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Original Article

Ki-Energy (Life-Energy) Protects Isolated Rat Liver Mitochondria from Oxidative Injury

S. Tsuyoshi Ohnishi[1], Tomoko Ohnishi[2] and Kozo Nishino[3]

[1]Philadelphia Biomedical Research Institute, King of Prussia, PA 19406, [2]Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA and [3]School of Nishino Breathing Method, Shibuya-ku, Tokyo 150, Japan

We investigated whether 'Ki-energy' (life-energy) has beneficial effects on mitochondria. The paradigm we developed was to keep isolated rat liver mitochondria in conditions in which they undergo heat deterioration (39°C for 10 min). After the heat treatment, the respiration of the mitochondria was measured using a Clarke-type oxygen electrode. Then, the respiratory control ratio (RC ratio; the ratio between State-3 and State-4 respiration, which is known to represent the integrity and intactness of isolated mitochondria) was calculated. Without the heat treatment, the RC ratio was >5 for NADH-linked respiration (with glutamate plus malate as substrates). The RC ratio decreased to 1.86–4.36 by the incubation at 39°C for 10 min. However, when Ki-energy was applied by a Japanese Ki-expert during the heat treatment, the ratio was improved to 2.24–5.23. We used five preparations from five different rats, and the significance of the differences of each experiment was either $P < 0.05$ or $P < 0.01$ ($n = 3$–5). We analyzed the degree of lipid peroxidation in the mitochondria by measuring the amount of TBARS (thiobarbituric acid reactive substances). The amount of TBARS in heat-treated, no Ki-exposed mitochondria was greater than that of the control (no heat-treated, no Ki-exposed). However, the amount was reduced in the heat-treated, Ki-exposed mitochondria (two experiments; both $P < 0.05$) suggesting that Ki-energy protected mitochondria from oxidative stress. Calcium ions may play an important role in the protection by Ki-energy. Data also suggest that the observed Ki-effect involves, at least, near-infrared radiation (0.8–2.7 μm) from the human body.

Keywords: infrared radiation – Ki-energy – lipid peroxidation – mitochondria – oxidative stress – reactive oxygen species – respiratory control ratio – TBARS

Introduction

We are demonstrating that so-called 'Ki-energy' is a natural phenomenon, and therefore, it can be analyzed by rigorous scientific and objective investigations. A 'breathing method' developed by a Japanese Ki-expert, Kozo Nishino (1–4), stimulated immune activity of practitioners and lowered their stress levels (5). We then reported that his Ki-energy inhibited cell division of cultured human carcinoma cells (6). Since 'breathing' is directly related to oxygen respiration, he has long proposed that mitochondria may play a key role in maintaining vitality and health (3,4). This led us to undertake the project to explore a possible relationship between Ki-energy and mitochondrial function.

Isolated rat liver mitochondria is a well-established model for studying biophysical and biochemical aspects of energy metabolism. The simplest marker for the intactness of mitochondria is a respiratory control ratio (RC ratio, which is the ratio between State-3 and State-4 respiration) (7). Using this model, we found that a heat treatment (incubation at 39°C for 10 min) decreased the RC ratio by ∼60%. Therefore, we examined whether Ki-energy emitted from the fingers of the Japanese Ki-expert (K.N.) could inhibit the decrease.

For reprints and all correspondence: S. Tsuyoshi Ohnishi, PhD, Philadelphia Biomedical Research Institute, suite 250, 100 Ross Road, King of Prussia. PA 19406, USA. Tel: +1-610-688-6276; Fax: +1-610-254-9332; E-mail: stohnishi@aol.com

We also attempted to find the mechanism for the Ki-effect. After the early work by Boveris and Cadenas (8), reactive oxygen species (ROS) has been recognized as an important factor to damage mitochondrial functions, and numerous papers were published on this subject for the past 30 years. In order to test whether Ki-energy could reduce the ROS production, we measured the amount of mitochondrial lipid peroxidation after the heat treatment (with and without exposure to Ki-energy) using a well-known assay technique for TBARS (thiobarbituric acid reacting substances).

