学　位　論　文

Negative chronotropic and inotropic effects of lubiprostone on iPS cell-derived cardiomyocytes via activation of CFTR

（ルビプロストンはCFTRチャネルの活性化を介してiPS細胞由来心筋細胞において陰性変時作用および陰性変力作用を示す）

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論　文　内　容　要　旨（和文）

学位論文題名	Negative chronotropic and inotropic effects of lubiprostone on iPS cell-derived cardiomyocytes via activation of CFTR ルビプロストンは CFTR チャネルの活性化を介して iPS 細胞由来心筋細胞において陰性変時作用および陰性変力作用を示す

背景：ルビプロストンは、腸管や鼻粘膜において CFTR チャネルや ClC-2 チャネルを活性化させることが報告されている。特に腸管においては、ルビプロストンは CFTR チャネルを活性化させ体液分泌を促進させることから、慢性便秘薬として使用されている。しかしながら、これらのチャネルの発現が確認されている心筋細胞において、ルビプロストンの効果は検証されていない。

iPS 細胞は自己複製能と多分化能を持つため、心筋細胞をはじめとする多種多様な細胞に分化できる能力を持っている。さらに、自己由来の iPS 細胞を用いることで、一塩基多型による個体差を考慮した薬効評価が可能である。これらの理由から、iPS 細胞から分化誘導させた心筋細胞は実験動物の代替えになり、ルビプロストンの効果を明らかにするための有効なツールになり得ると考える。

本研究の目的は、iPS 細胞から分化誘導した心筋細胞を利用することで、心筋細胞におけるルビプロストンの効果を明らかにすることである。

方法:iPS 細胞から三胚葉を形成できる胚様体を介して心筋細胞へ分化誘導させ、RT-PCR と免疫染色によって分化細胞の性状解析を行った。また、βアドレナリン作動薬であるイソプロテレノールと Ca^{2+}チャネルブロッカーであるニトレンジピンを添加し、心筋細胞の自発的拍動数及び収縮力の変化を動画や ImageJ で解析することで、iPS 細胞由来心筋細胞の生理機能を解析した。さらに、自発的拍動数及び収縮力におけるルビプロストンの作用を動画や ImageJ で解析すると共に、GlyH（CFTR チャネルブロッカー）と $CdCl_2$（ClC-2 チャネルブロッカー）を用いることで、ルビプロストンの標的になっている Cl^-チャネルも検証した。

結果：iPS 細胞から分化誘導した心筋細胞は、心筋マーカーである GATA4、心筋トロポニン I、心筋トロポニン T、コネキシン 43 が発現していた。さらに、ルビプロストンの標的クロライドチャネルである CFTR チャネルや ClC-2 チャネルの発現も確認された。また、イソプロテレノールやニトレンジピンは、iPS 細胞由来心筋細胞の自発的拍動数及び収縮力を変化させたことから、生理的に機能性を有する心筋細胞であることが確認された。さらに、ルビプロストンは iPS 細胞由来心筋細胞の自発的拍動数及び収縮力を低下させ、これらの効果は GlyH の添加によって抑制された。また、ルビプロストンは、マウスから採取した胎仔心筋細胞や成体心筋細胞においても自発的拍動数及び収縮力を低下させ、GlyH の添加によってその効果を抑制した。

結論：ルビプロストンは、心筋細胞において CFTR チャネルを活性化させ、陰性変時作用及び陰性変力作用を示したことから、心不全、虚血、不整脈などの心疾患の治療に活用できる可能性を示す。

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ABSTRACT

Background

Lubiprostone (LBP) is a novel chloride channel opener that has been reported to activate chloride channel protein 2 (ClC-2) and cystic fibrosis transmembrane conductance regulator (CFTR). LBP facilitates fluid secretion by activating CFTR in the intestine and is used as a drug for treating chronic constipation. While ClC-2 and CFTR expression has been confirmed in cardiomyocytes (CMs), the effect of LBP on CMs has not yet been investigated. Thus, the present study aimed to investigate the effect of LBP on CMs using mouse-induced pluripotent stem (iPS) cell-derived CMs (iPS-CMs).

Methods

Mouse iPS cells were induced into CMs through embryoid body formation. The iPS-CMs were compared with CMs isolated from adult and fetal mice in terms of gene expression, spontaneous beating rate, and contraction ratio in response to LBP.

Results

In the iPS-CMs, the mRNA expression of the undifferentiated cell markers, *Rex1* and *Nanog,* decreased, whereas the expression of the unique cardiomyocyte markers, *GATA4*, *cardiac troponin I (cTnI*), and *cardiac troponin T*, increased. Immunostaining showed that the localization of cTnI and connexin-43 (Cx43) in the iPS-CMs was similar to that in the primary fetal CMs (FCMs) and adult CMs (ACMs). Furthermore, the expression of *CFTR* and *ClC-2* was confirmed in iPS-CMs. LBP decreased the spontaneous beating rate of the iPS-CMs and FCMs, and decreased the contraction ratio of the iPS-CMs and ACMs. The reduction in the beating rate and contraction ratio caused by LBP was inhibited by glycine hydrazide, which is a CFTR inhibitor.

Conclusion

These results suggest that LBP activates CFTR in CMs and has negative chronotropic and inotropic effects on CMs. LBP may be useful for treating cardiac diseases such as heart failure, ischemia, and arrhythmia.

Keywords: iPS cells; Cardiomyocytes; Lubiprostone; ClC-2; CFTR

Background

Lubiprostone (LBP) is an activator of the chloride channel protein 2 (ClC-2) and cystic fibrosis transmembrane conductance regulator (CFTR) channels. In the intestinal epithelium, LBP binds to prostanoid receptor and increases intracellular cyclic adenosine monophosphate (cAMP), and CFTR activated by cAMP-dependent protein kinase A (PKA) promotes intracellular Cl^- and fluid secretion to the intestinal tract; therefore, LBP is used as a chronic constipation drug [1]. On the other hand, it has also been reported that LBP directly binds to ClC-2, and ClC-2 activated by LBP causes intracellular Cl^- and fluid secretion to the intestinal tract [2]. However, Bijvelds et al. has indicated that LBP is not involved in the activation of ClC-2, since LBP fails to induce Cl^- secretion in human and mouse colon when CFTR is not present [3]. In murine nasal airway epithelia, LBP independently activates both ClC-2 and CFTR, but the activation mechanism of ClC-2 and CFTR caused by LBP has not been demonstrated [4, 5]. Furthermore, LBP has been reported to bind to the prostaglandin E receptor 1, which is one of the prostanoid receptor, and increase the contraction of smooth muscle through the prostaglandin E receptor 1 signaling pathway in the small intestine [6]. While ClC-2 and CFTR expression has been confirmed in cardiomyocytes (CMs), the effect of LBP on CMs has not yet been determined.

