Heat induces adenosine triphosphate release from mast cells in vitro: a putative mechanism for moxibustion

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Abstract

OBJECTIVE: To investigate the role of adenosine triphosphate (ATP) purinergic signaling in mast cells (MCs) modulated by heat to further understand the molecular mechanisms of moxibustion.

METHODS: Skin temperatures induced by monkshood cake moxibustion were evaluated by measuring the Neiguan acupoint (PC 6) from 31 participants with a digital thermocouple thermometer. Temperatures of 43 ℃ and 52 ℃ were applied to cultured human leukemia mast cell line HMC-1 in vitro. Calcium fluorescence was applied to detect intracellular Ca²⁺ ([Ca²⁺]i). Extracellular ATP contents were measured by luciferin-luciferase assay.

RESULTS: Maximum skin temperatures mostly ranged from 40-45 ℃, but some reached up to 50 ℃. Both 43 ℃ and 52 ℃ induced MC degranulation, which was accompanied by an increase in [Ca²⁺], and ATP release. Complexing extracellular Ca²⁺ with 5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) inhibited the noxious heat-induced elevation of [Ca²⁺]i and prevented the enhanced ATP secretion by those cells at 52 ℃, but not 43 ℃.

CONCLUSION: Monkshood cake moxibustion can generate heat sufficient to trigger cellular events of MCs, including degranulation, [Ca²⁺]i elevation, and ATP release, suggesting that purinergic signals originating from MCs are possibly the initiating response of acupoints to moxibustion.

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Key words: Fire toxin syndrome; Mast cells; Cell degranulation; Calcium; Adenosine triphosphate; Moxibustion
INTRODUCTION

Moxibustion is a common therapy in Traditional Chinese Medicine (TCM). Its main purpose is to stimulate acupoints with heat. The thermal properties of different kinds of moxibustion have been evaluated in several previous studies. For moxibustion with warming needles, finite element analysis showed that the maximum temperature that the skin reaches is in excess of 50 °C. This indirect analysis was further confirmed by direct temperature measurements of the warming needles. For direct moxibustion, the highest temperatures of the skin were > 45 °C with a distance of < 3 cm between the moxa and acupoint. For indirect moxibustion, monkshood cake moxibustion is mostly applied in clinics. The characteristic maximum temperature of 57 °C, measured at the bottom of mediator cakes, has been found for monkshood cake moxibustion. However, the actual skin temperature generated by this treatment remains unknown.

Mast cells (MCs) have been shown to represent a component of morphological characteristics of acupuncture points. With respect to their functional specificity, MCs participate in the analgesic effects in response to needling acupuncture, moxibustion, and laser acupuncture in vivo. Transient receptor potential vanilloid 2 (TRPV2)-mediated degranulation, increase of whole-cell membrane currents, and elevation of intracellular calcium ([Ca\(^{2+}\)]\text{in}) activity were induced by a temperature of 52 °C in cultured MCs in vitro. Aside from TRPV2, MCs also express TRPV1 channels that are activated by temperatures higher than 43 °C. Considering the temperature characteristics of moxibustion described above, both TRPV1 and TRPV2 expressed on MCs are likely activated during heat treatment.

Increasing evidence suggests that extracellular adenosine triphosphate (ATP) and purinergic signaling participate in various physiological and pathophysiological processes. The purinergic signaling pathway was hypothesized to be involved in the mechanism of pain relief induced by acupuncture. It was recently demonstrated that adenosine, a metabolic product of ATP, participates in the analgesic effect of needling acupuncture by binding to the A1 purinergic receptor at acupuncture points. MCs express purinergic receptors and are capable of releasing ATP after physical stimuli. However, whether the ATP purinergic signal of MCs is involved in moxibustion remains unknown. The interaction between TRPV channels and purinergic signals was reported in previous studies. TRPV3 in keratinocytes transmits thermal information to neurons by releasing ATP. It is unclear whether high temperatures can activate TRPV1 or TRPV2 channels to trigger ATP purinergic signals in MCs.

The aim of this study was to understand the role of ATP purinergic signaling of MCs during moxibustion. We measured the temperature of human skin on Neiguan (PC 6) generated by monkshood cake moxibustion and found that maximum temperatures were usually in the range of 40-45 °C, and occasionally exceeded 50 °C. Temperatures of 43 °C and 52 °C were applied to cultured human leukemia mast cell line HMC-1 \textit{in vitro} to detect their responses in morphology and function. The two temperatures induced degranulation of HMC-1 cells, elevated the intracellular calcium, and potentiated ATP release.