From the test of comparing the effects of ethylene diamine tetraacetic acid (EDTA) and ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA) during the preparation of mitochondria, we tried to find whether or not calcium ions would play a role in the protection mechanism by Ki-energy. Since we already demonstrated that Ki-energy would involve infrared radiation (6), we used optical filters to examine the range of wavelengths with which Ki-energy was effective in protecting mitochondria.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Preparation of Mitochondria

Rat liver mitochondria were prepared from male Sprague–Dawley rats (body weight ~200 g) by the method of Hagihara (9) with a slight modification. In brief, the preparation buffer contains 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, 1 mg ml^{-1} bovine serum albumin (BSA) and 10 mM HEPES (pH 7.4). In this buffer, liver was minced and homogenized with a teflon-glass homogenizer. After homogenization, the mitochondrial fraction was separated as a fraction which was sedimented by centrifugation between 600 *g* for 10 min and 6000 *g* for 10 min. The pellet was wash-centrifuged with a washing buffer consisting of 10 mM HEPES (pH 7.4) and 150 mM KCl. The final pellet was suspended in the same buffer at a protein concentration of ~30 mg ml^{-1} and stored at 0°C. The protocol was approved by the institutional animal care and use committee.

Application of Ki-energy

This is essentially similar to the method we used for the cultured cancer cell experiments (6), except that for a mitochondrial suspension (0.05–0.1 ml) was kept in a thermostatic air chamber. The reason why we did not use a conventional water bath to keep the temperature of the mitochondrial suspension constant was that the infrared radiation is absorbed by water. In the chamber, the suspension was sandwiched between two borosilicate glass plates (diameter 40 mm and the thickness 0.17 mm; the transmittance of infrared radiation is above 90% up to the wavelength of 5 μm) which were separated by spacers (0.1 mm thick). The assembly was placed in a air chamber (made of 1.6 mm acrylic plates) with two borosilicate glass windows (40 m diameter and 0.17 mm thick) through which Ki-energy was applied (Fig. 1A). By circulating water from a thermostatic water bath into two coils made of Tygon tubings, the temperature inside the air chamber was kept at ~37°C. Temperature of the mitochondrial suspension was heated to 39°C with two heating elements (each 5 Ω, 2 W) and by applying the electric voltage (adjustable between 3.6 and 6 V) across two heating elements (total of 10 Ω). The air inside the chamber was circulated by two small motor-driven fans. The temperature was regulated by using a thermocouple thermometer and a K-type (small bead-type) thermocouple (Fisher Scientific, Pittsburgh, PA) connected with a Honeywell 700 regulator-relay system (Cole-Parmer Instrument Co., Vernon Hills, IL). The diameter of the bead is 0.8 mm, and the bead was attached onto the borosilicate glass plate with heat-conducting grease (which is used to increase heat conduction between a semiconductor chip and air-fins). The output of the thermometer (1 mV per centigrade) is connected to a recorder (5 mV full scale) (Fig. 1B). Figure 1C shows an inside view of the air chamber. Figure 1D and E show how Ki-energy was applied through the windows. The actual temperature recordings are shown in Fig. 2.

Optical Filters

Two visible range filters (IR-absorption filter) with a bandpass wavelength range of 360–760 nm and two infrared filters with a bandpass wavelength range of 0.8–2.7 μm were purchased from Edmond Optics (Barrington, NJ). When they were used, one filter was placed on top of the chamber, and the other under the chamber (Fig. 1E). The transmission spectrum for visible wavelength was measured using Hitachi spectrophotometer model U-2000. That for infrared filter was measured using Bruker infrared spectrophotometer model IFS 66 at the laboratory of Dr J. Vandakooi, Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine (see Fig. 3).

Assay for Mitochondrial Respiration

Oxygen consumption of isolated rat liver mitochondria (4 mg protein per ml) was measured using a Clark-type oxygen electrode at 25°C (the chamber volume of 0.3 ml) in a reaction medium which consists of the washing buffer plus the additions of 4 mM potassium phosphate, 0.1 mM EDTA and 1 mg ml^{-1} BSA. Since NADH is not permeable to the intact mitochondrial inner membrane, we used a mixture of glutamate and malate, both of which are permeable to the inner membrane and produce NADH in the matrix space of mitochondria. A 80 μl mitochondrial suspension (30 mg ml^{-1}) in 150 mM KCl, 10 mM HEPES (pH 7.4) and 20 mM glutamate-malate was kept in the chamber for 10 min at 39°C with or without the application of Ki-energy. After incubation, 40 μl of the suspension was collected and added to a polarographic

