Biophysical Journal Volume 106 June 2014 2458–2464

Temperature Changes in Brown Adipocytes Detected with a Bimaterial Microcantilever

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ABSTRACT Mammalian cells must produce heat to maintain body temperature and support other biological activities. Methods to measure a cell's thermogenic ability by inserting a thermometer into the cell or measuring the rate of oxygen consumption in a closed vessel can disturb its natural state. Here, we developed a noninvasive system for measuring a cell's heat production with a bimaterial microcantilever. This method is suitable for investigating the heat-generating properties of cells in their native state, because changes in cell temperature can be measured from the bending of the microcantilever, without damaging the cell and restricting its supply of dissolved oxygen. Thus, we were able to measure increases in cell temperature of <1 K in a small number of murine brown adipocytes ($n = 4$–7 cells) stimulated with norepinephrine, and observed a slow increase in temperature over several hours. This long-term heat production suggests that, in addition to converting fatty acids into heat energy, brown adipocytes may also adjust protein expression to raise their own temperature, to generate more heat. We expect this bimaterial microcantilever system to prove useful for determining a cell's state by measuring thermal characteristics.

INTRODUCTION

In mammals, brown adipocytes are important for maintaining body temperature, and for raising body temperature when waking from hibernation, through nonshivering thermogenesis (for a review, see Cannon and Nedergaard (1)). Through a series of signal transduction reactions, norepinephrine and other hormonal signals prompt the decomposition of neutral fats in the adipocyte to produce fatty acids. These fatty acids interact with and activate uncoupling protein 1 (UCP1), which is specifically expressed in brown adipocytes in the mitochondrial inner membrane, and this causes the energy of the proton gradient in the mitochondrial inner membrane to dissipate as heat (Fig. 1 *A*). Although the proton gradient is reformed through the β-oxidation of fatty acids in the mitochondria, it continues to dissipate heat once UCP1 has been activated. Thus, brown adipocytes convert fatty acids into heat energy to maintain body temperature homeostasis in mammals.

Conventional methods to estimate cells' thermogenic capacity include microcalorimetry and measuring oxygen consumption, both of which require 10^5 or more cells (2–6), and measuring temperature changes in adipose tissue with a thermocoupled probe (7,8). Results from these methods indicate that individual cells generate heat on the order of nanowatts, and that UCP1 is important for this process. However, most studies have monitored cell activity over short periods of time, because dissolved oxygen is quickly exhausted in a closed vessel. Moreover, large numbers of cells are required because of the low sensitivity of the measuring devices, making it difficult to clarify the characteristics and heat-generating mechanism of individual cells.

Recently, techniques for measuring the temperature of mammalian cells in culture have been developed (9–15) that are sensitive to changes of <1 K in response to chemical stimulation, including a temperature-sensitive gel (9–11), Q-dots (12), a fluorescent-dye-filled glass microcapillary (13), and chemically synthesized fluorescent beads (14,15). However, these techniques require the introduction of some kind of thermometer into the cell or the detection of fluorescence intensity, and are not suitable for long-term measurements of cellular physiological temperature changes. Examining the properties of a small number of cells in their native state over a period of several hours requires a highly sensitive, noninvasive technique that does not involve special cell treatments. Toward that end, we focused on using a temperature-sensing technique based on bimaterial microcantilevers. The materials in each layer of such microcantilevers have different coefficients of thermal expansion, which cause the device to bend in response to changes in temperature. Temperature changes are calculated from the amount of bending.

Various kinds of bimaterial cantilevers have been reported (16–18), but they have not been used for biological applications in aqueous conditions. Recently, a micrometer-sized bimaterial cantilever was developed by our group (19) that can be used to detect changes in cell temperature. This microcantilever's high thermal conductivity and micrometer-sized structure reduce its heat capacity, making it a highly sensitive reporter for temperature changes. In this article, we adopted the recently published bimaterial microcantilever design to detect heat production

Submitted February 24, 2014, and accepted for publication April 28, 2014.

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Editor: Hiroyuki Noji.

