《Original Articles》

$P2X_5$ receptor is functionally expressed on mouse brown adipocyte and can be a cell surface marker

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Abstract

Brown adipocytes expressed a large variety of purinergic receptor subtypes. We investigated about the expression of $P2X_5$ receptor subtype among purinergic receptor subtypes expressed in mouse adipose tissue. Ca^{2+} imaging showed that applied 10 μM ATP or 1 μM 2MeSATP (a $P2X_5$, $P2Y_1$ and $P2Y_{11}$ agonist) increased intracellular Ca^{2+} concentration in brown adipocytes. RT-PCR and real-time PCR studies revealed that $P2X_5$ receptor mRNA was expressed much in mouse brown adipocytes but less in white adipocytes. The expression levels of $P2X_5$ receptor mRNA in brown adipocytes were about nine times higher than that of white adipocytes. Western blotting studies suggested the expression of $P2X_5$ receptor protein was detected in brown adipocytes but not in white adipocytes. Immunohistostaining studies indicated the expression of $P2X_5$ receptor protein was shown on brown adipocytes but not on fibroblasts. Thus, $P2X_5$ receptors have a function for Ca^{2+} rises and it can be available as tools to identify and target brown adipocytes.

Keywords: brown adipocytes, white adipocyte, $P2X_5$, cell marker

Introduction

Brown adipocytes are present in all mammalian especially in neonates or cold-acclimated adult which are located in interscapular and armpit depots and mixed together with white adipocytes and fibroblasts. In response to cold exposure, heat is produced by uncoupling of respiration by the activation of uncoupling proteins under the enhanced hydrolysis of neural lipids by the β-adrenergic activation of noradrenaline which is released from sympathetic nerves [1, 2, 3]. Simultaneously, sympathetic nerves are thought to release ATP [4]. Extracellular ATP also evokes a number of responses in brown adipocytes [5, 6]. ATP plays a variety role of signal transductions in many cells. Mammalian purinergic receptors consist of seven P2X subtypes (ionotropic receptors: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$) and eight P2Y subtypes (G-protein-coupled receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). Last year, we showed the functional expression of $P2X_1$ and $P2X_7$ on mice brown adipocytes, with a variety of techniques including Ca^{2+}-imaging, reverse transcriptase-mediated polymerase chain reactions (RT-PCR) and Western blotting [7]. Although we also showed the expression of $P2X_5$ using RT-PCR techniques, there was no conclusive proof of the expression of $P2X_5$ in the protein levels. $P2X_5$ receptor is known to be expressed in coeliac ganglia [8] or in trigeminal mesencephalic nucleus neurons [9] and plays roles of neural signal transductions.

In this study, we have investigated whether $P2X_5$ receptor was expressed on mouse brown adipocytes and white adipocytes. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) were measured by fluorometry with Fura-2. The results demonstrated that the activation of $P2X_5$ by 2MeSATP stimula-

tion causes Ca^{2+} influx from extracellular space. And we detected the expression of $P2X_5$ in mice brown adipocytes using RT-PCR, western blotting and immunostaining techniques. Also we compared the amount of $P2X_5$ receptor mRNA expressed in mouse brown adipocytes with white adipocytes using real-time PCR method. The roles of these purinergic receptors are discussed in terms of Ca^{2+} dynamics in mouse brown adipocytes.

Method

Cell isolation

Preparations and solutions are essentially similar to those in the previous study [7, 10]. Mice (C57BL/6J supplied from SLC Japan: Male, 4 weeks old) were anesthetized and killed. All procedures were performed in accordance with the Animal Experimentation Guide lines of Nagoya University of Arts and Sciences. Interscapular brown adipose tissues were isolated. Brown adipocytes were isolated by treatment with collagenase type-2 and DNase-I and cultured for 7 days. Culture medium was composed of D-MEM, 10% fetal bovine serum, penicillin and streptomycin. Adipocytes cultured from mice for 2–7 days were used for imaging of $[Ca^{2+}]_i$.