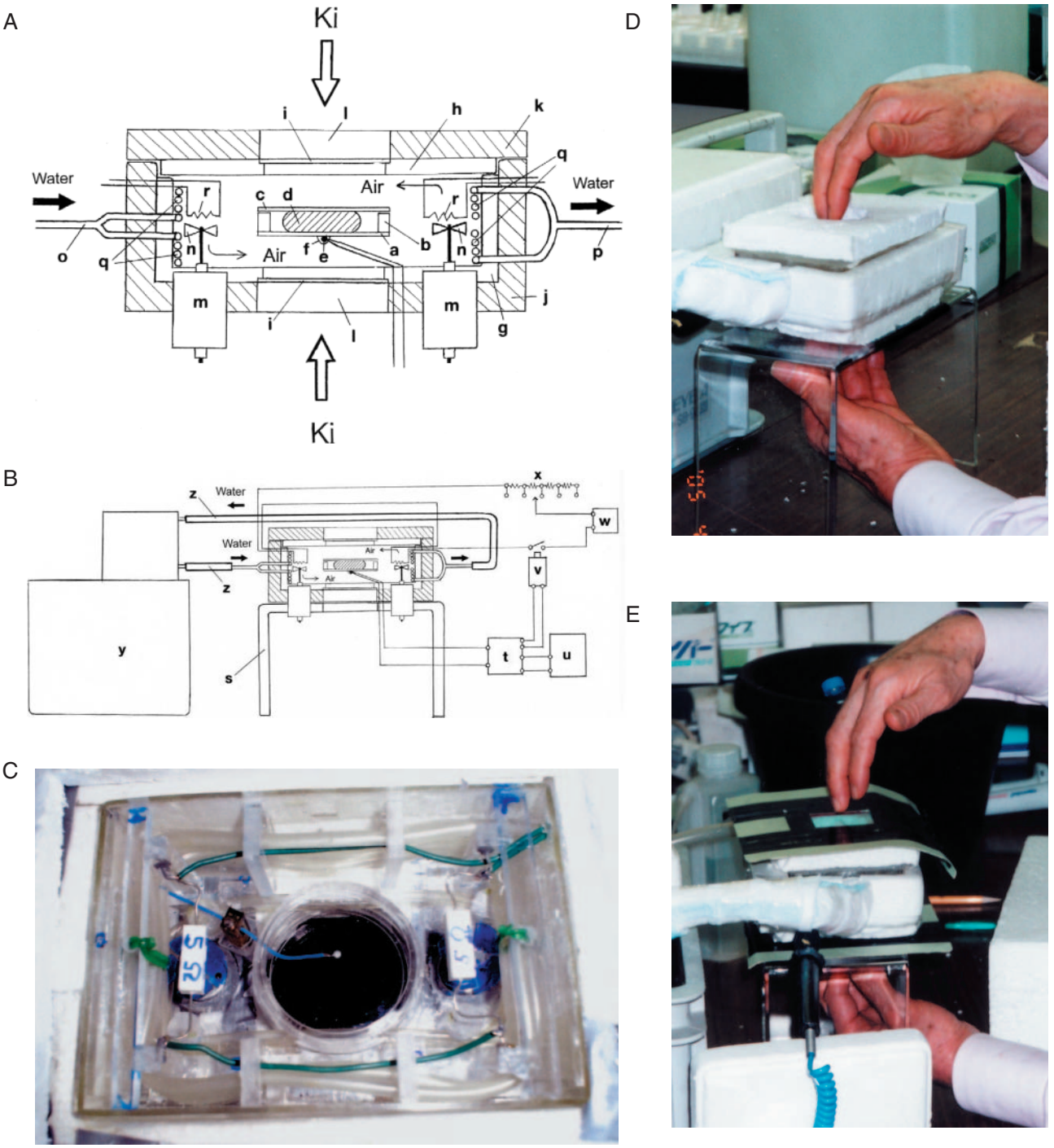

Figure 1. (**A**) Schematic illustration of how Ki-energy is applied to a mitochondrial suspension kept between two glass plates (0.17 mm thick), which are separated by thin spacers (0.1 mm thick), and placed inside a thermostatic air chamber. The drawing is not in proportion to the actual size. *Abbreviations*: a, borosilicate glass plate (0.17 mm thick); b, spacer (0.1 mm thick); c, lid made of borosilicate glass plate (0.17 mm thick); d, mitochondrial suspension; e, bead-type thermocouple (diameter, 0.8 mm); f, heat-conducting silicone grease; g, box made of acrylic plates (1.6 mm thick); h, lid made of acrylic plate (1.6 mm thick); i, borosilicate glass window (40 mm diameter and 0.17 mm thick); j, heat-insulating box made of polystyrene foam (8 mm thick); k, heat-insulating lid made of polystyrene foam (8 mm thick); l, 50 mm diameter holes made in the heat insulators; m, small toy-motor; n, fan; o, circulating water inlet; p, circulating water outlet; q, two coils made of thin Tygon tubings; r, heating elements (each 5 Ω, 2 W). (**B**) Schematic illustration of the constant temperature system. *Abbreviations*: s, plastic stand with a 50 mm diameter hole on the top plate; t, thermometer; u, recorder; v, relay-regulator; w, constant DC voltage supply (6 V); x; resistors