0006-3495/14/06/2458/7 $2.00

http://dx.doi.org/10.1016/j.bpj.2014.04.044

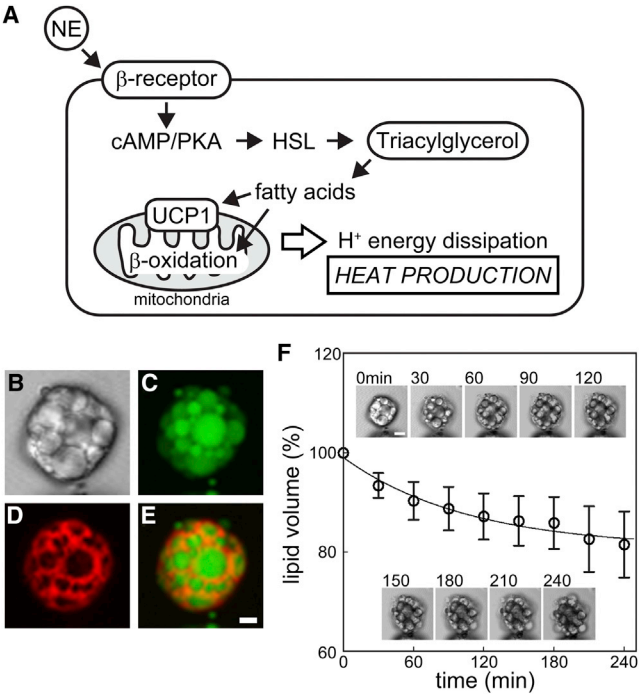

FIGURE 1 Decomposition of lipid droplets in a norepinephrine-stimulated brown adipocyte. (*A*) Heat production in brown adipocytes: (NE) norepinephrine; (cAMP) cyclic adenosine monophosphate; (PKA) protein kinase A; (HSL) hormone-sensitive lipase. (*B–E*) A brown adipocyte, observed with a confocal microscope. (*B*) A differential interference contrast image. (*C*) Fluorescence images of BODIPY 493/503-labeled lipid droplets; (*D*) mitochondria, labeled with MitoTracker Red CMXRos (Life Technologies); and (*E*) the merged images of panels *C* and *D*. Bar = 5 μm. (*F*) Changes in the cell's lipid volume over time. Bars indicate ± SD (n = 5–8). The data were fitted to single-exponential decay; the rate constant was 9.4×10^{-3} min^{-1}. (*Insets*) Differential interference contrast images of the cell at each time point. Scale bar (*inset*, 0 min) = 10 μm. To see this figure in color, go online.

by norepinephrine-stimulated mouse brown adipocytes, and found that these cells produced heat for much longer than has been previously observed.

MATERIALS AND METHODS

Unless otherwise noted, all operations were performed at 25 ± 1°C.

Preparation of brown adipocytes

Male 8-week-old ICR mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). The mice were euthanized with CO_2; the interscapular brown adipose tissue was then removed and digested by collagenase solution (D-MEM high glucose; Life Technologies, Carlsbad, CA) containing 2 mg/mL collagenase (Wako Pure Chemical Industries, Osaka, Japan) and 20 mg/mL bovine serum albumin (Sigma-Aldrich, St. Louis, MO) for 40 min at 37°C. The digested tissue was filtered through 200-μm nylon mesh to remove large debris. The filtered solution was centrifuged (1000×g for 1 min) and cells floating on the surface were collected and triple-washed with wash buffer (D-MEM containing 10 mg/mL bovine serum albumin). Cells were counted using a hemocytometer under a microscope. The viability of the prepared brown adipocytes was checked by the Trypan Blue (Sigma-Aldrich) exclusion test. All experimental procedures and mouse care followed the regulations for animal experiments and related activities at Tohoku University (Sendai, Japan).

Fluorescence imaging of a brown adipocyte

Mitochondria in the adipocytes were stained by 100 nM MitoTracker Red CMXRos (Life Technologies) for 30 min at 37°C. The cells were placed on a 35-mm glass-bottomed dish and stained with 0.5 μM BODIPY 493/503 (Life Technologies) for 10 min. Unbound cells and staining reagents were washed away with 1 mL of D-MEM three times, and the dish was refilled with 5 mL of D-MEM. The reaction for heat production was started by adding 1 μM norepinephrine (Sigma-Aldrich) to the dish (0 min). Every 30 min, differential interference contrast images of the cells were taken simultaneously with fluorescence images of the mitochondria and lipids using a confocal laser microscope system (FV1000-D; Olympus, Tokyo, Japan) equipped with a 60× objective (UplanSApo 60×/1.35; Olympus). Cross-sectional images of each cell were taken every 1 μm, and the diameter of each lipid droplet was measured from the cross-sectional images. The volumes of 4–10 randomly selected lipid droplets in a cell were calculated, and the average rate of volume change was considered to be the rate of fatty acid consumption in the cell.

Observing bending in the bimaterial microcantilever

Bimaterial microcantilevers (500 × 20, 750 × 20, or 750 × 40 μm^2) with a gold layer (0.1-μm thick) and a silicon nitride layer (0.2-μm thick) were made for this study, as previously described in Toda et al. (19). The bimaterial microcantilever was fixed to the bottom of a 35-mm glass-bottomed dish by attaching it to a perpendicular piece of glass. The dish was placed on an inverted microscope (IX70; Olympus) equipped with a charge-coupled device camera (Neptune 100; Watec, Yamagata, Japan) and a 50×/0.35 SLMPlan N objective (Olympus). Bright-field images of the bimaterial microcantilever were recorded at 30 frames/s with a personal computer, using a video-to-USB converter (DFG/USB2pro; The Imaging Source Europe, Bremen, Germany). The position of the centroid of the microcantilever tip was calculated with our own programs, written in the software LabVIEW 2009 (National Instruments, Austin, TX).

Confirmation of correlative temperature change and bimaterial microcantilever bending

The displacement of the tip of the microcantilever was monitored while adding hot water to the dish, and the water temperature was recorded at 1 s intervals using a thermocouple probe (ST series type K; Anritsu, Tokyo, Japan) and thermocouple input module (NI 9211; National Instruments). We compared the relationship between the displacement of the tip and the temperature change of the water, to confirm that the displacement followed the model of uniform temperature change of a bimaterial microcantilever (16),

$$\text{Displacement} = -3(\gamma_1 - \gamma_2)\frac{t_1 + t_2}{t_2^2 K}\Delta T \times L^2, \quad (1)$$

where γ_1 and γ_2 are the thermal expansion coefficients of gold (14.2×10^{-6} K^{-1}) (20) and silicon nitride (3×10^{-6} K^{-1}) (21), respectively, and t_1 and t_2 are the thickness of the layers of gold (0.1 μm) and silicon nitride (0.2 μm), respectively. The value ΔT is the temperature change of the bimaterial microcantilever, L is the length of bimaterial microcantilever, and

$$K = 4 + 6\left(\frac{t_1}{t_2}\right) + 4\left(\frac{t_1}{t_2}\right)^2 + \left(\frac{E_1}{E_2}\right)\left(\frac{t_1}{t_2}\right)^3 + \left(\frac{E_2}{E_1}\right)\left(\frac{t_2}{t_1}\right),$$

where E_1 and E_2 are Young's modulus of gold (78 GPa) (20) and silicon nitride (271 GPa) (21), respectively.

