Hyperthermal Influence on Intracellular Free Calcium Concentration in Cultured Macrophages of Mice

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In this study, we have determined the fluorescence value of intracellular free calcium ($[Ca^{2+}]_i$) in macrophages from mice cultured in different temperatures (37°C, 38°C and 39°C) by laser cytometer. The results indicate that hyperthermia caused elevations in the levels of $[Ca^{2+}]_i$. The characteristic effects of hyperthermia were, 1) rapid changes in $[Ca^{2+}]_i$ concentration, that is to say, the fluorescence value of $[Ca^{2+}]_i$ rose a few sec after hyperthermia; 2) a detectable relationship between the fluorescence value and temperature during defined temperature (38°C–39°C) and time (0–

3600 sec), 3) non-synchronous responses of cells, in other words, the extent and time of elevation of $[Ca^{2+}]_i$ fluorescence value were different among the cells of the same group. We thought $[Ca^{2+}]_i$ elevation induced by heat happened through the mechanism of IP_3 -induced calcium release (IICR) and calcium-induced calcium release (CICR). The heparin and procaine might lower the concentration of $[Ca^{2+}]_i$ during hyperthermia through the mechanism that IICR and CICR were inhibited respectively. Free calcium influx from the extracellular medium was not thought to be necessary for heat-induced $[Ca^{2+}]_i$ elevation.

Key words: Macrophage, Hyperthermia, Intracellular free calcium, Fluorescence value

I. Introduction

The influence of hyperthermia on living organisms has been of great interest for some time. Many investigators have physiologically, biochemically and structurally studied either the $[Ca^{2+}]_i$ or thermally-induced membrane potential changes in various organisms [2, 9, 13, 16, 20, 21, 24, 26, 27]. Previously, changes in intracellular free calcium ($[Ca^{2+}]_i$) after heat or cold stimulation was reported in the cells of salivary glands [5], myocytes [11, 12], cardiocytes [17], hair cells [18], sensory neurones [8, 15] and tumor cells [19]. However, up to now, the detailed effects of hyperthermia on living cells still remain to be clarified. In this study we measured the level of fluorescence values of $[Ca^{2+}]_i$ in macrophages cultured from mice at 37°C, 38°C and 39°C using a laser cytometer, Adhesive Cell Analysis and Sorting Interactive Laser Cytometer 570 (ACAS 570, Meridian Instruments, Inc., U.S.A.). The goals of this study were to explore the characteristic features and the mechanisms of $[Ca^{2+}]_i$

elevation in macrophages during hyperthermic conditions.

II. Materials and Methods

Cultures of mouse peritoneal cavity macrophages

Forty-nine male Kunming mice weighing between 18 to 20 g were used in the present study. The animals were reared under ordinary laboratory conditions and fed a normal diet until use. In every experiment, three mice were decapitated and bled, and then immersed in a beaker with 75% alcohol for 3 min. The mouse peritoneal cavities were exposed on a clean bench. The peritoneal fluid containing macrophages was aspirated after the peritoneal injection of 5 ml RPMI-1640 culture medium and centrifuged at 1000 rpm for 5–10 min. The supernatant was discarded and the pellet was resuspended in the culture medium. The cell concentration was adjusted to 2×10^6 cells/ml RPMI-1640 culture medium. Subsequently, 0.2 ml of the suspension was dropped into the central portion of a modified Petri dish already containing 0.5 ml of culture medium and 10% fetal bovine serum. After the cells were cultured for 24 hr in a CO_2 incubator containing 5% CO_2 , the culture medium was changed two times with 1 ml RPMI-1640 to remove the non-adherent cells. The

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macrophages still attached in Petri dishes were continuously cultured for 48 hr in a CO₂ incubator containing 5% CO₂.