Cation channels, such as sodium, potassium, and calcium channels in CMs have been well studied, and antiarrhythmic or cardiotonic drugs have been developed that modulate these channel activities [7–9]. Although the function and role of anion channels have also been investigated in CMs using knockout mice, the function and role of chloride channels have not been as extensively investigated as those of other anion channels. For example, it has been previously reported that the role of the ClC-2 channel is to control the activation of cardiac pacemaker and heart rate under pathological conditions [10]. Additionally, it is well known that CFTR, which is a chloride channel responsible for cystic fibrosis, is expressed in CMs. A previous study reported that the infarct size induced by ischemia/reperfusion injury of the heart in CFTR knockout mice was larger than that in wild-type mice, and that the cause was loss of cell volume regulation due to CFTR dysfunction [11]. However, the role of ClC-2 and CFTR in CMs has not

yet been fully elucidated.

Induced pluripotent stem (iPS) cells can be reprogrammed by the effect of several stemness factors, such as Oct3/4, Nanog, Sox2, Klf4, c-Myc, and Lin28 [12–14], and have the ability of self-renewal and pluripotency. Thus, they are similar to embryonic stem (ES) cells, which are isolated from the inner cell mass of a blastocyst [15, 16]. ES cells can be created by destroying the early embryo, and raise ethical issues. Furthermore, cells differentiated from ES cells may cause rejection after cell transplantation. Therefore, the use of iPS cells generated from individual somatic cells can overcome the disadvantages of using ES cells; iPS cells are also expected to be used as powerful tools for regenerative medicine to repair damaged tissues [13]. Furthermore, genomic integration-free iPS cells produced using episomal vectors [17], Sendai viruses [18], or the piggyBac system [19] are regarded as safer because they avoid the risk of tumor formation. iPS cells can be differentiated into various cell types under the appropriate conditions, and CMs differentiated from iPS cells are effective tools not only for cell and tissue replacement, but also for pharmacological and toxicological testing. The use of self-derived iPS cells also does not cause differences in drug effects among individuals due to genetic single nucleotide polymorphisms. For these reasons, iPS cell-derived cardiomyocytes (iPS-CMs) are considered to be an effective source of cells for studying the effects of LBP on CMs and the roles of ClC-2 and CFTR. However, to accurately demonstrate the effects of LBP on CMs using mouse iPS-CMs, iPS-CMs should be compared with primary CMs isolated from mouse hearts. Many researchers have reported that the characteristics of cells differentiated from mouse iPS cells are similar to those of primary cultured cells isolated from mice, and they have evaluated the pharmacological action of drugs on both types of cells [20–25]. For example, Kuzmenkin et al. reported that CMs generated from mouse iPS cells have the same functional properties as *in vivo* CMs. In their study, mouse iPS cells were differentiated into CMs *in vitro* and compared with primary CMs isolated from mouse hearts using immunocytochemistry, electrophysiology, and drug response. The characteristics of the iPS-CMs were similar to those of the primary CMs, and the response of the iPS-CMs treated with several drugs, such as the Na^+ channel blocker lidocaine

and the Ca^{2+} channel blocker nifedipine, was comparable to that of the primary CMs [20]. Sancho-Bru et al. induced mouse iPS cells into hepatocytes and examined the gene expression and functional characteristics of mouse iPS cell-derived hepatocytes by comparing them with those of primary hepatocytes isolated from mouse livers [22]. Yoshie et al. investigated whether mouse iPS cell-derived airway epithelial cells have the characteristics of native airway epithelial cells by comparing their ciliary beating frequency with that of native airway epithelial cells isolated from mouse airways [24].

The aim of the current study was to examine the effect of LBP on CMs using mouse iPS-CMs and primary CMs isolated from fetal and adult mouse hearts.

Methods

Animals

The present study was performed following the approval of the Animal Care and Use Committee in accordance with the Guidelines for Animal Experiments of Fukushima Medical University. Nine- to twelve-week-old BALB/cAJc1 mice (25–28 g) (CLEA Japan, Inc., Tokyo, Japan) and BALB/cAJc1 mice (CLEA Japan, Inc.) that had been pregnant for 17 days (29–34 g) were used in this study. There were 12 mice in each group. The mice had free access to food and water and were maintained in a temperature- (24–25 °C) and humidity- (50%) controlled room. Animals were also subjected to a twelve-hour shift of light and dark cycle.

Mouse iPS cell culture

The culture of mouse iPS cells (20D17), carrying Nanog promoter-driven GFP/IRES/puromycin-resistant gene, was performed as described previously [26]. Briefly, mouse iPS cells were cultured on mouse primary embryonic fibroblast feeder cells (Oriental Yeast Co., Ltd., Tokyo, Japan) in Dulbecco's Modified Eagle Medium (DMEM; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 15% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Massachusetts, USA), 2 mM L-glutamine (FUJIFILM Wako Pure Chemical Corporation), 100 μM non-essential amino acids (FUJIFILM Wako Pure Chemical Corporation), 100 μM 2-mercaptoethanol (2-ME; Thermo Fisher Scientific Inc.), 10^3 units/mL of leukemia inhibitory factor (FUJIFILM Wako Pure Chemical Corporation), and penicillin-streptomycin (FUJIFILM Wako Pure Chemical Corporation).