METHODS AND MATERIALS

**Human tests**

Thirty-one healthy undergraduate students, 17 males and 14 females, were recruited, aged 22-24. Each subject’s left Neiguan (PC 6) was heated by monkshood cake moxibustion. Commercial moxa-cigars (Φ = 24 mm) (Hanyi, Hanyi Airong Limited Company, Nanyang, China) were cut into 2 g cakes at 8 mm thick. The cut monkshood was fired from the top during moxibustion. The digital thermocouple thermometer (model: UT-325, UNI-T, Shanghai Uni-Trend Electronics Company, Shanghai, China) was placed on the left Neiguan (PC 6) and was completely covered by the monkshood cake. The thermometer was connected to a computer and data were recorded every 10 s with UT32X software (Shanghai Uni-Trend Electronics Company, Shanghai, China). Participants were given informed consent and the study was performed in accordance with the guidelines of the Clinic Trial Committee of Shanghai University of TCM (Certificate No. ChiECRCT-20110022).

**Cell culture**

Human mast cell line 1 (HMC-1) was kindly provided by Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN, USA). Cultivation was performed as previously described. In brief, the cells were incubated in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco Life Technologies, Grand Island, NE, USA) without phenol red, supplemented with 2 mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Sigma, Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) fetal bovine serum (Gibco, Life technologies, Grand Island, NE, USA), 1% penicillin and streptomycin (Gibco, Life technologies, Grand Island, NE, USA), in a 95% humidity-controlled incubator (Model: 310, Thermo, Thermo Electron, Waltham, MA, USA) with 5% CO\(_2\) at 37 °C. Cell density was about 1×10\(^5\)/mL.

**Reagents and solutions**

The bath solution (BS) for HMC-1 cells was composed of the following (in mM): 150 NaCl, 5 KCl, 2 CaCl\(_2\), 5 MgCl\(_2\), 5 D-sorbitol, 10 HEPES, and pH 7.4 (adjusted with NaOH). Osmotic pressure of the solutions was 310 mOsm/L. (Model: 3300, Micro Osmometers, Advanced Instruments Inc., Norwood, MA, USA)
USA). In some experiments, 5 mM ethylene glycol-bis (β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA) was added into BS to chelate trace Ca^{2+}. Probenecid (Sigma, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a minimum volume of 1 M NaOH and diluted into 250-mM stock solution with distilled water. 5 mM Calcium Green-1 AM was dissolved in 20% (w/v) Pluronic F-127 (Invitrogen, Life Technologies, Carlsbad, CA, USA). 100 mM N-ethylmaleimide (NEM) (Sigma, Sigma-Aldrich, St. Louis, MO, USA) was prepared in 100% ethanol.

**Light and fluorescence images**

Coverglass coated with poly-L-lysine (Sigma, Sigma-Aldrich, St. Louis, MO, USA) was fixed into a heating chamber (Model: HCMIS, ALA science, New York, NY, USA). Then, HMC-1 cells were transferred into the chamber and grew on the coated glass for 30 min. The temperature of the chamber was controlled by temperature control device (Model: PTC-20, NPI electronic GmBH, Tamm, Baden-Württemberg, Germany). To investigate the effects of heat on MC degranulation, micrographs of HMC-1 cells were captured by CCD (charge-coupled device) camera (Orca-ER, Hamamatsu photonics, Hamamatsu, Shizuoka, Japan) attached to an inverted light microscope (Model: TE2000-U, Nikon, Japan). Calcium Green-1 AM (acetoxymethyl ester) fluorescence was measured to estimate \([\text{Ca}^{2+}]_i\), as described previously. In brief, HMC-1 cells were transferred into a heating chamber as described above and loaded in phenol red-free IMDM containing 4 μM Calcium Green-1 AM (Invitrogen) for 30 min. The loaded cells were washed three times with BS. To keep the recording chamber with cells at desired temperature, it was pre-warmed to 28 °C before recording and perfusion was stopped during the experiment. All solutions used in the fluorescence experiments contained 2.5 mM probenecid. Photos were taken every minute. Images were digitized and averaged (5 frames), background corrected, and analyzed by an image-processing system (Wasa-Software, Hamamatsu photonics, Hamamatsu, Shizuoka, Japan). Fluorescence intensities of all cells in the viewing field were ascertained. Graphs were colored by Image J software (National Institutes of Health, Bethesda, MD, USA).

