The effect of a 50-Hz sinusoidal magnetic field on nitric oxide (NO) production by human umbilical vein endothelial cells (HUVECs)

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Abstract. To investigate the effect of magnetic fields on living systems, we analyzed the effect of a 50-Hz, 1-mT sinusoidal magnetic field on nitric oxide (NO) production in human umbilical vein endothelial cells (HUVECs). Statistically significant differences in NO production were identified between exposed and unexposed (sham) cells. This finding confirms previous studies showing that magnetic fields can influence NO production; however, unlike previous studies, our results demonstrate two-way deviation of NO production under the influence of a magnetic field. Thus, in some cases, NO production was higher for the exposed group than the sham group, while in other cases the reverse was true. We suggest that the magnetic field affected the homeostasis of NO levels in a complex manner. Thus, the resulting NO level was dependent on the condition of the cell in each experiment or cells in each experiment were at a different stage in the cell cycle and therefore exhibited a different response when exposed to the magnetic field.
1. Introduction

Low-frequency (50/60 Hz) magnetic fields have been shown to interact with biological systems in a number of ways; for example, they promote healing of fractured bones and skin wounds [1]. Another study showed that a magnetic field causes single- and double-strand breaks in the DNA of brain cells of mice [2]. A magnetic field also caused frog arteriole vasodilation, a process which is regulated by nitric oxide (NO) produced by the endothelial cells that form the walls of blood vessels [3].

NO is synthesized from the amino acid L-arginine by an enzyme called nitric oxide synthase (NOS). NO is a key molecule that plays various roles in biological situations such as the immune response of macrophages, signal transduction in neurons and the regulation of blood pressure [4]. In human umbilical vein endothelial cells (HUVECs), an isoform of NOS (endothelial NOS; eNOS) is constitutively expressed and synthesizes NO (figure 1). The NO produced by HUVECs inhibits the contractility of the cell, thereby regulating blood pressure.

Several previous studies imply an intimate but apparently complex connection between magnetic fields and NO production. For example, in mouse macrophage cell lines, application of a pulsed magnetic field (10–250 Hz, 4.4 mT max) for 20 min elevated the level of NO production [5]. Similarly, in HUVECs, exposure for 20 min to an oscillating magnetic field (20 Hz, 20 mT max; generated by rotating permanent magnet) promoted NO production [6]. In epidermal keratinocytes, application of a sinusoidal magnetic field (50 Hz, 1 mT r.m.s.) for 3 h increased NOS and NO production [7]. However, in human monocytes, overnight exposure to a sinusoidal magnetic field (50 Hz, 1 mT r.m.s.) decreased the level of NOS expression [8]. The reasons for these discrepant results remain unclear. The importance of NO in many facets of biological activities and the existence of many accessible methods for the measurement of NO prompted us to study the effect of a magnetic field on NO production by HUVECs. In the present study, we measured the level of NO produced by HUVECs using a fluorescence method and evaluated the effect of a 50-Hz sinusoidal magnetic field.

2. Materials and methods

2.1. Cells and reagents
HUVECs, culture medium and additives were purchased from Lonza (Cat. No. CC-2517A; Walkersville, MD, USA). Cells from the same lot were used throughout the study. For experiments, $5 \times 10^5$ cells were plated in a 6-cm-diameter plastic culture dish (Greiner, Tokyo, Japan) and were cultured in a CO$_2$ incubator (Model 5410, Precision Scientific, Chicago, IL, USA) at 37 °C (controlled to within ±0.1 °C) in 5% CO$_2$ for 3 or 4 days prior to the experiments.

Diaminofluorescein (DAF-2) that was used to measure the level of NO was purchased from Cayman Chemicals (Ann Arbor, MI, USA) or Sekisui Medical (Tokyo, Japan). L-arginine and other chemicals (analytical grade) were obtained from Wako (Osaka, Japan) and were used without further purification.

2.2. Apparatus and measurements

Four sets of Helmholtz coils (inner diameter, 115 mm; thickness, 40 mm; Model U21901, 3B Scientific GmbH, Hamburg, Germany) were used for each experiment (figure 2A and B). Two sets were placed side-by-side in incubator 1 and the other two sets were placed in incubator 2. As shown in figure 2C, sinusoidal current generated by a function generator (Model WF1943A, NF Co., Yokohama, Japan) was fed to each coil through an electric amplifier (Model PMA390AE, DENON, Kawasaki, Japan). A Tesla meter (Model 421, Lake Shore Cryotronics, Inc., Westerville, OH, USA) was used to measure the magnetic flux density at the center of the coil pair.

Thermometers consisting of a thermistor probe and a data logger (TR-77Ui, T&D Co., Matsumoto, Japan) were used to monitor and record transient temperature changes in each coil. The thermistor was placed near the bottom of the culture dish (figure 3A). Data of transient temperature changes such as those shown in figure 3B were used to calculate the temperature difference between the exposed and the sham coils (see Data analysis).

The mechanical vibration of the coil base was measured using a motion sensor (sensor 2304A connected to the recorder 1332B; Showa Sokki, Tokyo, Japan; figure 4). The sensor was attached with a double-sided adhesive tape to the front edge of the coil base (figure 4, cross symbol) and the motion of the support was recorded for 4.4 sec at 50 kHz.