Generation of CMs *from iPS cells*

Differentiation of CMs from mouse iPS cells was performed as previously described for ES cells [27]. Mouse iPS cells were treated with 0.25% trypsin (FUJIFILM Wako Pure Chemical Corporation) and dispersed into a single-cell suspension in embryoid body (EB) medium, which consisted of knockout DMEM (FUJIFILM Wako Pure Chemical Corporation),

20% FBS, 2 mM L-glutamine, 100 μM non-essential amino acids, 100 μM 2-ME, and penicillin-streptomycin. Next, the cells were cultured in a 24-well plate (Greiner Bio-One Co., Ltd., Tokyo, Japan) to allow EB formation using the hanging drop method. After five days, the formed EBs were transferred to a gelatin-coated 24-well plate (Greiner Bio-One Co., Ltd.) and cultured in EB medium. The EB medium was exchanged every two days.

Reverse transcription polymerase chain reaction

Total RNA was isolated using an RNeasy Mini kit (Qiagen K. K., Hilden, Germany) according to the manufacturer's protocol. For cDNA synthesis, Superscript Reverse Transcriptase (TaKaRa Bio Inc., Shiga, Japan) was used, and PCR was performed using Ex Taq (TaKaRa Bio Inc.). The PCR cycling conditions were as follows: 30 or 35 cycles at 94 °C for 30 s, annealing at the temperatures specified for each primer set for 30 s; 72 °C for 30 s; and a final cycle at 72 °C for 7 min. The primer sets and annealing temperatures are shown in Table 1.

Immunocytochemistry

Mouse iPS-CMs and primary CMs were immersed in 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation) for 30 min at room temperature, and then washed with phosphate buffered saline (PBS; Thermo Fisher Scientific Inc.). The cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich Co. LLC, Missouri, USA) for 30 min and then washed with PBS. The cells were then treated with 4% Block Ace Powder (DS Pharma Biomedical Co., Ltd., Osaka, Japan) in PBS for 30 min, followed by staining via incubation at 4 °C overnight with specific antibodies against cardiac troponin I (cTnI) and connexin-43 (Cx43) (Abcam plc., Cambridge, UK). Following this incubation, the cells were incubated with Alexa Fluor 488, 568, and DAPI (Thermo Fisher Scientific Inc.). Fluorescent images were captured with a confocal laser scanning microscope A1R (Nikon Instech, Co., Ltd., Tokyo, Japan).

Isolation and culture of CMs from adult mouse hearts

The CMs of adult mice (ACMs) were isolated using the Langendorff-free method, as described in a previous study [28]. The buffers and medium for the isolation and culture of the CMs were also prepared according to the same report. In brief, the mice were euthanized with an anesthetic containing 5 mg/kg butorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), 0.3 mg/kg medetomidine (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), and 4 mg/kg midazolam (Astellas Pharma Inc., Tokyo, Japan), after which their chests were opened. EDTA buffer (130 mM NaCl, 10 mM taurine, 10 mM glucose, 10 mM HEPES, 10 mM 2,3-Butanedione monoxime, 5 mM EDTA, 5 mM KCl, and 0.5 mM NaH_2PO_4; These reagents were purchased from FUJIFILM Wako Pure Chemical Corporation and Sigma-Aldrich Co. LLC) was injected into the right ventricle, following which the heart was excised and placed in a 60 mm dish (Greiner Bio-One Co., Ltd.). EDTA buffer, perfusion buffer (130 mM NaCl, 10 mM taurine, 10 mM glucose, 10 mM HEPES, 10 mM 2,3-Butanedione monoxime, 5 mM KCl, 1 mM MgCl2, and 0.5 mM NaH2PO4; MgCl2 was purchased from FUJIFILM Wako Pure Chemical Corporation), and collagenase buffer (0.5 mM collagenase type II, 0.5 mM collagenase type IV, and 0.05 mg/ml protease type XIV; These enzymes were purchased from Worthington Biochemical Corporation, New Jersey, USA and Sigma-Aldrich Co. LLC) were continuously injected into the left ventricle, and then the heart tissues were dissociated by gentle pipetting. Next, stop buffer (perfusion buffer and 5% FBS) was added to the 60 mm dish, and the cell suspension was transferred to a 50 mL tube (Greiner Bio-One Co., Ltd.) through a 100 μm cell strainer (Corning Inc., New York, USA). After the dissociated CMs were gradually exposed to calcium, they were plated and cultured on a cell culture dish. The culture medium was changed every two days.

Isolation and culture of CMs from fetal mouse hearts

Isolation of CMs from fetal mice (FCMs) was performed according to a previous study [29]. Briefly, 17-day pregnant BALB/cAJc1 mice (CLEA Japan, Inc.) were euthanized by cervical dislocation, and the uterus was isolated. Euthanasia by cervical dislocation has been widely used in animal experiment, since it can cause a fast and painless death and avoid damage

to the fetus [29–36]. Furthermore, it does not chemically contaminate tissues. The excised uterus was then washed with PBS containing penicillin-streptomycin, followed by removal of the fetus. The heart was removed from the fetus, washed with PBS, then minced and incubated in PBS containing 0.05% trypsin for 30 min at 37 °C. The digested heart tissue was homogenized by vortexing and then placed into a 15 mL tube (Greiner Bio-One Co., Ltd.) containing culture medium, which consisted of DMEM with 10% FBS and penicillin streptomycin. The 15 mL tube was centrifuged, and the cell pellet was suspended in culture medium. The cell suspension was then plated and cultured on a cell culture dish, and the culture medium was changed every two days.

Analysis of spontaneous beating rate

The spontaneous beating rates of the iPS-CMs and FCMs were analyzed using several chemical compounds. Isoproterenol (ISP) (0.4, 4, 40, and 400 nM) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), nitrendipine (NDP) (10 μM) (FUJIFILM Wako Pure Chemical Corporation), LBP (0.05, 0.5, and 5 μM) (Toronto Research Chemicals, Inc., North York, Canada), glycine hydrazide (GlyH) (5 μM) (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), and/or $CdCl_2$ (Cd) (10 μM) (FUJIFILM Wako Pure Chemical Corporation) were added to the culture medium. The iPS-CMs and FCMs treated with these chemical compounds were incubated for 20 min at 37 °C in a 5% CO_2 incubator (Panasonic Corporation, Osaka, Japan), and spontaneous beating of the iPS-CMs and FCMs was captured at 16 frames per second using an ORCA-ER EMCCD camera (Hamamatsu Photonics K.K., Shizuoka, Japan) connected to an IX-71 microscope (Olympus Corporation, Tokyo, Japan). Changes in beats per minute (bpm) were then analyzed.