and a rotary switch to adjust the voltage applied to the heating elements; y, thermostatic water bath; z, rubber tubings. (**C**) The inside of the air chamber showing the coil made of Tygon tubings. A blue wire is the conductors for the thermocouple. The white material at the tip of the blue wire is a small lump of heat-conducting grease which covered the thermocouple. (**D**) Ki-energy is applied from Nishino's fingers through the holes made in the insulator. (**E**) Ki-energy is applied through two optical filters (one on top and the other under the chamber).

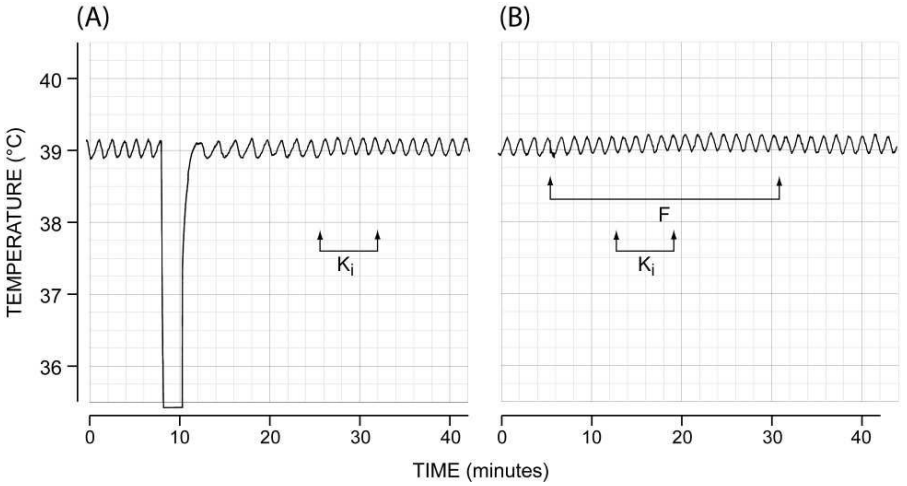

Figure 2. Temperature recording of a mitochondrial suspension (100 µl) as measured by thermometer with a bead-type thermocouple. (**A**) A dip and a rise of the recording at the beginning show the timing when a new suspension was applied between two borosilicate plates. Ki indicates the application of Ki-energy. (**B**) F indicates when two filters were placed, one on top and the other under the chamber. Ki indicates the application of Ki-energy through the filters. See text for details.

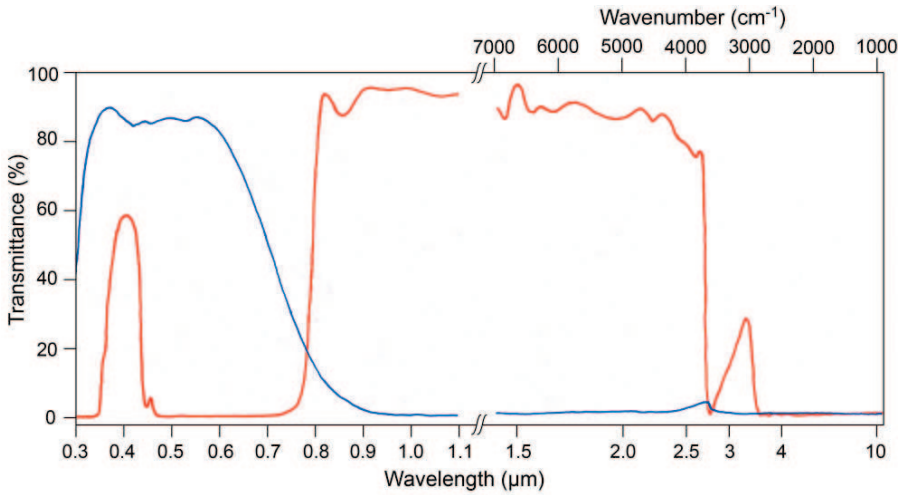

Figure 3. Transmission spectra recorded by Hitachi U-2000 (from 300 to 1100 nm) and Bruker IFS-66 (from 1.42 to 10 µm). Blue curve, IR-absorption filter; red curve, IR-bandpass filter.