Detecting the heat production of brown adipocytes with the microcantilever

We performed the microcantilever experiments at room temperature to avoid uncertain behavior of the microcantilever due to buffer vaporization and convection by local warming with a heating plate. We began by attaching arbitrarily chosen small groups of brown adipocytes to the tip of a glass rod coated with 0.01% (w/v) poly-L-lysine, and immersing the cells in the dish that contained the microcantilever. Because we could not accurately control the number of cells that attached, we selected a rod with 4–7 cells for each experiment. To keep the solution in the dish from evaporating during the experiment, we covered the dish with a glass plate, leaving enough open space to insert the glass rod, and filled the dish with 9 mL of D-MEM. The cover also prevented water-surface disturbances from destabilizing the bimaterial microcantilever, and the space left for inserting the rod also allowed oxygen to reach the D-MEM.

Next, unstimulated cells or cells stimulated with 1 μM norepinephrine were manipulated with a water-pressured manipulator (PD-5; Narishige, Tokyo, Japan). We added norepinephrine (this was set as 0 min) and used a pipette to draw up and release 1 mL of medium 10 times to distribute the norepinephrine uniformly. We allowed 30 min for the microcantilever and the temperature of whole measurement system including experimental room to stabilize the disturbance caused by a series of operation of adding norepinephrine before measuring thermal bending in the microcantilever. The cells were manipulated to a distance ~400 μm from (distant) or within several microns (close) of the tip of the gold layer of the microcantilever. The resulting displacement of the microcantilever tip due to thermal bending was then observed. The difference between the microcantilever's average position when the cells were close or distant was considered to be the displacement. The distance between the microcantilever and the cell's surface was determined from video images using the software ImageJ (http://rsb.info.nih.gov/ij/). To prevent any drift in microcantilever position from affecting our measurements, we manipulated the cells at regular intervals to confirm that a displacement of the microcantilever was observed only when cells were placed close to the microcantilever.

Calculating cell temperature from the displacement of the bimaterial microcantilever

Cellular temperature was calculated from the relationship between the bending of the bimaterial microcantilever and the temperature distribution along the microcantilever, as (16)

$$\frac{d^2z}{dx^2} = 6(\gamma_2 - \gamma_1)\frac{t_1 + t_2}{t_2^2 K}[T(x)], \qquad (2)$$

where z is the bending displacement of the microcantilever tip, x is a position along the microcantilever (measured from its tip), and $T(x)$ is the distribution of temperature elevation along the microcantilever. Because $T(x)$ depends on heat transferred from the cells, each possible value for cell temperature as a boundary condition produced one candidate for $T(x)$ using the finite-element method and COMSOL Multiphysics software (COMSOL AB, Stockholm, Sweden), as described in Fig. S1 in the Supporting Material. Arbitrary cell temperature values were tested to find the temperature that best fit Eq. 2 with the measured displacement z.

The order of the temperature change estimated with finite-element method calculation was verified by examining the relationship between the displacement of the microcantilever and temperature change using a U-shaped microheater (~200-μm length, 5-μm diameter of platinum/rhodium wire) and a source meter (model No. 2400; Keithley Instruments, Cleveland, OH) (see Fig. S2). The temperature elevation of the microheater when the electrical power was applied to the microheater was calculated from its electrical resistivity change and temperature coefficient (0.0165/K). Based on the resistivity at room temperature in water (2.87 Ω), we converted the resistivity change into temperature change of the heater. The displacement of microcantilever in response to the temperature elevation of the heater was measured, and by changing the heater temperature, the relationship between the displacement of the microcantilever and the temperature change was obtained.

RESULTS

Norepinephrine stimulation causes lipid droplet decomposition

The decomposition of lipid droplets in norepinephrine-stimulated brown adipocytes was observed under a confocal microscope (Fig. 1, *B–E*). Lipid droplets comprised 55 ± 16% (n = 8 cells, mean ± SD) of the initial cell volume (Fig. 1, *B*, *C*, and *E*), as estimated from cross-sectional images of cells stained with BODIPY 493/503. MitoTracker staining (Life Technologies) showed that mitochondria were interspersed between the droplets throughout the cell (Fig. 1, *D* and *E*). After the start of norepinephrine stimulation, we measured the volume of 4–10 randomly selected lipid droplets every 30 min. Over a 240-min period, 19.5 ± 6.7% (n = 5 cells, mean ± SD) of fatty acids in a norepinephrine-stimulated cell were consumed, at a constant rate of 9.4×10^{-3} min^{-1} (Fig. 1 *F*), and the cells lost their original spherical shape (Fig. 1 *F*, *insets*).