Loading of fluorescence probes

The loading method of the fluorescence probes was different according to the experimental purpose. The cells were only loaded with Fluo-3 AM (Fluo-3-acetoxymethyl ester) in studying the influence of $[Ca^{2+}]_i$ concentration treated with a different temperature. The process was as follows, 1) every modified Petri dish containing macrophages was washed 3 times with 5 mmol/L HEPES [N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)] buffer (pH 7.2–7.3), 2) 0.2 ml of staining solution, that is, 10 μ mol/L Fluo-3 AM dissolved in RPMI-1640, was added to each dish and retained for 30 min at 37°C or room temperature, and 3) after that, the dish was washed 3 times with HEPES buffer, and 0.5 ml buffer were kept in each dish at the last time. This process is known as Fluo-3 Test. In fluorescence ratiometric measurement of $[Ca^{2+}]_i$, the cells were dually loaded with Fluo-3 AM and Fura-red. The two kinds of fluorescence probes loaded, namely, 0.2 ml 10 μ mol/L Fluo-3 AM and 10 μ mol/L Fura-red were simultaneously added to each dish in step 2 compared with Fluo-3 Test. The experiment of $[Ca^{2+}]_i$ ratiometric measurement was named Fluo-3 and Fura-red Test. Its characteristic was to use the two laser detectors of ACAS 570 to survey the changes of $[Ca^{2+}]_i$ more precisely.

Fluorescence measurements of $[Ca^{2+}]_i$

The ACAS 570 is a precision instrument which can determine the fluorescence value representing some special substances by means of laser scanning, on which there is an accessory, mini-incubator. It is a metal box with a lid and there is a round hole which can hold a Petri dish on the bottom. The mini-incubator connects with the mains and temperature-controlled knob of the apparatus, and the regulated range of the temperature is 35°C to 45°C. When measured, the mini-incubator was set on the scanning stage of the fluorescence microscope of the apparatus, then the mains was put through and the temperature was controlled by a temperature-controlled knob. The temperatures of 18 experiments in this study were all controlled by the mini-incubator. Among these, the process of Fluo-3 Test was: 1) the wavelength of the chosen excitation ray was 488 nm, 2) the dish with cells loaded by Fluo-3 AM was placed in the mini-incubator and covered with a lid, 3) temperature was controlled at 37°C, 4) to observe cellular conditions in the Petri dish and choose cells grown well and with moderate density to measure; 5) regulating the test-parameters through computer and assigning a file name; and 6) the fluorescence values of the chosen cells were measured continuously for 3000–3600 sec and the data were saved to computer. After the measurement was completed at 37°C, using another dish containing cells, the temperature was elevated to 38°C and another test was begun as above. After the test at 38°C,

the third dish was measured at 39°C. At least three Petri dishes containing macrophage were tested during each experiment. This experiment was repeated 5 times, during which, there were 3 times that the temperature was lowered to 37°C and the fluorescence value of the cells in the same dish was tested continuously for 360 sec after measuring at 39°C. In the 4 times that Fluo-3 and Fura-red Tests were conducted, the cells of the same dish were measured at different temperatures. The measured time of each cell group (37°C, 38°C and 39°C groups) was respectively 1500 sec, 1100 sec and 1000 sec.

To study further the influence mechanism of hyperthermia on $[Ca^{2+}]_i$, the heparin and procaine were added into the culture medium in hyperthermia. It was named for Heparin and Procaine Test and was repeated 3 times. In the tests, the cells which were cultured in the same Petri dish and loaded with Fluo-3, were measured for 300–360 sec in 37°C, then measured in 38°C for 360 sec with 0.5 mg heparin in each dish. After completing the measurement the culture medium with heparin was changed into the culture medium with 20 μ g procaine in each dish, the temperature was changed to 39°C and measured for 600 sec.

Expression and statistics of experimental data

The main expressions of experimental data were average fluorescence values and fluorescence graphics of cells. Mean $\pm$ SD of averaged fluorescence values were taken as statistical data and compared between the control group (37°C) and the experimental group (38°C and 39°C). The graphics were photographed and printed with the camera and printer.

III. Results

Most of the cultured macrophages adhered tightly to the surface of the dishes after 72 hr. The cells were short and shuttle-shaped. They crowded into many areas of the dishes. Individual cells were easily distinguished by two dimensional fluorescence images.