cell (total volume 0.3 ml), which was incubated with the reaction medium at 25°C. After a few minutes of stabilization, 2.5 µl of a mixture of 0.5 M glutamate and 0.5 M malate was added to start measurements. Then, ADP was repeatedly added to the suspension (20 mM, 2 µl each) and the RC ratio was calculated from the ratio of the slope of State-3 respiration (during ATP synthesis from ADP) to that of the subsequent State-4 (after ADP was consumed) (7). We calculated the ratio from the average of ratios for three successive ADP additions.

Assay for Lipid Peroxidation

The method of Ohkawa *et al*. (10) which involves the extraction of TBARS by *n*-butyl alcohol was used. In brief, the substrates (10 mM glutamate and 10 mM malate) were added to a 80 µl mitochondrial suspension (suspended in 150 mM KCl, 10 mM HEPES, pH 7.4) and it was incubated in the air chamber for 10 min at 39°C (with or without Ki-application). Then, 40 µl was collected and the amount of TBARS was assayed. The amount of TBARS was measured using Hitachi fluorescent spectrophotometer Model 650 with the excitation wavelength of 533 nm and emission wavelength of 550 nm. As a standard for lipid peroxide, 1,1,3,3, ethoxy propane was used.

Statistics

All data are shown as the means ± standard deviations. We used a computer software (StatView) for statistical analysis. When two sets of data were compared, the Student's *t*-test

was used to assess the statistical significance. When multiple sets of data were compared, they were analyzed by ANOVA with the Fisher's PLSD test. In both cases, $P < 0.05$ was considered to be statistically significant.

Results

Temperature Stability of the Chamber

As shown in Fig. 2, the temperature of the mitochondrial suspension was regulated at 39 ± 0.12°C. When Ki-energy was applied by K.N. through two 40 mm holes made in the heat insulator box (Fig. 1D), the average temperature was raised by 0.03°C (because of the infrared radiation), but it returned to the original temperature a few minutes after the finger was removed (Fig. 2A). When two near-infrared range filters were placed above and below the windows (Fig. 1E), the temperature was raised by 0.02°C (because of a heat-insulating effect of the filter). When Ki was applied through the filter, the temperature was further raised by 0.02°C. When both fingers and filters were removed, the temperature did not quite return to the original value, but it was ~0.02°C higher than the original value (Fig. 2B). Therefore, the fluctuation of the temperature of the whole system was ±0.17°C. The temperature response of the system was satisfactory. When we applied a mitochondrial suspension (100 µl), the temperature returned to 39°C within 2 min if the 4.5 V was applied across 10 Ω of heating elements (Fig. 2A). When temperature reached to this level, the voltage was decreased to 3.6 V, because this gave a smaller fluctuation of the chamber temperature.

Oxygen Polarographic Analysis

The original mitochondria maintained a high degree of integrity as shown by an example of Fig. 4 curve (a) which had the RC ratio of 6.3. This ratio was maintained for at least 8 h after preparation if stored in ice. When mitochondria were incubated for 10 min at 39°C without Ki-energy, they deteriorated as shown in Fig. 4 curve (b); the RC ratio was 4.33. When Ki-energy was applied during the entire incubation time, the deterioration was inhibited as shown in Fig. 4 curve (c); the RC ratio became 5.23.

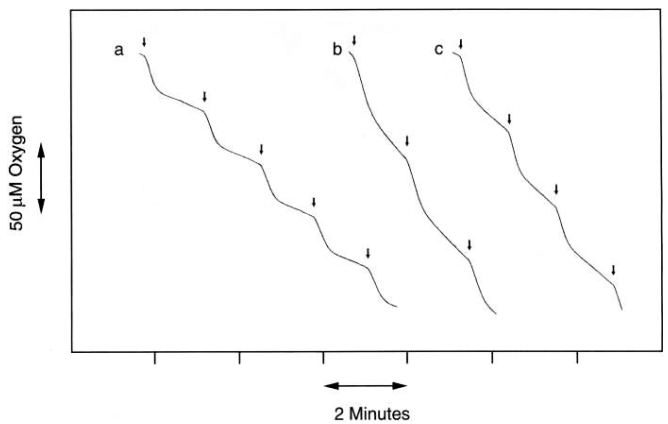

Figure 4. Example of polarographic records. (a) Control Rat liver mitochondria; (b) mitochondria were incubated without Ki-energy for 10 min at 39°C; (c) mitochondria were incubated for 10 min at 39°C while Ki-energy was applied. The final concentration of mitochondria was 4 mg ml^{-1}. See text for details.