Analysis of the contraction ratio

The iPS-CMs and ACMs treated with ISP, LBP, GlyH, or Cd were stimulated with monophasic pulses at 2 to 5 V/cm and a frequency of 0.5 Hz with pulse durations of 20 ms using

an electronic stimulator 3F46 (Nippon Avionics Co., Ltd., Tokyo, Japan). The contraction of iPS-CMs and ACMs was captured at 16 frames per second with an ORCA-ER EMCCD camera connected to an IX-71 microscope. The difference between the relaxation and contraction lengths (ΔL) of each iPS-CMs and ACMs was analyzed using ImageJ (National Institutes of Health, Maryland, USA) [37], and the ratio of ΔL to relaxation length was analyzed to determine the contraction ratio.

Statistical analysis

All statistical analyses were performed with EZR (Jichi Medical University Saitama Medical Center, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [38]. All experiments were performed independently at least five times. Data are expressed as the mean ± SD. All statistical significance was verified using Wilcoxon signed-rank sum tests and ANOVA. P values of < 0.05 were considered statistically significant.

Results

Analysis of the iPS-CMs characteristics

I induced iPS cells into CMs through EB formation (Fig. 1A). To confirm the state of the iPS-CMs, a Nanog-GFP reporter system was used as an efficient marker to mimic endogenous Nanog gene expression. Undifferentiated iPS cells showed GFP expression, which is controlled by the Nanog promoter. On the other hand, GFP expression was not observed in the beating region of iPS-CMs (Fig. 1B). I further investigated whether the expression of cardiomyocyte markers could be detected in iPS-CMs. The CM-specific markers *GATA4*, *cTnI*, cardiac troponin T (*cTnT*), and *Cx43* were expressed in iPS-CMs. Conversely, the expression of the undifferentiated markers *Rex1* and *Nanog* was weak in the iPS-CMs compared to that in the iPS cells. The expression of *CFTR* and *ClC-2* was successfully confirmed in iPS-CMs, FCMs, and ACMs (Fig. 1C). Next, to precisely examine whether iPS-CMs expressed CM markers, these cells were evaluated by double immunostaining. cTnI- and Cx43-positive cells were clearly observed in the iPS-CMs, FCMs, and ACMs (Fig. 1D). These results suggest that iPS-CMs had the characteristics of CMs.

Analysis of iPS-CMs and FCMs beating after LBP treatment

First, CMs were evaluated for their responses to ISP, which is a β-adrenergic agonist, and NDP, which is a calcium channel blocker. However, almost all of the ACMs isolated from the adult hearts lacked spontaneous beating. Therefore, spontaneous beating analysis was performed using iPS-CMs and FCMs. Spontaneous beating rate of the iPS-CMs treated with 400 nM ISP increased from 78.0 ± 34.3 to 120.0 ± 45.2 ($P = 0.0355$, Fig. 2A). Additionally, the bpm of the FCMs treated with 400 nM ISP increased from 123.9 ± 19.7 to 141.1± 22.4 ($P = 0.0138$, Fig. 2C). On the other hand, spontaneous beating of the iPS-CMs and FCMs treated with 10 μM NDP stopped (bpm was 0) ($P = 0.00013$, Fig. 2B and $P = 0.00000022$, Fig. 2D). Next, the effect of LBP on spontaneous beating was examined. The bpm of the iPS-CMs treated with 5 μM LBP decreased from 48.8 ± 7.1 to 34.5 ± 13.9 ($P = 0.00707$, Fig. 2E). To determine which chloride channels might contribute to the reduction of the beating rate, I examined the beating rate using Cd, a ClC-

2 channel blocker [39], and GlyH, a CFTR channel blocker [40]. While the addition of 10 μM Cd did not change the bpm reduction of the iPS-CMs treated with 5 μM LBP ($P = 0.00701$, Fig. 2F), the bpm of the iPS-CMs treated with 5 μM LBP and 5 μM GlyH slightly increased from 28.8 ± 11.6 to 35.8 ± 8.7 ($P = 0.0104$, Fig. 2G). When the FCMs were also treated with the same chemical compounds, the changes in bpm were similar to those of the iPS-CMs ($P = 0.00391$, Fig. 2H, $P = 0.0271$, Fig. 2I, and $P = 0.00781$, Fig. 2J). These results indicate that LBP decreased bpm not through the ClC-2 channel, but through CFTR.

Contraction analysis of iPS-CMs and ACMs after LBP treatment

The contraction ratio of iPS-CMs and ACMs was investigated. Since FCMs tightly adhered to the cell culture dish, their relaxation and contraction lengths could not be accurately measured. First, the contraction ratio of the iPS-CMs treated with ISP at various concentrations was investigated. The contraction ratio of the iPS-CMs significantly increased in the presence of 4 nM or 40 nM ISP ($P = 0.0048$ and $P = 0.0219$, respectively, Fig. 3A). Similarly, the contraction ratio of the ACMs treated with 4 nM or 40 nM ISP increased ($P = 0.0095$ and $P = 0.0276$, respectively, Fig. 3B), but decreased at 400 nM ISP. Next, the effect of LBP on the contraction ratio was examined. The contraction ratio of the iPS-CMs and ACMs treated with 5 μM LBP significantly decreased compared to that of the control ($P = 0.041$ for both, Fig. 3C and D). I found that Cd did not affect the contraction ratio of the iPS-CMs and ACMs treated with LBP, although GlyH abolished the reduction of the contraction ratio in response to LBP (Fig. 3E and F). These results indicate that LBP decreased the contraction ratio not through the ClC-2 channel, but through CFTR (Fig. 4).