Temperature-dependent bending of the bimaterial microcantilever

To confirm that the bimaterial microcantilever made by our group worked properly in aqueous conditions, we measured the displacement of the microcantilever tip and the temperature of the buffer around the microcantilever while adding hot water to a dish. A 750×40 μm^2 microcantilever, constructed of a 0.1-μm-thick layer of gold and a 0.2-μm-thick layer of silicon nitride (Fig. 2 *A*), was fixed to a glass-bottomed dish, and the position of the centroid of the microcantilever tip was measured. Changes in the buffer temperature produced observable bending in the microcantilever (Fig. 2 *B* and *insets* in Fig. 2 *C*). There was a linear relationship between the displacement of the microcantilever tip and the temperature change of the buffer, with a slope of 9.15 ± 0.01 μm/K (mean ± SE) (Fig. 2 *C*). This value was comparable to the theoretical value of 9.46 μm/K (calculated from Eq. 1 as described in Materials and Methods, for a 750-μm-long bimaterial microcantilever). Given the spatial resolution in our system of 6.4 nm (see Fig. S3), the temperature resolution of the 750-μm-long microcantilever was estimated as 0.7 mK. In the case of heating the tip of the microcantilever, the temperature resolution of the microcantilever was ~20 mK, as described in Discussion and Fig. S4.

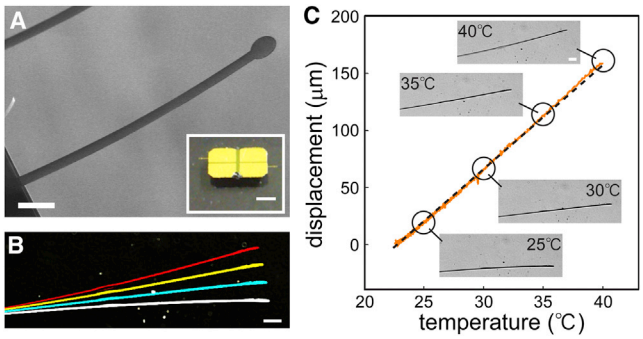

FIGURE 2 Bimaterial microcantilever bending associated with temperature changes in the environment. (*A*) A scanning electron microscope image of a 750 × 40 μm^2 bimaterial microcantilever. Scale bar = 100 μm. (*Inset*) An overview of the bimaterial microcantilever and its base. Bar = 1 mm. (*B*) Merged images of the side view of the bimaterial microcantilever at 40°C (*red*), 35°C (*yellow*), 30°C (*blue*), and 25°C (*white*). Bar = 50 μm. (*C*) The relationship between ambient temperature change and displacement of the tip of a 750 × 40 μm^2 bimaterial microcantilever in water (*orange line*). The slope (*dotted line*) was 9.15 ± 0.01 μm/K (mean ± SE). (*Insets*) The bimaterial microcantilever at each temperature point. (*Inset*) Scale bar at 40°C is 50 μm. To see this figure in color, go online.

Bending of the bimaterial microcantilever in proximity to norepinephrine-stimulated cells

We measured displacement at the tip of a 750 × 20 μm^2 bimaterial microcantilever when a group of seven cells was placed close to the gold layer of the microcantilever (1.8–2.4 μm from the tip) (Fig. 3) and stimulated with norepinephrine or left unstimulated (Fig. 3 *A*). In the absence of norepinephrine, there was no noticeable displacement of the microcantilever tip, whether the cells were close to it (Fig. 3 *B*, (*iii*)) or distant (Fig. 3 *B*, (*i*) and (*v*), ~400 μm apart), although the microcantilever was disturbed briefly as the cells were moved (Fig. 3 *B*, (*ii*) and (*iv*)). In contrast, the tip was displaced from its original position when the same manipulations were performed after norepinephrine was added (Fig. 3, *C–I*, (*iii*)), and the displacement increased over time (Fig. 3 *J*). The displacement of the microcantilever tip continued to increase for a few hours (Fig. 4 *A*) before starting to decrease (this tendency was remarkable in Fig. 4 *A*; *orange*, *purple*, and *black plots*).