**ATP measurements**

ATP content was quantified by bioluminescence assay measuring light output from luciferin-luciferase reactions. Cultured HMC-1 cells grown in clusters were isolated into individual ones by pipette sucking and blowing. Cell density was adjusted to about 3.5 × 10^7/mL. 100-μL aliquots of dispersed cell suspensions were placed in 1.5 mL Eppendorf tubes and incubated for 2 h to equilibrate. After equilibration, to determine temperature dependence of ATP release, the samples were kept at room temperature (about 24 °C) or placed into a water bath for 3 min at 43 °C and 52 °C. After, the heated samples were cooled down to room temperature within 1 min. 10 μL luciferin-luciferase assay (Sigma, Sigma-Aldrich, St. Louis, MO, USA) was added into each sample, and light emission was measured immediately by luminometer (GloMax® 20/20, Promega, Madison, Wisconsin, USA). Manipulation was gentle, to avoid irritation of cells by mechanical stimulation. In some experiments NEM or EGTA was applied to cells. 10 min ahead of adding the luciferin-luciferase assay, 50 μL of upper supernatant in each sample was removed and replaced with 50 μL IMDM containing 200 μM NEM or 10 mM EGTA to obtain the final concentration of 100 μM or 5 mM, respectively. Luciferin-luciferase luminescence was calibrated versus ATP standards before and after all sample measurements. Extracellular ATP was quantified as nM/10^6 cells.

**Data analysis**

For data analysis and presentation, ORIGIN software (OriginLab, Northampton, MA, USA) was used. Data are expressed as mean ± standard error of mean. The \(n\) values give the number of measurements obtained from different samples of cells; the \(N\) values present the number of independent experiments. Differences between sample means were determined using one-way analysis of variance test, and \(P < 0.05\) was considered statistically significant.

**RESULTS**

**Maximum temperatures of skin on acupoints during monshodo cake moxibustion**

Figure 1 illustrates the maximum temperatures recorded from 31 participants. 23 out of the 31 maximum temperatures reached or exceeded 40 °C. 18 temperatures ranged from 40-45 °C. Two temperatures reached up to 50 °C. Monshodo cake moxibustion generates moderate noxious heat compared with other types of indirect moxibustion, which generate higher temperatures.

**Degranulation of HMC-1 cells induced by noxious heat**

Figure 2 demonstrates the effects of noxious heat on MC morphology. When HMC-1 cells were equilibrated at 28 °C, the cell membrane was smooth and intact. The cell membrane became rough and some cytoplasmic granules were observed at 43 °C, indicating exocytotic degranulation. When the temperature was further increased to 52 °C, the degranulation became more evident.

**[Ca^{2+}], elevation in HMC-1 cells induced by noxious heat**

Heat elevated the [Ca^{2+}], in HMC-1 cells as demonstrated by the increase in Calcium Green-1 fluores-
cence with increasing temperatures (Figure 3). The elevation at 43 °C amounted to 3.2% ± 1.5% (N = 8 independent experiments) of control (28 °C) and continuously increased to 4.8% ± 2.0% (N = 4 independent experiments, P = 0.044) at 52 °C. The presence of 5 mM EGTA for 10 min in BS effectively inhibited the enhancement of Ca\(^{2+}\) fluorescence induced by heat (Figure 3). This observation suggests that calcium entry from the extracellular space contributed to the elevation of [Ca\(^{2+}\)]\(_i\) in HMC-1 cells during heat treatment.

Enhanced ATP release from MCs induced by heat
Extracellular ATP content in untreated control cells (about 24 °C) was (8.3 ± 3.3) nM (n = 42 samples in N = 11 independent experiments). Thermal stimulation promoted ATP release and elevated extracellular ATP to (20.2 ± 9.7) nM (P = 0.0256, n = 11, N = 8) and (40.9 ± 13.6) nM (P < 0.0001, n = 24, N = 10) at 43 °C and 52 °C, respectively (Figure 4). 100 µM NEM, an inhibitor of exocytosis,\(^{29}\) effectively prevented the enhancement of ATP release induced by heat, suggesting that it was because of exocytosis and did not involve cell damage by high temperature (Figure 4). In the presence of 5 mM EGTA, the heat-induced ATP secretion was partially reduced at 52 °C, but was not affected at 43 °C. This suggests that extracellular calcium entry partially contributed to ATP release at 52 °C.