Discussion

In the present study, I demonstrated that LBP decreased the spontaneous beating rate and contraction ratio of iPS-CMs, FCMs, and ACMs through CFTR, but not CIC-2. While several research groups have reported that LBP activates CFTR and ClC-2 in the intestinal and nasal airway epithelium [1–5], there have been no studies on the roles of LBP in CMs. To the best of my knowledge, the present study is the first to focus on the effect of LBP on CMs.

First, I induced iPS cells into CMs according to a previously-described simple protocol [27]. GATA4 is expressed in the early fetal heart, and is known to regulate the expression of several genes, such as atrial natriuretic peptide, brain-type natriuretic peptide, and α-type myosin heavy chain [41, 42]. Thus, GATA4 is an important factor in cardiac development, and is a specific marker of CM differentiation [43]. Additionally, cTnI and cTnT, which are present in myocardial fibers, are components of the troponin complex, and they control the contraction of CMs in response to changes in calcium ion concentration. Moreover, gap junctions formed from the connexin family have the function of synchronizing cardiac contraction with adjacent CMs. Cx43, which is a member of the connexin family, is the major protein expressed in the mammalian heart [44]. These markers are often used to evaluate the differentiation of CMs from iPS cells [45–48]. Therefore, I examined the expression of *GATA4*, *cTnI, cTnT*, and *Cx43* mRNA by RT-PCR and the expression of the cTnI and Cx43 proteins by immunostaining. The expression of these genes could be successfully detected in the iPS-CMs, and the cTnI and Cx43 proteins were localized to the appropriate region (Fig. 1C and D). Furthermore, the expression of CFTR and ClC-2, which are target chloride channels of LBP, was also examined by RT-PCR. The expression of these chloride channels was detected in iPS-CMs, FCMs, and ACMs (Fig. 1C). These results clearly indicate that iPS-CMs have the characteristics of native CMs.

ISP stimulates β-adrenergic receptors on CMs and increases the beating rate due to the increase in intracellular cAMP and calcium ion concentration [49, 50]. The calcium channel blocker NDP inhibits increases in intracellular calcium concentration. Therefore, I analyzed whether iPS-CMs responded to these chemical compounds. ISP increased the spontaneous

beating rate, and NDP completely suppressed the spontaneous beating of iPS-CMs (Fig. 2A and B). It has been reported that ISP increases not only the beating rate but also the contraction force [49, 50], and in the current study, the contraction ratio of iPS-CMs treated with ISP was also increased (Fig. 3A). The changes in the beating rate and contraction ratio of the FCMs and ACMs treated with these compounds were also similar to those of the iPS-CMs. The response of CMs treated with these chemical compounds is consistent with that reported in previous studies [51–53]. Therefore, my results indicate that iPS-CMs had the physiological and functional characteristics of native CMs.

The spontaneous beating rate of iPS-CMs and FCMs, and the contraction rate of iPS-CMs and ACMs decreased with LBP treatment (Fig. 2E, 2H, 3C, and 3D). These results are not consistent with those of a previous study [10] that used anti-ClC-2 antibody or ClC-2 knockout mice. The activation of ClC-2 in the heart has been reported to play important roles in the diastolic depolarization and firing of pacemaker cells [10]. However, LBP is known to activate not only ClC-2 but also CFTR in the nasal airway epithelium [4, 5]. Thus, I analyzed the spontaneous beating rate and contraction ratio after treatment with Cd and GlyH to investigate which chloride channels mainly contributed to the reduction of the spontaneous beating rate and contraction ratio. The CFTR blocker GlyH inhibited the effect of LBP, while the ClC-2 blocker Cd did not (Fig. 2H–J and 3E and F); therefore, LBP activated CFTR in CMs. It has been reported that the target chloride channel of LBP is different depending on the tissue type. For example, while LBP activates CFTR on the intestinal epithelium [1], it activates both CFTR and ClC-2 on the nasal epithelium [4, 5]. In the present study, I clarified that LBP activated CFTR in CMs. It raises possibility that the expression levels of CFTR and prostanoid receptor, which is one of the target molecules of LBP, might lead to dominant activation of CFTR (Fig. 4). The expression of prostanoid receptor has also been confirmed in CMs [54]. On the other hand, LBP has been reported to increase intracellular cAMP concentration in the intestinal and nasal epithelium. It is well known that an increase in intracellular calcium concentration due to an increase in intracellular cAMP concentration results in an increase in the beating rate and contraction ratio

of CMs. However, the results of the current study indicated that the spontaneous beating rate and contraction ratio of the CMs treated with LBP decreased compared to those of the untreated CMs. On the other hand, LBP has also been demonstrated to increase CFTR protein levels at the apical membrane through prostanoid receptor signaling [1, 55]. Judging from these reports, LBP bound to the prostanoid receptor and synthesized cAMP. PKA activated by cAMP caused the phosphorylation of CFTR and also increased CFTR protein at cell membrane. Thus, the depolarization and inward Ca^{2+} currents in LBP-treated CMs were disturbed by actively inward flowing Cl^- currents mediated by CFTR (Fig. 4). As a result, I consider that LBP decreased the spontaneous beating rate and contraction ratio of CMs. Further investigation is required to elucidate the more detailed mechanisms of action of LBP.

Conclusion

The results of the current study suggest that LBP decreased the spontaneous beating rate and contraction ratio of iPS-CMs by activating CFTR. Similarly, LBP had negative chronotropic and inotropic effects on FCMs and ACMs. Thus, iPS-CMs are considered to be an effective cell source for investigating the effects of chemical compounds and channel function. I tried to induce human iPS cells into CMs in order to investigate the effect of LBP on human CMs, but could not stably generate CMs from human iPS cells. In a future study, I will examine the effect of LBP on human CMs using CMs generated from human iPS cell by a stable induction method.

