19H03981).

Data availability statement

Data included in article supplementary material/referenced in article.

Declaration of interests statement

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Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2020.e05816.

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