Imaging with Fura-2 and analysis

Mouse brown adipocytes were loaded with Fura-2/AM (5 μM) for 40 min at 37°C. Changes in $[Ca^{2+}]_i$ were measured from adipocytes with conventional Ca^{2+}-imaging system (CCD camera, C4742-12R, Hamamatsu photonics, Shizuoka, Japan) set on an inverted microscope (ECLIPSE Ti with an objective, 40 x water, numerical aperture 1.15, NIKON, Tokyo, Japan). Fura-2 was excited alternatively at 340 nm (D340, Chroma Technology Corp., Vermont, USA) and 380 nm (D380, Chroma Technology Corp.) using a filter changer (EFC-1, Nikon, Tokyo, Japan) for the inverted microscope. Fura-2 fluorescence was recorded through a band-pass filter (D535/30, Chroma Technology Corp.). Fluorescence intensity was averaged over the contour of each cell by software (Aquacosmos, Hamamatsu photonics) in the experiments of single cell culture, while it was averaged over the area containing several numbers of cells in the experiments of adipose tissue. The ratio of fluorescence excited at 340 nm to that at 380 nm, F340/380, was converted to a $[Ca^{2+}]_i$ value using the dissociation constant of 145 nM, the ratio of the maximum F340/F380 to the minimum (14.1) and the fluorescence ratio of the free to the Ca-bound form (9.59).

RNA isolation and RT-PCR

Total RNA was extracted with the RNeasy Lipid Tissue mini kit (Qiagen, Germany) from brown and white adipose tissues isolated from 4 weeks mice under anesthesia and homogenized at 20,000 rpm (Ultra-Turrax T-25). After incubation at room temperature for 5 min and vortexed with addition of chloroform (0.2 ml homogenate /ml QIAzol, Qiagen, Germany) for 15 sec, samples were centrifuged at 12,000 rpm and 4°C for 15 min. The upper aqueous phase of centrifuges was mixed with 70% ethanol, incubated at room temperature for 10 min, and centrifuged in RNeasy Min Spin Column at 20°C and 12,000 rpm for 15 sec, adsorbing RNA to the silica gel membrane. After addition of 350 μl RW1 buffer, the column was recentrifuged at 20°C and 12,000 rpm for 15 sec. 10 μl DNase I and RDD buffer were added to the silica gel membrane of the column. The column was washed with 350 μl RW1 buffer with centrifugation at 20°C and 12,000 rpm for 15 sec, then washed twice with 500 μl PRE buffer with centrifugation at 20°C and 12,000 rpm for 15 sec and 2 min, and finally centrifuged at 15,000 rpm for 1 min for drying the silica gel membrane. For extraction of RNA, 20 μl RNase free water was added to the silica gel membrane, then left for 2 min and finally centrifuged at 15,000 rpm and 20°C for 1 min. After repetition of this procedure twice, the collected RNA in aqua solution was stored at –80°C.

Reverse transcription was performed with the First-Strand cDNA Synthesis kit (Applied Biosystems, Inc., California, USA). cDNA was stored at –20°C and subjected to the standard RT-PCR. For the

standard RT-PCR, 0.5 μl First Strand cDNA reaction mixture was added to a 49.5 μl PCR reaction mixture consisting of 0.5 μM of each primer, PCR buffer (provided in the kit), 2 mM $MgCl_2$, 200 μM each dNTP mix, and 1.25 units Taq DNA polymerase. The sense and antisense oligonucleotide primers specific for different purinergic receptor subtypes are listed in Table 1. Each fragment of cDNA was amplified in a DNA thermal cycler (model 2400 GeneAmp PCR system; California, USA). The mixture was first denatured at 94°C for 2 min and then cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec were repeated for 40 cycles. This was followed by a final extension at 72°C for 2 min to ensure complete product extension. The PCR products were electrophoresed through a 2% agarose gel, and stained with ethidium bromide. For real-time PCR, the reaction mixture containing 0.2 μl cDNA, 12.5 μl SYBR Green I, 2 μl of each primer (5 mM) and 8.3 μl water was subjected to PCR analysis (Miniopticon, Bio-Rad, Tokyo, Japan). The oligonucleotide primers used for the detection or quantification of $P2X_5$ receptor were sense 5'- TCCACCAATCTCTACTGC -3' and antisense 5'- CCAGGTCACAGAAGAAAG -3'. These sequences of primers for $P2X_5$ receptor were the same as used before [7].