Abbreviations

LBP: lubiprostone

ClC-2: chloride channel protein 2

CFTR: cystic fibrosis transmembrane conductance regulator

cAMP: cyclic adenosine monophosphate

PKA: protein kinase A

CMs: cardiomyocytes

iPS: induced pluripotent stem

ES: embryonic stem

iPS-CMs: iPS cell-derived cardiomyocytes

DMEM: Dulbecco's Modified Eagle Medium

FBS: fetal bovine serum

2-ME: 2-mercaptoethanol

EB: embryoid body

PBS: phosphate buffered saline

cTnI: cardiac troponin I

Cx43: connexin-43

ACMs: adult cardiomyocytes

FCMs: fetal cardiomyocytes

ISP: isoproterenol

NDP: nitrendipine

GlyH: glycine hydrazide

Cd: $CdCl_2$

cTnT: cardiac troponin T

bpm: beats per minute

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Table 1. Primer sequences for RT-PCR

Gene	Accession	Sense primer sequence (5'-3')	Antisense primer sequence (5'-3')	Annealing temperature [°C]
Rex1	NM_009556	ACGAGTGGCAGTTTCTTCTTGGGA	TATGACTCACTTCCAGGGGGCACT	65
Nanog	AB903574	GCTTACAAGGGTCTGCTACT	CCTCAGGACTTGAGAGCTTT	60
GFP	NC_011521	AGAAGAACGGCATCAAGGTG	CTCGTTGGGGTCTTTGCTCA	65
GATA4	NC_000080	GCAGCAGCAGTGAAGAGATG	GCGATGTCTGAGTGACAGGA	60
cTnI	NC_000073	CTCCTCTGCCAACTACCGAG	CTCAAACTTTTTCTTGCGGC	60
cTnT	NC_000067	ATCCCCGATGGA	TTCCCACGAGTTTTGGAGAC	65
Cx43	NC_000076	TTGACTTCAGCCTCCAAGG	AATGAACAGCACCGACAGC	65
CFTR	NM_021050	AGTTTCCTGGACAGCTCA	CTAATGGCCTGCTGGAAGAT	60
ClC-2	NM_009900	CTTAGAGTGGGAAGAACA	CTCCTTTAGGGTGACAATCC	60
β-actin	NM_007614	TTCCTTCTTGGGTATGGAAT	GAGCAATGATCTTGATCTTC	60

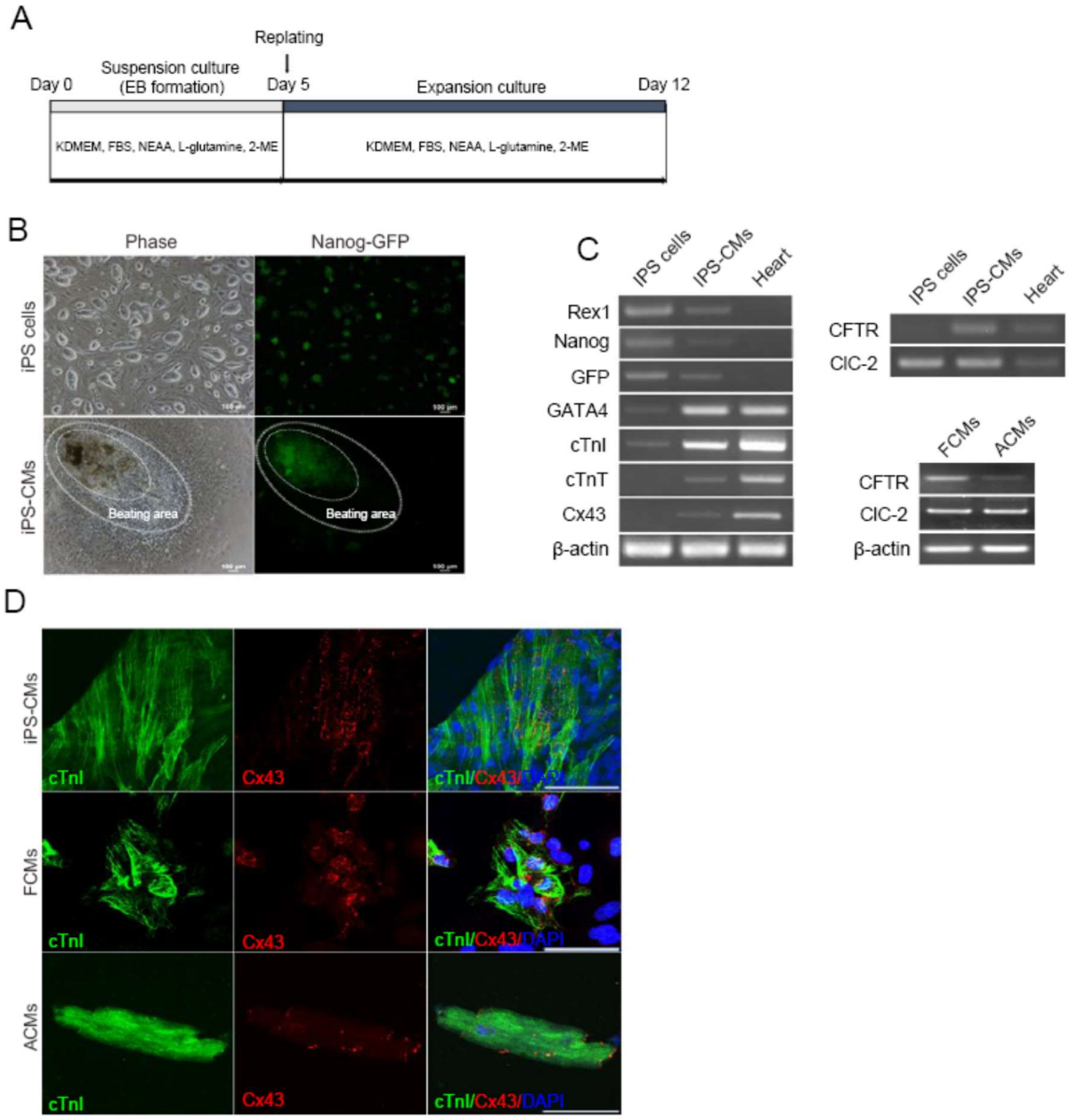

Fig. 1. Generation of CMs from iPS cells. A: Time course for the generation of CMs. B: Undifferentiated mouse iPS cells and iPS-CMs. Left panel: phase contrast, Right panel: Nanog-promoter-driven GFP. Bars indicate 100 μm. C: RT-PCR analysis of gene expression in undifferentiated iPS cells, iPS-CMs, and the adult mouse heart, using specific primers to identify undifferentiated markers, CM markers, chloride channels, and a ubiquitous housekeeping gene, β-actin, as indicated in the left column. D: Immunocytochemistry of iPS-CMs, FCMs, and ACMs using anti-cTnI and Cx43 antibodies. Bars indicate 50 μm.