Fluo-3 Test

In the 37°C group, 428 cells were measured totally, the highest fluorescence value of $[Ca^{2+}]_i$ in macrophages was 136.45 arbitrary units, the lowest was 106.95 arbitrary units, and the average fluorescence value was 121.7 ± 29.5 arbitrary units (mean $\pm$ SD) (Fig. 1). In the 38°C group, 461 cells were measured, the average fluorescence value of $[Ca^{2+}]_i$ in cells was 301 ± 92.5 arbitrary units (mean $\pm$ SD) (Fig. 2). The values obtained from the 38°C group cells showed that there was a gradual increase in fluorescence value with time. Fig. 2 is a result of one experiment. The values were 248 arbitrary units at the beginning (0 sec) and 409 arbitrary units at the end (3600 sec) of scanning. If the curve was divided into four parts by placing marks at 1000, 2000, 3000 and 3600 sec respectively, it could be seen that the slope of the curve was

Fig. 1. Curve of average fluorescence value of $[Ca^{2+}]_i$ in 37°C group cells.

Fig. 2. Curve of average fluorescence value of $[Ca^{2+}]_i$ in 38°C group cells. The values rose gradually with time. However, no great changes were found in the last 600 sec. Lines were drawn at the 1000, 2000, 3000 and 3600 sec marks.

greatest during the first 1000 sec (about 60 arbitrary units). Values increased only 40 arbitrary units in the second and third 1000 sec intervals respectively. No great changes were observed during the last 600 sec (Fig. 2). Statistical comparison (student's test) of average fluorescence values of $[Ca^{2+}]_i$ between 37°C and 38°C, 37°C and 39°C group cells showed significant differences ($p < 0.01$).

In the 39°C group, 406 cells were measured. The slopes of the measured values increased rapidly over time. Fig. 3 was the result of one experiment. The fluorescence value arrived at 492 arbitrary units once the cells were

Fig. 3. Curve of average fluorescence value of $[Ca^{2+}]_i$ in 39°C group cells. The value increased approximately 3-fold from the beginning (0 sec) to the end (3600 sec).

cultured at 39°C and increased 3-fold approximately in the end, therefore, the two dimensional fluorescence intensity of the cells got progressively darker from the beginning (0 sec) to the end (3600 sec) (Figs. 4, 5). However, elevation of the values was not synchronous among the loaded cells. Fig. 6 represents the curves from six cells reflecting the $[Ca^{2+}]_i$ changes during 3600 sec in same cell group. Not only did the range of the fluorescence values among them differ greatly (for example, No. 3 cell: 319–807 arbitrary units and No. 4 cell: 642–2107 arbitrary units) but the slope of each line was distinct (for example, No. 1, 2 and 4 cells dropped 300–400 arbitrary units at 1500 sec). When the temperature returned to 37°C, the average fluorescence value in the cells came down, but until

Fig. 4. These were fluorescence value graphics of 39°C group cells at the beginning of the test (0 sec). The fluorescence intensity of six cells surrounded with polygonal rings was lower at the beginning of scanning (0 sec) than at the end (3600 sec, Fig. 5). $\times 400$.

Fig. 5. The cells are the same as in Fig. 4 but at 3600 sec. The fluorescence intensity of the cells was obviously elevated at the end (3600 sec) of scanning. $\times 400$.

Fig. 6. The six curves represented fluorescence value of $[Ca^{2+}]_i$ of the six cells in 39°C group cells. They were non-synchronous on the response of hyperthermal influence. Abscissa: Time (sec). Ordinate: Average fluorescence value (Arbitrary unit).

measurement was completed, it did not decline to the beginning degree of 37°C. Fig. 7 represents the fluorescence value of 3 measurements.

Fluo-3 and Fura-red Test

247 cells were tested, with the increase of temperature, the fluorescence value of $[Ca^{2+}]_i$ showed different forms. Fig. 8 is the result of ratiometric measurement of $[Ca^{2+}]_i$ in one test. The fluorescence value of $[Ca^{2+}]_i$ in the cells loaded with Fluo-3 increased after hyperthermia, but the fluorescence value of $[Ca^{2+}]_i$ in the cells loaded with Fura-red declined with increase of temperature. The two curves crossed at 111,000 arbitrary unit.