Protein isolation and western blotting analysis

Brown adipose tissues isolated from mice were homogenized in 20 mM Tris buffer (pH 7.4) with 1 mM EGTA containing protease inhibitors (mix tablet; Roche Diagnostics, Indiana, USA) with Potter-Elverhjem homogenizer and then centrifuged at 4°C and 25,000 rpm (50,000 g) for 60 min. After removing the supernatant including lipid fraction, the precipitate was placed in 1 ml TE buffer. Precipitated fractions were denatured and separated by SDS-PAGE calibrated with prestained protein molecular weight markers (Bio-Rad, California, USA). Separated proteins were transferred to nitrocellulose membranes (Hybond-C, Bio-Rad). After being blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 (Sigma Aldrich, Missouri, USA), membranes were stained with affinity-purified polyclonal antibodies (1/500) specific for $P2X_5$ at 20°C for 1 hour and then at 4°C over night. After washing the primary antibody, membranes were stained with anti-rabbit horseradish peroxidase-conjugated IgG (1/10,000) for 1 hr. An enhanced luminol-linked chemical luminescence detection system (ECL; Amersham, New Jersey, USA) was used to detect each protein.

Immunohistostaining

Immunohistostaining of $P2X_5$ receptor was observed in optical slices obtained with confocal laser scanning microscopy (FLUOVIEW, Olympus, Japan). We fixed brown adipocytes and fibroblasts overnight with a fixation solution, washed with six 10 min changes of a phosphate-buffered solution (PBS). After a brief wash with PBS, brown adipocytes and fibroblasts were incubated in a blocking solution for 1 h. These cells were probed using indirect immunofluorescence. A polyclonal antibody to mouse $P2X_5$ receptor (1:100, Abcum, Cambridge, UK) served as the primary antibody. Alexa 488-conjugated donkey anti-rabbit IgG (1:1000, Molecular Probes,Eugene, OR, USA) served as secondary antibody. Each antibody was diluted with the blocking solution to the concentration indicated, respectively, above.

Statistical analyses

Statistical data were shown by the mean ± S.E. of the mean. Significant differences among groups were assessed by Student's t test.

Drugs

Collagenase type-2 (class 2) was obtained from Worthington Biochemical (New Jersey, USA), DNase-I from Roche Diagnostics (Indiana, USA). D-MEM (low glucose type, 11885-084), penicillin and streptomycin were from Applied Biosystems, Inc. (California, USA). Fetal bovine serum was from Thermo Electron (Melbourne, Australia). ATP and 2MeSATP were from Sigma Aldrich (Missouri, USA). Fura-2/AM was from Molecular Probes, Inc.

(Oregon, USA).

Solutions

The composition of normal Krebs Ringer solution was (mM): NaCl, 150; KCl, 5; CaC12, 2.0; $MgSO_4$, 1.0; HEPES, 20.0; glucose, 5 (pH 7.4 adjusted with NaOH). A nominally Ca^{2+} -free Krebs solution was made by subtracting 5 mM $CaC1_2$ from Krebs solution, in which total $CaC1_2$ was replaced with 5 mM NaCl. Drugs were applied by changing a perfusing solution to the solution containing a drug(s). The blocking solution comprisesd: 3% normal donkey serum dissolved in the PBS and supplemented with 1% bovine serum albumin and 0.3% Triton X.