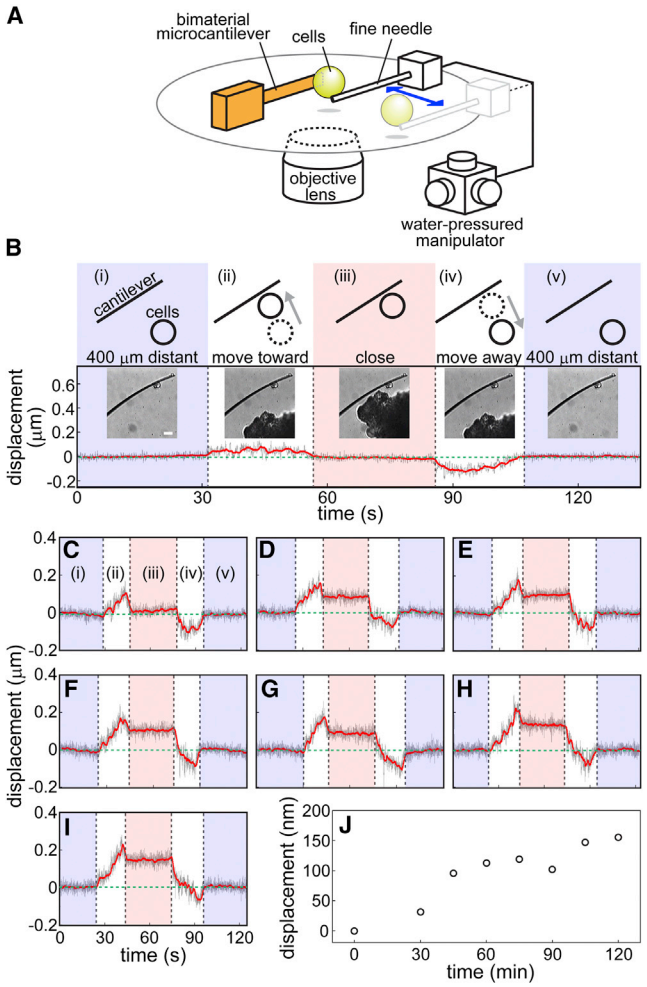

FIGURE 3 Bimaterial microcantilever displacement over time. (*A*) A diagram of the experimental setup for detecting temperature changes in brown adipocytes. (*B*) Displacement traces of a 750 × 20 μm^2 bimaterial microcantilever, just before adding norepinephrine (0 min). Over a period of 120 s, seven cells on the end of a fine needle were moved from (*i*) a position ~400 μm from the microcantilever (distant), (*ii*) toward (*iii*) and to within 1.8–2.4 μm of the microcantilever (close), (*iv*) and then away (*v*) to a distance of ~400 μm (distant). The difference between the microcantilever's average position when the cells were close or distant was considered to be the displacement. Bar = 10 μm. This was repeated (*C*) 30 min, (*D*) 45 min, (*E*) 60 min, (*F*) 75 min, (*G*) 90 min, (*H*) 105 min, and (*I*) 120 min after stimulation with norepinephrine. The experimental temperature was 25 ± 1°C. The color of vertical divisions in panels *C–I* corresponds to the states shown in panel *B*. (*Red lines*) Moving average for a set of 50 data points (calculated from the *gray trace*). (*J*) Plots of microcantilever displacement in panels *B–I* when cells were close to the tip (within 1.8–2.4 μm). To see this figure in color, go online.

DISCUSSION

Temperature changes in norepinephrine-stimulated cells

The temperature resolution in the measuring system with heating the tip of the microcantilever (Fig. 3, and see Fig. S4) is sufficient for detecting the temperature changes in brown adipocytes, considering the reported changes in cell temperature (in the range of 1 K or less; (9,10,13,15,22)). The results of measurements from microcantilevers of sizes 500 × 20, 750 × 20, or 750 × 40 μm^2 are shown in Fig. 4 *A*; microcantilevers of all of these sizes successfully detected cells' heat generation. The displacement of the bimaterial microcantilever (Fig. 4 *A*) was converted into temperature change per cell (Fig. 4 *B*), using the relationship between the displacement of the microcantilever and the temperature profile of the microcantilever (Eq. 2; see Materials and Methods).

In this calculation, the temperature distribution inside a cell was assumed to be uniform, because the mitochondria

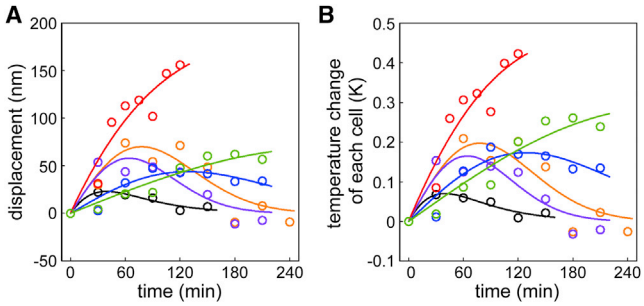

FIGURE 4 Changes in cell temperature during norepinephrine stimulation. Data were plotted using Eq. 3 (described in the Discussion). (*A*) Tip displacement, with the following bimaterial microcantilever sizes and cell numbers: (*red*) 750 × 20 μm^2 bimaterial microcantilever, with seven cells; (*orange*) 750 × 40 μm^2 with six cells; (*green*) 500 × 20 μm^2 with four cells; (*blue*) 500 × 20 μm^2 with five cells; (*purple*) 750 × 40 μm^2 with seven cells; and (*black*) 750 × 40 μm^2 with four cells. (*B*) Changes in cell temperature over time at the experimental temperature of 25 ± 1°C; colors correspond to those in panel *A*. The data were converted from panel *A* with conversion factors: 750 × 20 μm^2 with seven cells, 0.368 nm/mK; 750 × 40 μm^2 with six cells, 0.354 nm/mK; 500 × 20 μm^2 with four cells, 0.238 nm/mK; 500 × 20 μm^2 with five cells, 0.253 nm/mK; 750 × 40 μm^2 with seven cells, 0.350 nm/mK; and 750 × 40 μm^2 with four cells, 0.328 nm/mK (see Fig. S1 in the Supporting Material). Values shown for time 0 were measured immediately before adding norepinephrine. To see this figure in color, go online.

were distributed throughout the cell (between the lipid droplets) (Fig. 1, *D* and *E*). The maximum temperature change for each cell shown in Fig. 4 *B* was 0.217 ± 0.120 K/cell ($n = 6$, mean ± SD). The maximum changes in cell temperature, distance between the microcantilever and the cell surface, size of the microcantilever, and the number of cells are shown in Table S1 in the Supporting Material. The estimated cellular temperature change was comparable to that reported in our previous study (22) and in other studies measuring changes in cell temperature (in the range of 1 K or less) (9,10,13,15). The variation in estimated ΔTs in Fig. 4 *B* may be due to differences in the heat-generating ability of the individual adipocytes, which depends on their UCP1 and mitochondrial content.