Heparin and Procaine Test

In 3 experiments, 242 cells in all were determined. Before heparin and procaine were added, the average fluorescence value of $[Ca^{2+}]_i$ increased with temperature from 37°C to 38°C. After heparin was added, the average fluorescence value showed a slight decline for 360 sec.

Fig. 7. Curves of fluorescence value of $[Ca^{2+}]_i$ in macrophages (from 37°C, to 38°C, to 39°C, and returned to 37°C again). Three curves represented the results of 3 experiments. —◆—: No. 1, —■—: No. 2, —▲—: No. 3 experiment.

Fig. 8. Under hyperthermia, ratiometric measurement of $[Ca^{2+}]_i$ fluorescence values in macrophages. —◆—: fluorescence value of $[Ca^{2+}]_i$ loaded with Fluo-3, —■—: fluorescence value of $[Ca^{2+}]_i$ loaded with Fura-red.

Fig. 9. Three curves represent the average fluorescence value of macrophages in three experiments, in which the cells were treated with heparin and procaine respectively. H; Place of adding heparin, P; Place of adding procaine.

When procaine was added in 39°C, the fluorescence values still tended to drop (Fig. 9).

IV. Discussion

The present study showed that $[Ca^{2+}]_i$ obviously increased in the macrophages cultured under hyperthermic conditions and this change had the following characteristics: 1) the response of $[Ca^{2+}]_i$ concentration on hyperthermia was repaid. In several sec after hyperthermia the fluorescence value of $[Ca^{2+}]_i$ showed an obvious change (Figs. 2, 3) generally. 2) there was a detectable relationship between $[Ca^{2+}]_i$ concentration and temperatures at defined times (0–3600 sec) and temperatures (37°C–39°C). The higher the temperature was, the stronger the fluorescence value of $[Ca^{2+}]_i$ was, and 3) the response was non-synchronous. That is to say, although $[Ca^{2+}]_i$ concentration of macrophages became higher with the temperature, the response of each cell was not identical. For instance, the six cells in Fig. 6 were placed at the same environmental temperature but the extent and time of $[Ca^{2+}]_i$ concentration elevation in each macrophage was different.

The $[Ca^{2+}]_i$ concentration of elevation in macrophages cultured under hyperthermic conditions, theoretically, should return to a normal level with temperature

recovered (37°C) if there are no obviously secondary pathological changes. So we planned the experiments observing the relationship of recovered temperature and $[Ca^{2+}]_i$ concentration. The results (Fig. 7) showed that the fluorescence value declined 25% when the temperature was changed from 39°C to 37°C, although the value did not return to the former level in 37°C. Therefore, we thought, within a defined time and temperature, the change of $[Ca^{2+}]_i$ was reversible, that is to say, there existed a reversible regulation in the cell. As for the value not being able to return to a normal level, there might be three reasons, 1) laser beam illumination used to measure itself possibly had some influence upon $[Ca^{2+}]_i$ concentration. To test this assumption, we carried out 3 experiments with 3T3 cell line. The course of loading cells with fluorescence probe Fluo-3 was the same as that being introduced before. The fluorescence value of $[Ca^{2+}]_i$ in 3T3 cells was measured at room temperature for 3000 sec. The result showed that the fluorescence value increased 4–6% while being illuminated by scanning laser beam. This indicated that the laser beam illumination could elevate fluorescence value of $[Ca^{2+}]_i$ partly during testing. 2) It was only 360 sec after 39°C–37°C, the space of a few short min, it was not enough for recovery, and 3) it just suffered from heat. The regulated ability of the cells was influenced and insensitive temporarily.

In order to verify further that the elevation of intracellular calcium caused by hyperthermia was really $[Ca^{2+}]_i$, ratiometric measurement of the cells was carried out after being dually loaded with Fluo-3 and Fura-red. According to characteristics of these two kinds of calcium indicators, when they were loaded dually and excited at 488 nm simultaneously, the higher the concentration of $[Ca^{2+}]_i$ was, the stronger the fluorescence value given out by Fluo-3 was; conversely, the weaker the fluorescence value given out by Fura-red was. Our result (Fig. 8) was basically in accord with it, which provided powerful collateral evidence for the fact that the elevation of intracellular calcium caused by hyperthermia was really $[Ca^{2+}]_i$.