Results from five mitochondrial preparations (obtained from five different rats) are summarized in Table 1. The experiments (A) were performed in an old chamber in which temperature control was not as good as the one shown in Fig. 1. The fluctuation was ±0.6°C, whereas in the experiments (B), it was improved to ±0.17°C. Although the values of RC ratios changed somewhat from a preparation to the next, the tendencies were always the same. With glutamate+malate as substrates, the improvement of the RC ratios by Ki-energy was always observed and the differences were statistically significant ($P < 0.05$ or $P < 0.01$; $n = 3$–5).

Ki-energy Reduces Membrane Lipid Peroxidation

As shown in Table 2, the heat treatment increased the amount of TBARS in the mitochondria, but the amount was decreased

Table 1. Effect of Ki-energy on the heat-induced decrease of RC ratios of isolated rat liver mitochondria

Experiments	−Ki	+Ki
Experiment A		
Exp. 1 (n =4)	1.86 ± 0.15	2.24 ± 0.18*
Exp. 2 (n =3)	4.36 ± 0.06	5.23 ± 0.39*
Exp. 3 (n =3)	3.41 ± 0.15	4.55 ± 0.80*
Experiment B		
Exp. 4 (n =4)	2.96 ± 0.17	3.66 ± 0.18**
Exp. 5 (n =5)	2.86 ± 0.12	3.28 ± 0.09**

The RC ratios and the standard deviations for five different preparations (from 5 different rats) are shown. For experiment (A), the mitochondrial suspension was kept at 39 ± 0.6°C for 10 min in the presence of 5 mM glutamate and 5 mM malate. For experiment (B), the condition was the same as (A) except for the temperature which was 39 ± 0.17°C. The RC ratios of the control experiments (no heat treatment, no Ki-exposure) were between 5 and 6.5. In this table, -Ki and +Ki indicate that the experiments were done without and with Ki-exposure, respectively. Other experimental conditions are the same as those shown in Fig. 4. n is the number of measurements for each experiment. The '*' and '**' indicate that the statistical significance determined by Student's t-test is $P < 0.05$ and $P < 0.01$, respectively.

Table 2. Effect of Ki-energy on the amount of TBARS (nmoles mg^{-1} protein) of mitochondria during the heat treatment

Experiments	Control	−Ki	+Ki
Exp. 1	0.35 ± 0.01	0.60 ± 0.06*	0.44 ± 0.11#
Exp. 2	0.22 ± 0.02	0.35 ± 0.04*	0.27 ± 0.01#

The mean values and the standard deviations for the amount of TBARS. Experimental conditions are the same as those shown in Fig. 4.
Abbreviations are the same as in Table 1. '*' indicates that the significance of difference from the control experiments was $P < 0.05$ ($n = 3$).
'#' indicates that the significance of difference from the −Ki experiments was $P < 0.05$ ($n = 3$).

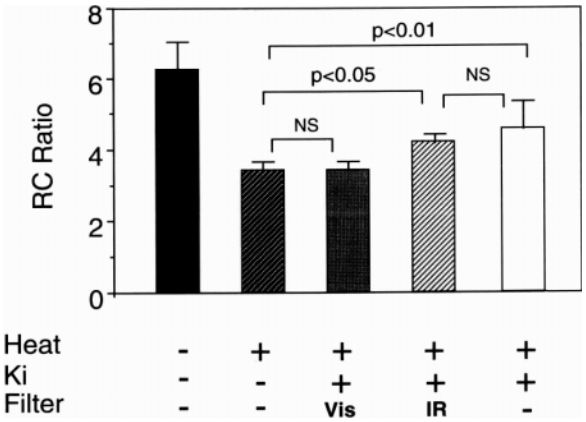

Figure 5. Effects of visible range filters (360–760 nm bandpass) and infrared filters (0.8–2.7 μm) on the RC ratios (bar graphs) with standard deviations (error bars). All data points were obtained using the same mitochondrial suspension prepared from one rat. The measurements were finished within 8 h after the preparation of mitochondria. *P*-values indicate the statistical significance of the difference. NS means that the difference was not significant. $n = 3$ for each category.

when Ki was applied during the 10 min incubation period. The difference was statistically significant for two preparations (for both experiments, $P < 0.05$; $n = 3$).