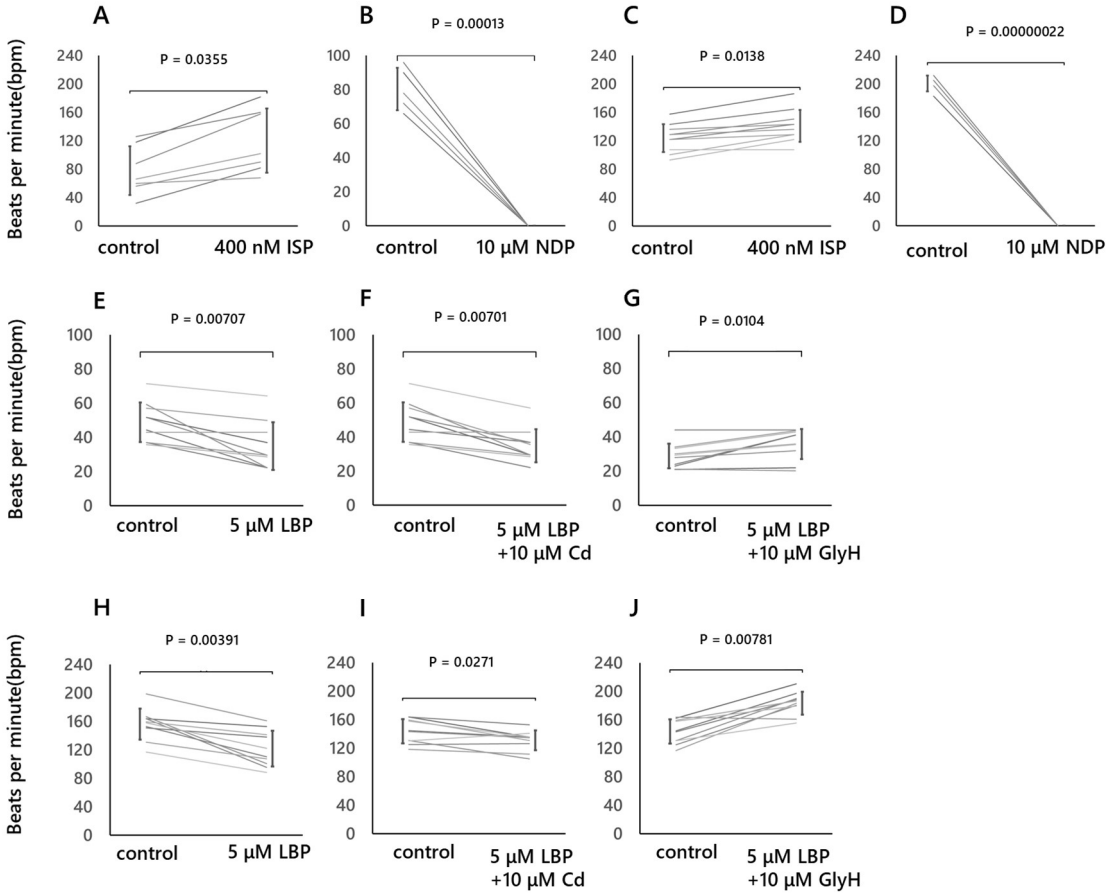

Fig. 2. Spontaneous beating rates in response to chemical compounds. A and B: Changes in the beating rate of iPS-CMs treated with 400 nM ISP (A) (n = 7) and 10 μM NDP (B) (n = 5). C and D: Changes in the beating rate of FCMs treated with 400 nM ISP (C) (n = 7) and 10 μM NDP (D) (n = 5). E–G: Changes in the beating rate of iPS-CMs treated with 5 μM LBP (n = 10), 5 μM LBP plus 10 μM Cd (n = 10), or 5 μM GlyH (n = 10). H–J: Changes in the beating rate of FCMs treated with 5 μM LBP (n = 10), 5 μM LBP plus 10 μM Cd (n = 10), or 5 μM GlyH (n = 10). Data are expressed as the mean ± SD. (P values vs. control by Wilcoxon signed-rank sum test).