Results

Ca^{2+} imaging

The application of ATP elicited Ca^{2+} responses in mouse brown adipocytes. $P2X_5$ receptor has been known to rise ($[Ca^{2+}]_i$) in response to 2MeSATP [11]. The averaged $[Ca^{2+}]_i$ of brown adipocytes was increased in response to the application of 1 μM 2MeSATP (113.2 ± 32.4 nM, n=22) (Fig. 1A). The irrigation with the nominally Ca^{2+}-free solution suppressed the magnitude of Ca^{2+} rises partially in response to the application of 1 μM 2MeSATP (22.6 ± 12.2 nM, n=12) (Fig. 1B). These results indicated that the most part of Ca^{2+} rises by the application of 2MeSATP was elicited by influx from extracellular Ca^{2+} via $P2X_5$ receptor. That is, $P2X_5$ receptor exists on mouse brown adipocytes functionally.

RT-PCR and real-time PCR analysis

RT-PCR reactions with specific primers for $P2X_5$ receptor subtype yielded cDNA fragments. This cDNA fragments showed that brown adipocytes expressed $P2X_5$ receptor mRNA (Fig. 2). We used brain and heart tissue as a control. They are already known to possess $P2X_5$ receptor [12, 13, 14]. We confirmed that $P2X_5$ subtypes occurred in mouse brown adipocytes and a little in white adipocyte. The detection of $P2X_5$ in brown adipocyte agreed with their Ca^{2+} responses to 2MeSATP and previous

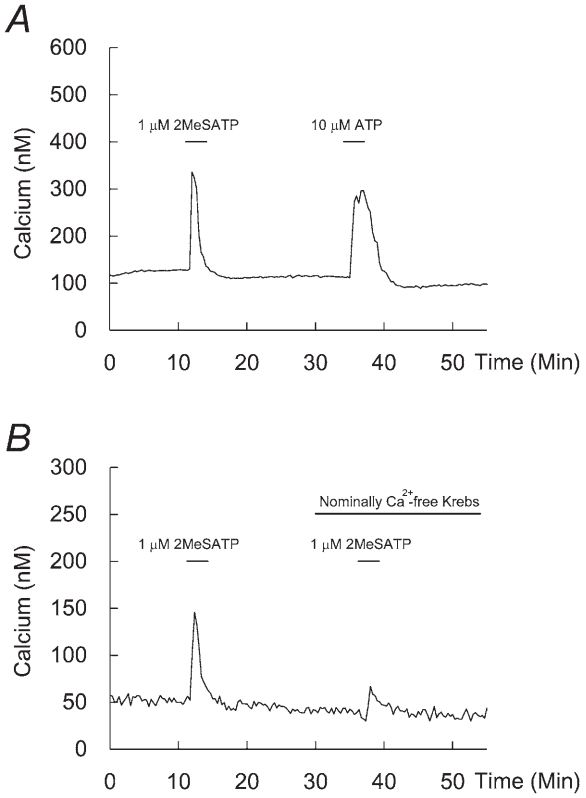

Figure 1

Ca^{2+} responses in mouse brown adipocytes by the effects of ATP and 2MeSATP.

A. Effects of application of 2MeSATP (a $P2X_5$, $P2Y_1$ and $P2Y_{11}$ agonist). 2MeSATP (1 mM) and ATP (10 mM) were applied during the period indicated by a black and grey horizontal bar in each. *B*. Effects of Nominally Ca^{2+}-free Krebs solution on 2MeSATP-induced Ca^{2+} response. 2MeSATP (1 mM) was applied during the period indicated by a black horizontal bar. A nominally Ca^{2+}-free Krebs solution was superfused to the bath for the period indicated by a grey horizontal bar.

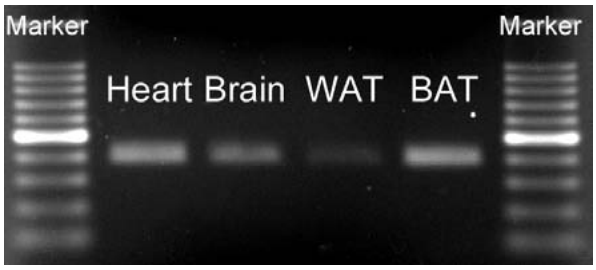

Figure 2

PCR-amplified products from $P2X_5$ cDNA reverse-transcribed from mRNA isolated from heart, brain, white adipose tissue (WAT) and brown adipose tissue (BAT).