Optical Filters to Determine Ki-wavelengths

As shown in Fig. 5, the effect of Ki-energy on mitochondrial respiration was lost when two visible range filters (IR-absorption filters) were placed to interrupt the Ki-energy. There was no significant difference between no Ki experiment and Ki with visible range filter experiments ($n = 3$). This shows that visible light is not involved in the observed Ki-effects. On the contrary, when two infrared filters (0.8–2.7 μm) were placed, Ki-energy was able to protect the mitochondria ($P < 0.05$; $n = 3$). The difference between the no-filter/Ki-exposure experiments and infrared filter/Ki-exposure experiments was not significant ($n = 3$).

A Role of Calcium Ions on the Ki-effect on Mitochondria

Mitochondria accumulate calcium ions in the presence of phosphate and the substrate. Since calcium accumulation uncouples mitochondrial function, it has been a custom to add a chelating agent, such as EGTA or EDTA, to the isolating buffer solution for mitochondria to chelate calcium ions. We compared mitochondrial function in two preparations, one homogenized and centrifuged in the presence of 1 mM EGTA, and the other in the presence of 0.1 mM EDTA. When mitochondria were prepared in the presence of EGTA, the protective effect of Ki-energy was lost within 3 h after preparation (Fig. 6A). On the other hand, when they were prepared in the presence of EDTA, they maintained the ability to respond to the Ki-effect even 8 h after the preparation (Fig. 6B).

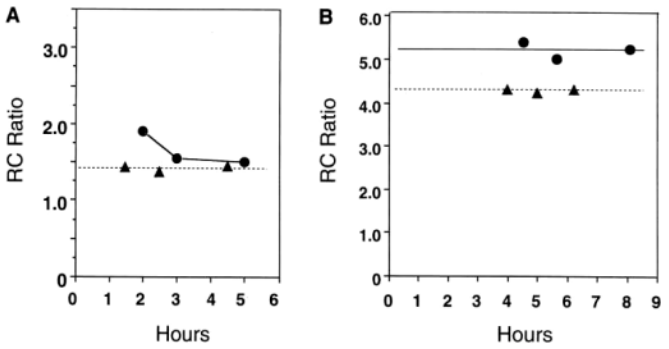

Figure 6. (**A**) Effects of EGTA (1 mM) and (**B**) EDTA (0.1 mM) added to the preparation medium on the Ki-protection effect. Closed circles represent data with heat/Ki treatment. Closed triangles are heat/no Ki treatment. Abscissa indicates the time after the preparation of mitochondria. Data in (**A**) were obtained using a separate mitochondrial preparation in which EGTA was used. Data in (**B**) were taken from Experiment 2 in Table 1.

Discussion

We report here that Ki-energy emitted by K.N. had significant effects on the function of isolated mitochondria. We have employed a heat deterioration treatment of isolated mitochondria (39°C for 10 min). This simple method was able to decrease the RC ratio by ~60% from the original ratio. While incubating mitochondria at 39°C, K.N. sent his Ki-energy to the mitochondrial suspension through his fingers for the entire period. As shown in this article, mitochondria were protected from the heat deterioration effects. In order to quantitatively analyze the Ki-effects, we measured the RC ratio, a well-known index for the integrity and intactness of isolated mitochondria. We found that mitochondria were damaged by the heat treatment as revealed by a decrease of the RC ratio, but that mitochondria of the same preparation were protected when they were exposed to Ki-energy during the heat treatment.

Table 2 indicates that lipid peroxidation was increased during the heat deterioration, suggesting that mitochondria were exposed to oxidative stress. Lipid peroxidation is known to damage the mitochondrial membrane. This may be the cause for the decrease of the RC ratio. The heat treatment of mitochondria may activate phospholipase A_2 and decompose some of the membrane phospholipids to produce free fatty acids. Free fatty acids are known to cause uncoupling between the electron transport and proton translocation (11). Free fatty acids are also prone to lipid peroxidation; therefore, they may further damage mitochondrial membranes. However, as we observed, lipid peroxidation was inhibited by Ki-energy, and the mitochondrial integrity was preserved.