**DISCUSSION**
Moxibustion has been found to induce degranulation of MCs residing in acupoints in vivo.\(^6\) Heat is the main stimulus during moxibustion. In the present study, most maximum temperatures ranged from 40-45 °C in humans, which includes the threshold for TRPV1 at 43 °C.\(^9\) TRPV1 is a thermosensitive vanilloid-sensitive channel protein expressed on MCs.\(^8\) We found that a temperature of 43 °C induced activation of HMC-1 cells, manifested by degranulation, [Ca\(^{2+}\)]\(_i\) increase, and ATP secretion. In a few cases, maximum temperatures reached 50 °C during monkshood cake moxibustion. Because the heat stimulation by monkshood cake moxibustion is moderate compared with other types of indirect moxibustion\(^17\) and warming needling moxibustion,\(^1,2\) the maximum temperature are expected to be higher with other forms of moxibustion. TRPV1 and TRPV2 are also thermosensitive ion channels in MCs,\(^8\) but they have a threshold temperature of 52 °C.\(^9\) TRPV2-mediated degranulation, increased whole-cell currents, and [Ca\(^{2+}\)]\(_i\) elevation have been reported to be induced by temperature of 53 °C.\(^8\) We found that the response of HMC-1 cells, including degranulation, [Ca\(^{2+}\)]\(_i\) increase, and ATP release were more pronounced at 52 °C than 43 °C. The presence of EGTA in the extracellular medium partially inhibited elevation of [Ca\(^{2+}\)]\(_i\) in HMC-1 cells,
suggesting that entry of calcium from the external medium contributed to the [Ca$^{2+}$]i increase induced by the threshold temperatures of 43 °C and 52 °C for TRPV1 and TRPV2, respectively. TRPV1 and TRPV2 form ion channels that are permeable to calcium and sodium ions. Therefore, activation of these channels will allow entry of extracellular calcium. The extent to which the influx contributes to the increase in [Ca$^{2+}$], still needs further investigation.

Heat-induced ATP release has also been reported for some other cell types. Erythrocytes secrete more ATP at temperatures of 39 °C and 43 °C. Temperatures of about 40 °C promoted keratinocytes to release ATP by activating membrane TRPV3. We found that a similar temperature of 43 °C also potentiated ATP release from HMC-1 cells. Usually, an elevation of [Ca$^{2+}$], is the trigger signal for ATP release. At 43 °C, the presence of EGTA could effectively inhibit [Ca$^{2+}$]i elevation, but could not prevent the enhancement of ATP release. This suggests that ATP release induced at 43 °C did not completely depend on the entry of external Ca$^{2+}$. TRPV1 channels might be activated at 43 °C and induce the entry of external Ca$^{2+}$, but this process cannot account for the entire ATP release. At 52 °C, the presence of EGTA effectively inhibited the increase of [Ca$^{2+}$]i and ATP release. Therefore, we hypothesize that heating to 52 °C activates of TRPV2 channels and allows the influx of extracellular calcium, which triggers cellular ATP release. SKF96365, a specific inhibitor for TRPV2, could partially inhibit the effects of temperature of 52 °C on ATP release from HMC-1 cells (data not shown). Obviously TRPV2 channels contributed to the ATP release induced by severe heat. The interaction between TRPV channels and purinergic signals has been reported previously. Extracellular ATP is the endogenous agonist for P2X receptors. MCs express purinergic receptors including P2X receptors, which are also non-selective calcium channels.

Therefore, there might be positive feedback between ATP secretion and [Ca$^{2+}$]i increase in MCs. Such a mechanism could amplify the heat effects at local areas to treat local disorders by moxibustion. The peripheral nervous system is the main domain for purinergic receptors, and MCs interact with nerve endings showing changes in morphology and function. The ATP released from MCs by heat may also bind the purinergic receptors expressed on nerve endings. The signal in response to heat is then transmitted to neurons in ganglia and further ascends to higher nervous structures to modulate organ functions.

Monkshood cake moxibustion generates heat sufficient to trigger MC degranulation, [Ca$^{2+}$]i elevation, and ATP release. This suggests that purinergic signals originating from MCs might be the initiating response of acupoints to moxibustion.

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