After the macrophages were treated with hyperthermia, where was the increased $[Ca^{2+}]_i$ from? That is, what was the mechanism of heat-induced change in calcium?

Generally considering, the intracellular calcium homeostasis depends on the influx of Ca^{2+} from the extracellular medium, and/or the release of Ca^{2+} from an internal store. The former includes five forms, that is, voltage-operated Ca^{2+} channels (VOC), receptor-operated Ca^{2+} channels (ROC), second messenger-operated Ca^{2+} channels (SMOC), mechanically operated Ca^{2+} channels (MOC), and leak Ca^{2+} or tonically active Ca^{2+} channels. In our experiments, before measurement, the cells had lived in HEPES buffer without Ca^{2+} . Therefore the influx from the extracellular medium might not happen. So we considered that $[Ca^{2+}]_i$ was also elevated although there were no calcium ions in the extracellular medium. That is to say, it is not necessary that heat-induced $[Ca^{2+}]_i$ eleva-

tion was caused by the influx of the extracellular medium. $[Ca^{2+}]_i$ elevated under hyperthermia might result from the intracellular calcium. The intracellular calcium included a small part of free calcium existing in the cytoplasmic matrix and the majority of combined calcium existing in the endoplasmic reticulum or the sarcoplasmic reticulum, which was called a calcium pool. The calcium pool included IP_3 -sensitive calcium pool (IsCaP) and IP_3 -insensitive calcium pool (IisCaP), and released Ca^{2+} to the cytoplasmic matrix through the IP_3 -receptor channel and ryanodine receptor channel respectively. The IP_3 receptors and ryanodine receptors were distributed mainly over the endoplasmic reticulum and/or sarcoplasmic reticulum, but the mechanism of causing Ca^{2+} to be released was different. The former release was induced by IP_3 (inositol triphosphate), which was called IP_3 -induced calcium release (IICR), and the latter was induced by the elevated Ca^{2+} , which was called calcium-induced calcium release (CICR) [6].

From our results, the elevation of Ca^{2+} caused by heat might be related to the two mechanisms above. The main reason was that after adding heparin, the average fluorescence value of cells was resisted although increasing temperature still did not elevate it after 360 sec. When the elevated temperature was at 39°C, treated with procaine, the fluorescence value was lower than that before adding procaine too (Fig. 9). Many scholars consider heparin to be a distinctive antagonist of IP_3 receptor [3, 4, 10, 14, 23], since heparin can control IP_3 -sensitive calcium pool and arrest Ca^{2+} flowing from calcium to cytosol. Despite living in a heated environment, cellular Ca^{2+} resource had dried up, so $[Ca^{2+}]_i$ concentration lowered. Procaine was an antagonist of ryanodine receptor (RYP) [1, 22, 25]. RYP could cause Ca^{2+} to be released from a IP_3 -insensitive calcium pool to cytosol, and this process provoked a positive feedback mechanism of CICR [25]. When cells contained procaine at hyperthermia there were extra-conditions for causing $[Ca^{2+}]_i$ to rise, but procaine could prevent Ca^{2+} in IP_3 -insensitive calcium pool from being released, and intra-conditions of $[Ca^{2+}]_i$ elevation were partly lost, so an increase of fluorescence value of $[Ca^{2+}]_i$ was not observed. The positive feedback mechanism of CICR was hard to find. Moreover, procaine could also block CICR caused by $[Ca^{2+}]_i$ concentration.

Generally speaking, the change of Ca^{2+} often showed calcium wave and calcium oscillation [7], but it did not respond definitely in our results. It is possible that because there was no Ca^{2+} in the extracellular medium, the calcium wave and calcium oscillation was not distinct and firm.

Some investigators consider that there is no correlation between the hyperthermal cell death and heat-induced alteration of calcium homeostasis [27]. We suggest that hyperthermal $[Ca^{2+}]_i$ elevation is a clear indication of the existence of metabolic disorders in cells. Heat-induced alteration of calcium homeostasis might cause cell death. Further understanding of the

law of elevation may be highly significant to gaining knowledge about the influence of hyperthermic conditions on the elevation of $[Ca^{2+}]_i$ in cells.

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VI. References

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