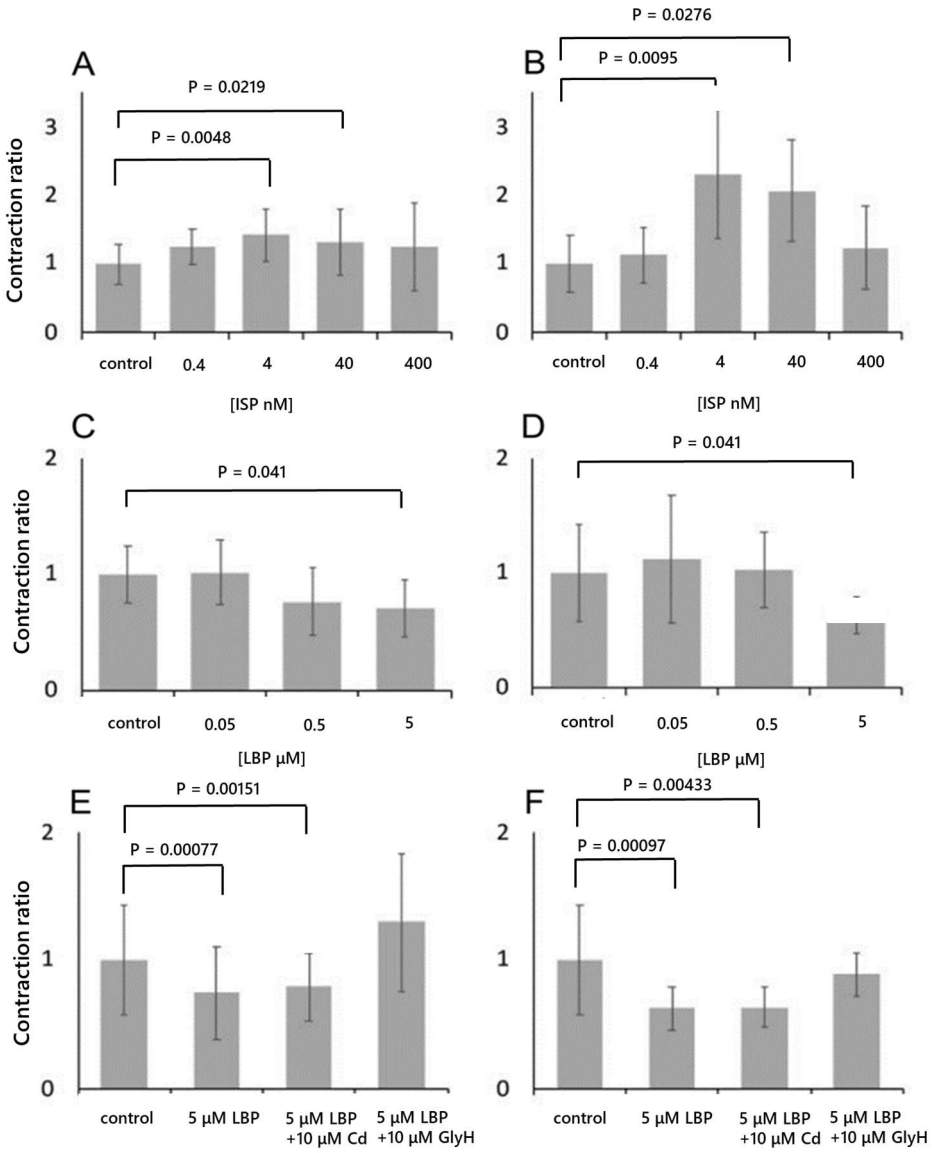

Fig. 3. Contraction response to chemical compounds. A and B: Changes in the contraction ratio of iPS-CMs (A) and ACMs (B) treated with ISP at various concentrations. The contraction ratio of the iPS-CMs significantly increased in the presence of 4 nM or 40 nM ISP. Similarly, the contraction ratio of the ACMs treated with 4 nM or 40 nM ISP increased. C and D: Changes in the contraction ratio of iPS-CMs (C) and ACMs (D) treated with various concentrations of LBP. The contraction ratio of the iPS-CMs and ACMs treated with 5 μM LBP significantly decreased compared to that of the control. E and F: Changes in the contraction ratio of iPS-CMs (E) and

ACMs (F) treated with 5 μM LBP, 5 μM LBP plus 10 μM Cd, or 5 μM GlyH. Data are expressed as the mean ± SD. (n = 10, P values vs. control by ANOVA).

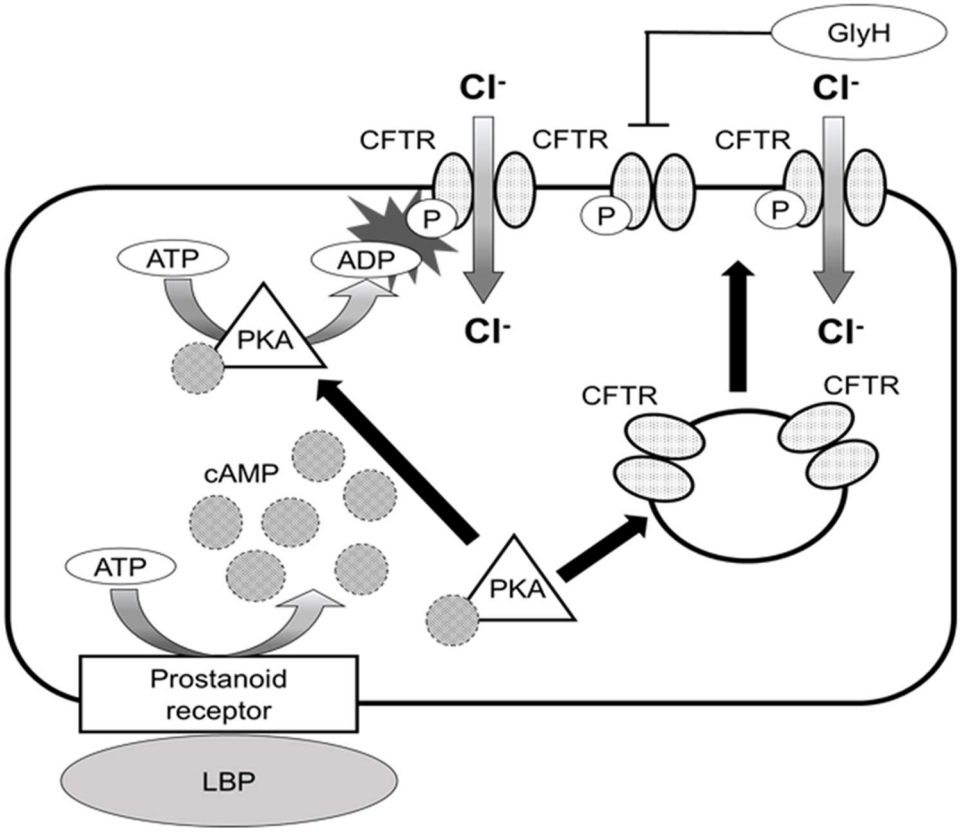

Fig. 4. Mechanisms of LBP action on CMs. LBP binds to the prostanoid receptor and synthesizes cAMP from ATP. PKA activated by cAMP causes the phosphorylation of CFTR and also increases CFTR protein at cell membrane. Inward flowing Cl^- currents mediated by CFTR disturb the depolarization and inward Ca^{2+} currents. As a result, LBP decreased the spontaneous beating rate and contraction ratio of CMs due to the activation of CFTR.

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