In our study, the cellular UCP1 and mitochondrial content could differ between experiments, resulting in the scattered ΔT of the cells. Because our experiment was conducted at a relatively low temperature (25 ± 1°C) compared to the cells' native condition in a living mouse (~37°C), and the activity of brown adipocytes depends on the temperature of their environment (23), it is to be expected that norepinephrine stimulation would produce a greater change in cell temperature in a living mouse than in our study.

Gradual, long-term increase in cell temperature

We showed that brown adipocytes can produce heat for several hours (Figs. 3 and 4). This appears to be an inherent ability that has not been observed through conventional microcalorimetry because of limitations in the experimental conditions. The number of cells necessary for microcalorimetry—on the order of 10^5—would consume all available dissolved oxygen in the chamber in a few tens of minutes, making it difficult to measure heat production over a prolonged period of time. However, the experimental bimaterial microcantilever system provided plenty of oxygen for the cells, allowing us to observe them for several hours and to discover their gradual, long-term increase in temperature.

To relate the timing of changes in cell temperature with chemical reactions occurring in the cell, the data in Fig. 4 were analyzed with the assumption that heat production can be divided into two steps, as follows:

Step 1. Cell activation, which begins with norepinephrine stimulation and produces fatty acid (see also Fig. 1 *A*),

$$[\text{OFF state}] \xrightarrow{k_1} [\text{ON state}], \text{ then}$$

Step 2. Heat production using proton energy in the mitochondria, with a rate constant proportional to the probability of the ON-state in cell activation

$$[\text{Energy}] \xrightarrow{k_2[\text{ON state}]} [\text{Heat}].$$

Under this assumption, the data were fitted using

$$\frac{d[\text{Heat}]}{dt} = -k_2\left\{\frac{k_2}{k_1} + k_2\left[-\frac{\exp(-k_1 t)}{k_1} - t\right]\right\} \times [\exp(-k_1 t) - 1], \quad (3)$$

where $k_1 \times k_2 = 1.1 \times 10^{-5}$–$5.8 \times 10^{-4}$ min^{-2} ($k_1 \times k_2$ for each plot is listed in Table S1). As the decomposition rate of lipid droplets was 9.4×10^{-3} min^{-1} (Fig. 1 *F*), k_2 is predicted to be 1.2×10^{-3}–6.2×10^{-2} min^{-1}. The gradual increase of cell temperature could occur as follows: fatty acids, which are released from lipid droplets in the cell, interact with and activate UCP1 (24,25). The cell's oxygen consumption rate depends on its UCP1 content (6), suggesting that the heat dissipation of energy from the proton gradient in the mitochondrial inner membrane also varies according to the UCP1 content. Brown adipocytes' UCP1 mRNA level increases with time after norepinephrine stimulation (26).

This indicates that norepinephrine stimulation might increase the level of UCP1 proteins as well, which could contribute to a gradual increase in cell temperature. Moreover, the elevated cell temperature might itself increase cellular activity. In the nervous system, the local temperature gradient generated by UCP may enhance cellular events such as synaptic transmission (Horvath et al. (27); and for review, see Andrews et al. (28)). Similarly, in brown adipocytes, increased cell temperature, whether in response to internal activity or heat from neighboring cells, would be expected to affect the rates of chemical reactions and the

diffusion of intracellular molecules. This could accelerate the signal transduction process described in Fig. 1 *A*, causing the cell to produce even more heat. These mechanisms may therefore account for the brown adipocytes' gradual, continuous elevation of cell temperature over a period of 2–4 h (Fig. 4 *B*).

During norepinephrine stimulation, the initially spherical cells (Fig. 1 *F*, *inset* 0 min) lost their distinct shape (Fig. 1 *F*, *inset* 30–240 min); this change was also observed during the bimaterial microcantilever experiments. This change in cell shape may be due, at least in part, to a loss of internal volume, as the lipid droplet volume inside the cell is consumed. It may also be caused by protein kinase A activation, which occurs as the norepinephrine signal is transduced to cause lipid decomposition (29) (Fig. 1 *A*). Protein kinase A is believed to affect cytoskeletal rearrangement and cell migration by targeting regulatory proteins for cell motility (for a review, see Howe (30)). We speculate that norepinephrine stimulation may have caused the change in the brown adipocytes' shape by rearranging the cytoskeleton—although the physiological significance of this change during heat production is still unclear.