Oligonucleotide primers specific for $P2X_5$ purinergic receptor subtypes were run in separate reactions and the products run on agarose gel electrophoresis. The expected product size of each reaction is 400 bp. Marker consists of 10 fragments between 100 bp and 1 kbp in multiples of 100 bp from the bottom.

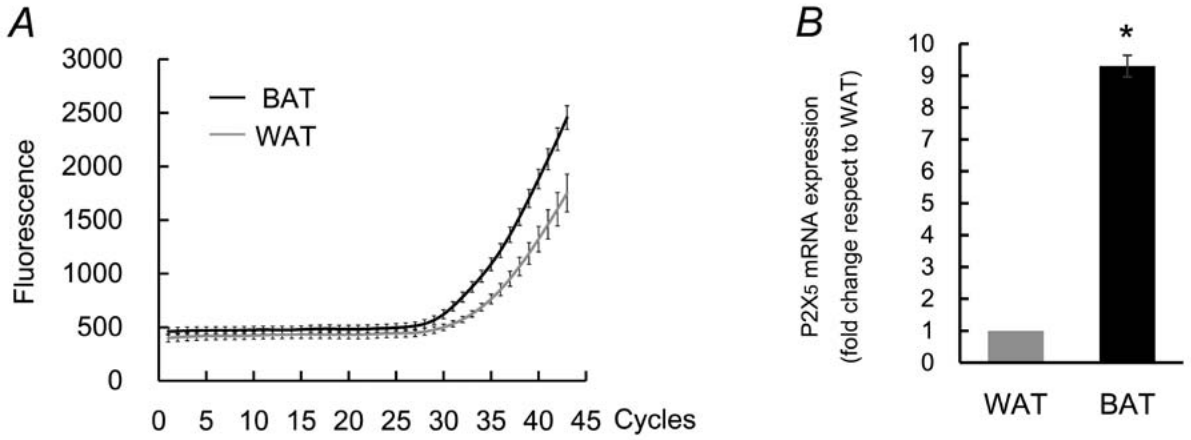

Figure 3

Real-time PCR analysis of mouse $P2X_5$ receptor of brown adipocyte and white adipocyte.

A. Changes in fluorescence were shown by a black line (brown adipose tissue: BAT) and a grey (white adipose tissue: WAT) line, vertical bars indicate S.E.M. of 10 mice. *B*. Comparison of the levels for $P2X_5$ receptor mRNA expression in brown adipocytes and white adipocytes. These results represented as mean values (fold change respect to WAT as control), vertical bars indicate S.E.M. of 10 mice adipose tissue. *$p < 0.05$ to the control.

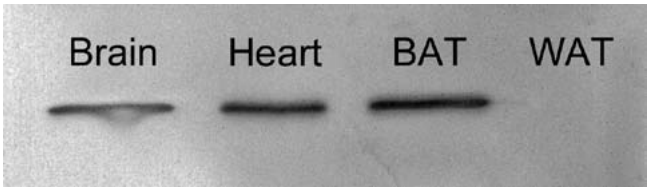

Figure 4

Western blotting of mouse purinergic signaling proteins subtype of $P2X_5$.

The results of western blotting analysis of $P2X_5$ receptor. We detected $P2X_5$ receptor proteins in mouse heart, brown adipose tissue (BAT) and brain but not in white adipose tissue (WAT).

paper submitted in last year [7]. In order to compare the amount of $P2X_5$ receptor expression in mouse brown with white adipocytes, we took advantage of real-time PCR analysis for quantification (Fig. 3). These results showed the expression levels of $P2X_5$ receptor on brown adipocytes are 9.3 ± 0.3 higher than that of white adipocytes (N=10, mean ± S.E.M.).

Western blotting and Immunohistostaining

In order to confirm the translation of mRNA for $P2X_5$ receptor subtypes in brown adipocytes, we applied western blotting analysis to detect $P2X_5$ receptor protein. Western blotting analysis revealed molecules involved in $P2X_5$ receptor in mouse brain, heart and brown adipocytes but not in white adipocyte (Fig. 4). Immunohistostaining analysis revealed that brown adipocytes were immunopositive for $P2X_5$ receptor antibody but fibroblasts were not (Fig. 5). These results suggested that proteins for $P2X_5$ receptors occurred only in mouse brown adipocytes but not in white adipocytes or fibroblasts.