Cellular and subcellular metabolisms are regulated by the network consisting of numerous cytokines, hormones, receptors, proteins, enzymes and chromophores (such as cytochromes). Various metal ions and ROS are also involved in these intricate metabolic networks. If Ki-energy could influence one of these components, then it would be possible that the effect may be multiplied by the cascade nature of reactions

to manifest as a measurable effect, for example, as an inhibition of lipid peroxidation.

We found two important clues for the observed Ki-effects on mitochondria. Namely, (i) mitochondria are constantly exposed to the danger of ROS-induced oxidative injury. The inhibition of lipid peroxidation suggests that Ki-energy may inhibit the generation of ROS in mitochondria. (ii) Our data suggest that Ca^{++} (calcium ions) may be involved in the Ki-triggered reactions. It has been known that EDTA cannot bind Ca^{++} in the presence of Mg^{++} (magnesium ions), because the binding constant between EDTA and Mg^{++} is higher than that for EDTA and Ca^{++}. We have to use EGTA to chelate Ca^{++} in the presence of Mg^{++} (12,13). Since mitochondria contains Mg^{++}, the addition of 0.1 mM EDTA in the preparation buffer may not be strong enough to remove Ca^{++}. On the contrary, the addition of 1 mM EGTA may be strong enough to remove protein-bound Ca^{++} from mitochondria. As shown in Fig. 6A, when we remove Ca^{++} from mitochondria, the protection from Ki-energy was lost within a few hours after the preparation of mitochondria. However, when mitochondria were prepared with 0.1 mM EDTA, the Ki-effect was maintained for at least 8 h (Fig. 6B).

Taken together, important players participating in the Ki-effect seem to be ROS and Ca^{++}. We hope to be able to dissect the protective mechanism of Ki-energy on mitochondria by analyzing the oxidative injury process of mitochondria with a special reference to the role of Ca^{++} and ROS.

From the standpoint of health and longevity, our results may have the following significance: (i) Ki-energy may protect mitochondria from oxidative injury. If the same reaction takes place in the practitioners' body, then mitochondria may produce more energy, and therefore, it has beneficial effects on cellular metabolism. (ii) Mitochondria are known to play key roles in apoptosis of many cell types. If cytochrome *c* and other apoptosis-inducing factors (AIF) are released from mitochondria, they activate a series of cascade reactions to cause apoptotic cell death (14–18). Although apoptosis is a fundamental feature of almost all animal cells and it is indispensable for the normal development of tissues, organs and immune systems (19), excessive apoptosis could cause diseases (20). Therefore, protecting mitochondrial integrity would help prevent cytochrome *c* release, thereby inhibiting inappropriate apoptosis from taking place.

Although there were several reports that Ki-energy may involve infrared radiation (21–26), the direct proof that infrared is a cause for the observed Ki-effects needs to be confirmed. We have provided evidence to suggest that the Ki-effect on cultured cancer cells involves infrared radiation (6). Using rat liver mitochondria, we now obtained new evidence that the observed Ki-effect on mitochondria involves, at least, infrared radiation between 0.8 and 2.7 μm. We used a thin borosilicate glass plate (0.17 mm thick), which has a high transmittance (>90%) for near-infrared (up to 5 μm), for our experimental chamber (Fig. 1A). In our previous article, we showed that Ki-energy did not increase the level of p53 (a tumor suppressor gene) (6), as opposed to high-energy X-ray radiation, which would increase the level (27,28). This suggests that the level of Ki-energy from the fingers of Ki-expert is relatively week. Machi (24) estimated that the energy of infrared radiation from a Chinese Qigong healer is on the order of 10 μW. A speculative mechanism on how such a week infrared radiation could induce measurable changes in biological systems is proposed (29).

In conclusion, Ki-energy maintains mitochondrial membrane integrity during the heat deterioration process. The effect of Ki seems to be related to the inhibition of oxidative injury on mitochondrial membranes caused by ROS. Therefore, Ki would have a beneficial effect on protecting mitochondria; thus, it would maintain efficient cellular metabolism and decrease the chance of unnecessary apoptosis. Our data suggest that calcium ions may play an important role in the protection mechanism by Ki-energy. From experiments with infrared and visible range filters, we demonstrated that observed Ki-effects involve, at least, near-infrared radiation with wavelength range between 0.8 and 2.7 μm.

Acknowledgments

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