Heat energy required for cell temperature changes

In our bimaterial microcantilever experiments, we estimated the temperature change of a single brown adipocyte to be 0.217 ± 0.120 K. Although intracellular temperature changes of 0.1 K or more have been reported in cells of various species (9–13,15), these changes have rarely been discussed in terms of the heat energy required to elevate the cell's temperature. Yang et al. (12) estimated the amount of heat by assuming a very small heat source; they calculated that 0.96–9.6 μW is required to raise the temperature 1.0–10 K at a heat source with a radius of 100 nm. Using the parameters in this study, with a 10-μm cell radius and a temperature rise of 0.01–0.1 K, the same calculation suggests a cellular production of 0.96–9.6 μW. Thus, the amount of heat estimated by the theory of heat conduction alone was on the order of 1–10 μW/cell. This calculation was unexpected, because a heat requirement on the order of microwatts per cell is much higher than the nanowatts per cell estimated from microcalorimetry (2–5). In addition, if a cell generates microwatts of heat, the lipids inside the cell may be exhausted within a few minutes (calculated with the following parameters: 10-μm-radius lipid, with 0.9 g/cm^3 and 38×10^3 J/g for specific gravity of the lipid (31) and calorific value of fat (32), respectively).

Other cell types that are reported to increase cell temperature by 0.1 K or more (9–13,15) are also unlikely to produce heat on the order of microwatts per cell, because their lipid volume, which provides the energy source, is smaller than that in brown adipocytes. Therefore, we think that a nanowatt per cell-order heat requirement to elevate cell temperature 0.1 K or more is a general property shared by various types of cells. Intriguingly, Okabe et al. (10), using a temperature-sensitive gel, found that a steep temperature change formed between the nucleus and cytoplasm in COS-7 cells. To achieve such a steep temperature change, the cellular membrane and/or cytoplasmic components would need strong heat-insulating properties. We speculate that such highly insulated surroundings may be a factor in cell-temperature increases of 0.1 K or more with heat generation on the order of nanowatts. This possibility should be addressed in a future study to elucidate heat transfer at the single-cell level (15).

CONCLUSION

Conventional microcalorimetry, which uses a cell suspension in a closed vessel, cannot avoid limitations such as the exhaustion of dissolved oxygen in the suspension and damage to the cells by stirring. These limitations hamper cells' ability to produce heat. In contrast, the bimaterial microcantilever can be used to measure the temperature in a small number of cells. This makes the microcantilever method particularly suitable for evaluating the heat-producing ability of cells that cannot be obtained in large numbers, such as human primary cells. Because our system allows temperature changes to be measured by simply observing the bending of the microcantilever when cells are placed near it in an open system, the cells are not damaged by thermometer insertion or close contact with measurement devices. Moreover, because plenty of solution is provided for the small number of cells involved, the exhaustion of dissolved oxygen, a limitation in this study (22), is not a concern. The system in this study allows sufficient dissolved oxygen to support cell activity over an extended period.

Because cell temperature increased gradually over several hours in this study, new protein expression or another time-consuming process may be involved in the gradual increase. In mammals, brown adipocytes are continually stimulated with norepinephrine at times when the body temperature needs to increase. During arousal from hibernation, raising the body temperature solely with the cells' initial capacity for heat production may take too much time. Thus, cells may be able to gradually increase their own temperature, to increase body temperature more efficiently in response to norepinephrine stimulation. For a detailed investigation of cell temperature and heat localization within the cell, reducing the size of the microcantilever even further to detect heat with higher sensitivity, and coupling a vacuum unit with a thermal sensor, to prevent heat loss to the surrounding water (22), will be effective. By exploring intracellular thermal distribution of numerous cell types, this method may be helpful in the future for the clinical identification of aberrant cells, such as cancerous or virus-infected ones.

SUPPORTING MATERIAL

Four figures and one table are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(14)00462-7.

We thank Prof. Masayuki Saito (Hokkaido University, Sapporo, Japan) for his helpful advice on undertaking this research.

This research was supported by the CREST grant from the Japan Science and Technology Agency (to A.I.); Grant-in-Aid for Scientific Research on Innovative Areas from Ministry of Education, Culture, Sports, Science and Technology/Japan Society for the Promotion of Science (to A.I.); Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology/Japan Society for the Promotion of Science (to A.I. and Y.I.); and the FIRST Program from the Japan Society for the Promotion of Science (to A.I. and Y.I.). This research was also supported in part by the Management Expenses Grants for National University Corporations from the Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES

1. Cannon, B., and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. *Physiol. Rev.* 84:277–359.
2. Nedergaard, J., B. Cannon, and O. Lindberg. 1977. Microcalorimetry of isolated mammalian cells. *Nature.* 267:518–520.
3. Clark, D. G., M. Brinkman, and S. D. Neville. 1986. Microcalorimetric measurements of heat production in brown adipocytes from control and cafeteria-fed rats. *Biochem. J.* 235:337–342.
4. Hansen, E. S., and J. Knudsen. 1986. Parallel measurements of heat production and thermogenin content in brown fat cells during cold acclimation of rats. *Biosci. Rep.* 6:31–38.
5. Tanaka, E., A. Yamakawa, …, H. Nakazawa. 1995. Regulation of heat production of brown adipocytes via typical and atypical β-adrenoceptors in the rat. *Jpn. J. Physiol.* 45:1043–1051.
6. Matthias, A., K. B. E. Ohlson, …, B. Cannon. 2000. Thermogenic responses in brown fat cells are fully UCP1-dependent. UCP2 or UCP3 do not substitute for UCP1 in adrenergically or fatty acid-induced thermogenesis. *J. Biol. Chem.* 275:25073–25081.
7. Inokuma, K., Y. Ogura-Okamatsu, …, M. Saito. 2005. Uncoupling protein 1 is necessary for norepinephrine-induced glucose utilization in brown adipose tissue. *Diabetes.* 54:1385–1391.
8. Inokuma, K., Y. Okamatsu-Ogura, …, M. Saito. 2006. Indispensable role of mitochondrial UCP1 for antiobesity effect of β_3-adrenergic stimulation. *Am. J. Physiol. Endocrinol. Metab.* 290:E1014–E1021.
9. Gota, C., K. Okabe, …, S. Uchiyama. 2009. Hydrophilic fluorescent nanogel thermometer for intracellular thermometry. *J. Am. Chem. Soc.* 131:2766–2767.
10. Okabe, K., N. Inada, …, S. Uchiyama. 2012. Intracellular temperature mapping with a fluorescent polymeric thermometer and fluorescence lifetime imaging microscopy. *Nat. Commun.* 3:705.
11. Tsuji, T., S. Yoshida, …, S. Uchiyama. 2013. Cationic fluorescent polymeric thermometers with the ability to enter yeast and mammalian cells for practical intracellular temperature measurements. *Anal. Chem.* 85:9815–9823.
12. Yang, J. M., H. Yang, and L. Lin. 2011. Quantum dot nano thermometers reveal heterogeneous local thermogenesis in living cells. *ACS Nano.* 5:5067–5071.
13. Suzuki, M., V. Tseeb, …, S. Ishiwata. 2007. Microscopic detection of thermogenesis in a single HeLa cell. *Biophys. J.* 92:L46–L48.
14. Oyama, K., M. Takabayashi, …, M. Suzuki. 2012. Walking nanothermometers: spatiotemporal temperature measurement of transported acidic organelles in single living cells. *Lab Chip.* 12:1591–1593.
15. Takei, Y., S. Arai, …, M. Suzuki. 2014. A nanoparticle-based ratiometric and self-calibrated fluorescent thermometer for single living cells. *ACS Nano.* 8:198–206.
16. Barns, J. R., R. J. Stephenson, …, M. E. Welland. 1994. A femtojoule calorimeter using micromechanical sensors. *Rev. Sci. Instrum.* 65: 3793–3798.
17. Varesi, J., T. Lai, …, A. Majumdar. 1997. Photothermal measurements at picoWatt resolution using uncooled micro-optomechanical sensors. *Appl. Phys. Lett.* 71:306–308.
18. Datskos, P. G., N. V. Lavrik, and S. Rajic. 2004. Performance of uncooled microcantilever thermal detectors. *Rev. Sci. Instrum.* 75:1134–1148.
19. Toda, M., T. Ono, …, I. Voiculescu. 2010. Evaluation of bimaterial cantilever beam for heat sensing at atmospheric pressure. *Rev. Sci. Instrum.* 81:055104.
20. Antonietti, J. M., J. Gong, …, C. Gerber. 2007. Micromechanical sensor for studying heats of surface reactions, adsorption, and cluster deposition processes. *Rev. Sci. Instrum.* 78:054101.
21. Mertens, J., E. Finot, …, E. Bourillot. 2003. Effects of temperature and pressure on microcantilever resonance response. *Ultramicroscopy.* 97:119–126.
22. Inomata, N., M. Toda, …, T. Ono. 2012. Pico calorimeter for detection of heat produced in an individual brown fat cell. *Appl. Phys. Lett.* 100:154104.
23. Kitao, N., and M. Hashimoto. 2012. Increased thermogenic capacity of brown adipose tissue under low temperature and its contribution to arousal from hibernation in Syrian hamsters. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 302:R118–R125.
24. Rial, E., A. Poustie, and D. G. Nicholls. 1983. Brown-adipose-tissue mitochondria: the regulation of the 32,000-M_r uncoupling protein by fatty acids and purine nucleotides. *Eur. J. Biochem.* 137:197–203.
25. Winkler, E., and M. Klingenberg. 1994. Effect of fatty acids on H^+ transport activity of the reconstituted uncoupling protein. *J. Biol. Chem.* 269:2508–2515.
26. Silva, J. E. 1988. Full expression of uncoupling protein gene requires the concurrence of norepinephrine and triiodothyronine. *Mol. Endocrinol.* 2:706–713.
27. Horvath, T. L., C. H. Warden, …, S. Diano. 1999. Brain uncoupling protein 2: uncoupled neuronal mitochondria predict thermal synapses in homeostatic centers. *J. Neurosci.* 19:10417–10427.
28. Andrews, Z. B., S. Diano, and T. L. Horvath. 2005. Mitochondrial uncoupling proteins in the CNS: in support of function and survival. *Nat. Rev. Neurosci.* 6:829–840.
29. Fredriksson, J. M., H. Thonberg, …, J. Nedergaard. 2001. Analysis of inhibition by H89 of UCP1 gene expression and thermogenesis indicates protein kinase A mediation of β_3-adrenergic signaling rather than β_3-adrenoceptor antagonism by H89. *Biochim. Biophys. Acta.* 1538:206–217.
30. Howe, A. K. 2004. Regulation of actin-based cell migration by cAMP/PKA. *Biochim. Biophys. Acta.* 1692:159–174.
31. Fidanza, F., A. Keys, and J. T. Anderson. 1953. Density of body fat in man and other mammals. *J. Appl. Physiol.* 6:252–256.
32. Maynard, L. A. 1944. The Atwater system of calculating the caloric value of diets. *J. Nutr.* 28:443–452.