Discussion

Some researchers investigated about the expression of ATP receptors on brown adipocytes, whereas there was no evidence how the activation of $P2X_5$ receptor affect thermogenesis in mouse brown adipocytes [5, 6, 7]. The present study showed that $P2X_5$ receptors were expressed only in brown adipocytes functionally among mouse adipose tissue and elicits Ca^{2+} responses by the application of ATP. In Ca^{2+}-imaging study, nominally Ca^{2+}-free Krebs decreased Ca^{2+} responses partially, not completely, elicited by the application of 2MeSATP. These results indicate

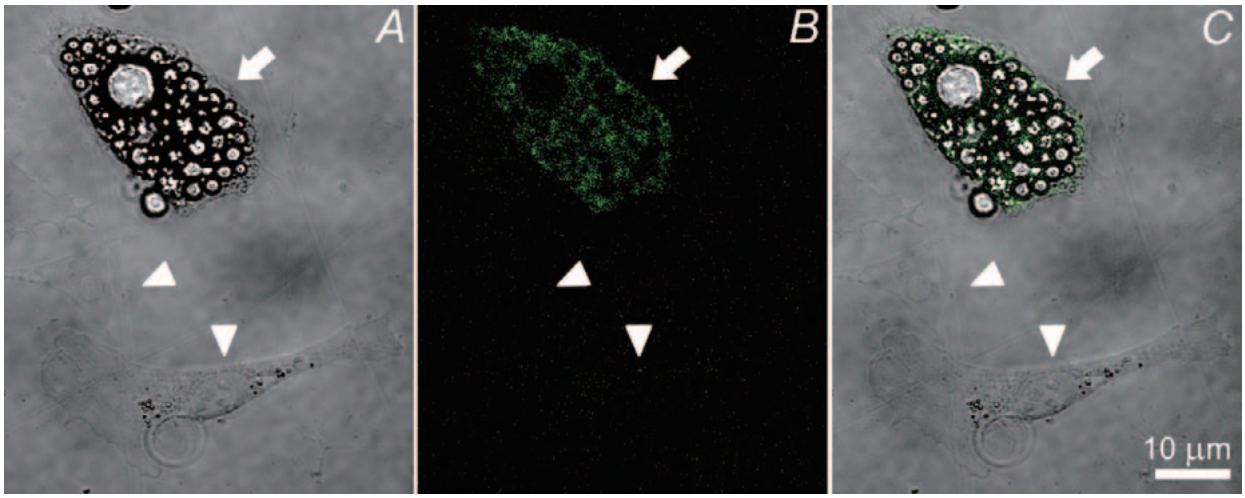

Figure 5
Immunohistostaining confocal images of $P2X_5$ receptor.

A. DIC image. A brown adipocyte and fibroblasts are pointed to a place by white arrow and arrow head, respectively. *B*. Immunoreactivity for $P2X_5$ receptor (green). *C*. Their overlay. Scale bar 10 μm.

that mouse brown adipocytes expressed both $P2X_5$ and $P2Y_1$ and/or $P2Y_{11}$ receptors. These results agree with the previous paper submitted in last year [7].

RT-PCR and real-time PCR studies suggested that brown adipocytes have a large amount of $P2X_5$ receptor mRNA than white adipocytes. Also, western blotting analysis indicated that brown adipocytes had much of $P2X_5$ proteins but white adipocytes had no signals for the existence of $P2X_5$ proteins. Immunohistostaining analysis revealed that $P2X_5$ receptors were expressed in brown adipocytes but not in fibroblasts. $P2X_5$ immunoreactivity was observed on the membrane of brown adipocytes in addition to intracellular spaces. We concluded that the components of $P2X_5$ immunoreactivity in intracellular spaces were endogenous $P2X_5$ proteins such as a transportation phase from ribosome to membrane.

UCP1 (uncoupling protein 1) is already known to be a marker of brown adipocytes, even though UCP1 exists only in the intracellular spaces. In the case we use UCP1 as a marker of brown adipocytes, it takes times and efforts to distinguish brown adipocytes from other cells because we must perforate the membrane of brown adipocytes for immunohistostaining. In this study, we showed that the existence of $P2X_5$ were only in and on mouse brown adipocytes but not in white adipocytes and not on fibroblasts. Brown adipocytes are present in all mammalian especially in neonates or cold-acclimated adult which are mixed together with white adipocytes and fibroblasts in interscapular and armpit depots. Among these tissues, we indicated that $P2X_5$ receptors expressed only in brown adipocytes. That is, $P2X_5$ receptors can be available as tools to identify and target white and brown adipocytes. Recently, Ussar et al reported that $P2X_5$ and PAT2 which is an amino acid transporter could be cell surface markers for classical brown adipocytes and beige adipocytes in mice [15]. Our results agree with their researches.

Last year, Rines et al reported that adenosine derived from ATP hydrolysis activates brown or beige adipocytes through the A_{2A} receptor [16]. We think adenosine also elicits Ca^{2+} rises by the activation of adenylyl cyclase via A_{2A} receptor in the same way as noradrenaline via the activation of β_3-adrenoceptor and it is important for thermogenesis in mouse brown adipocytes [10]. It is known that elevation of $[Ca^{2+}]_i$ increased oxygen consumption and thermogenesis in mouse brown adipocytes [17]. Our report will be the first time that confirm the functional expression of $P2X_5$ receptor due to intracellular Ca^{2+} elevation in mouse brown adipocytes. We concluded that ATP released from sympathetic nerves activates $P2X_5$ subtypes in addition to $P2X_1$ and $P2X_7$ of purinergic

receptor and causes Ca^{2+} influx from extracellular space. Simultaneously, ATP activate P2Y receptors and elicits a large phasic rise in $[Ca^{2+}]_i$ via Ca^{2+} release from the ER through IP_3 receptors [7]. We assume that these Ca^{2+} elicited by the ATP released from sympathetic nerve contribute to thermogenesis in mouse brown adipocytes.

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《原著》

マウス褐色脂肪細胞マーカーとしての $P2X_5$受容体の有用性

早戸亮太郎

要旨

褐色脂肪細胞は熱産生器官である。寒冷曝露により交感神経から放出されたノルアドレナリンを受容し、中性脂肪から遊離脂肪酸を生成する。この遊離脂肪酸がもつエネルギーを熱に変換する事で熱産生を行っている。その際、褐色脂肪細胞内では Ca^{2+} 濃度が上昇する。この Ca^{2+} は褐色脂肪細胞での熱産生量および酸素消費量を増大する事が知られている。交感神経はノルアドレナリンと同時に ATP を神経伝達物質として放出する。

昨年の私たちの研究により、マウス褐色脂肪細胞における複数の ATP 受容体サブタイプ発現が確認された。その中でも $P2X_5$受容体シグナルが遺伝子レベルにおいて強い事が示されたが、タンパクレベルでの発現の有無や、他の脂肪組織における $P2X_5$受容体の存在については明らかにされていなかった。

今回の研究により、マウス褐色脂肪細胞が $P2X_5$受容体タンパクを機能的に発現する事が確認された。また脂肪組織間における $P2X_5$遺伝子発現量を比較したところ、白色脂肪細胞に比べ褐色脂肪細胞では約 9 倍発現量が高い事が示された。白色脂肪細胞および繊維芽細胞には $P2X_5$受容体タンパクは確認できなかった。以上の結果から、脂肪組織において $P2X_5$受容体の分布を調べる事で、褐色脂肪細胞の分布を比較的容易に確認する事ができると思われる。これら $P2X_5$受容体は、交感神経から放出された ATP を受容する事で細胞内 Ca^{2+} 濃度を上昇させ、熱産生を促進している可能性が考えられる。