NO production was quantified using the fluorescent NO probe DAF-2, whose fluorescence intensity increases about 100-fold when it reacts with NO [9, 10]. The fluorescence intensity of DAF-2 was measured using a spectrofluorophotometer (Model FP-750, JASCO, Tokyo, Japan). The excitation and emission wavelength were 495 and 515 nm, respectively, with a bandwidth of 5 nm in both channels.

Figure 2. Apparatus used for the experiments.
A. A photograph of two sets of Helmholtz coils (white arrows) placed side by side in a CO2 incubator. The gray arrows indicate supports for cell culture dishes. B. A schematic representation of the arrangement of the Helmholtz coils and cell culture dishes in two CO2 incubators. C. Two sets of Helmholtz coils are connected in series to an amplifier and a function generator.

Figure 3. Measurement of transient temperature changes.
A. Measurement of temperature in the Helmholtz coil: the probe was attached to the lower side of the dish support, as indicated by a cross in the left panel. The white arrow in the right panel indicates the cable connecting the thermistor sensor to the data logger.
B. An example of a temperature record. Solid and dotted lines represent the record obtained in exposed and sham coils, respectively. The door of the incubator was opened at time zero. The average temperature was calculated during the period of temperature restoration (see Data Analysis).

Figure 4. Measurement of mechanical vibration.
A motion sensor (black arrow) was attached to the front of the base of the Helmholtz coil (cross) to detect vibration arising from the active coil.

2.3. Exposure

On the day of each experiment, the cells were washed twice in Krebs-Ringer phosphate buffer (KRP buffer). Then, KRP buffer containing 1 mM L-arginine and 1 mM DAF-2 was added to each culture dish. For exposure to the magnetic field, two or three culture dishes were placed on a dish support in each coil (figure 2A and B) in incubator 1 and an electric current was applied to the coil (figure 1C); dishes containing cells for the unexposed (sham) group were placed in a Helmholtz coil in incubator 2 without applying current. Cells were then left in the incubators for 1 h, during which time the temperature in the coil was monitored using the thermistor probe. The temperature difference between the two incubators in the steady state was maintained within 0.2 °C. After application of the magnetic
field, the culture medium in each dish was collected in separate test tubes and fluorescence intensities were measured using a spectrofluorophotometer.

2.4. Data analysis

The fluorescence intensities of individual exposed and sham samples were ensemble-averaged and are respectively designated as $<I_{\text{exp}}>$ and $<I_{\text{sham}}>$. Likewise, the ensemble-averaged fluorescence intensities of individual sham 1 and sham 2 samples (incubated in the incubator 1 and 2, respectively) were designated as $<I_{\text{sham}1}>$ and $<I_{\text{sham}2}>$. Fluorescence intensity was statistically analyzed using Welch’s test. The $p$ values representing statistical significance were calculated for each set of ensemble-averaged values, and $p < 0.05$ was judged as statistically significant.

In order to calculate the difference in temperature between exposed and sham coils, $T_{\text{Exp}}$ and $T_{\text{Sham}}$, or that between two sham coils, $T_{\text{Sham}1}$ and $T_{\text{Sham}2}$, we calculated the time-average of the temperature values, $T^*_{\text{Exp}}$, $T^*_{\text{Sham}1}$, $T^*_{\text{Sham}2}$, and the differences, $T^*_{\text{Exp}} - T^*_{\text{Sham}}$ and $T^*_{\text{Sham}2} - T^*_{\text{Sham}1}$ were taken.

The data of the displacement record of the coil base was Fourier-transformed and a power spectrum was calculated using the program supplied by the manufacturer of the motion sensor.

Figure 5. Effect of a magnetic field on nitric oxide production in HUVECs.
A, Summary of $<I_{\text{exp}}>/ <I_{\text{sham}}>$ values obtained in five independent experiments. Fluorescence intensity of the cell culture medium was assayed. The error bar was plotted using standard deviation. “Dishes”: number of dishes assayed per experiment; $p$: statistical significance of the difference between $<I_{\text{exp}}>$ and $<I_{\text{sham}}>$ values for each experiment. B, The result of three independent, all-sham experiments; ratios of $<I_{\text{sham}2}>$, obtained from incubator 2, to those of $<I_{\text{sham}1}>$ from incubator 1 are shown. The symbols are as defined in A, except that in the row labeled “Dishes” the numerals to the left and the right of the thrash indicate the number of samples for Sham 1 and for Sham 2, respectively.
C. Ratio of fluorescence intensity in the absence of L-arginine ($<I_{\text{NoArg}}>$) to that in the presence of L-arginine ($<I_{\text{Arg}}>$), obtained from three independent experiments. In the row labeled “Dishes” the numerals to the left and the right of the thrash indicate the number of samples with and without L-arginine, respectively.

Figure 6. Distribution of individual $I_{\text{Exp}}$ and $I_{\text{Sham}}$ values. A, Distribution of fluorescence intensities of exposed and sham samples. Open circles, exposed; crosses, sham. B, Distribution of fluorescence intensities of samples measured in incubator 1 (closed triangles) and in incubator 2 (open triangles), both of which were measured without exposure to a magnetic field.

3. Results and Discussion

Cellular NO production was assessed using a fluorescent method from cells incubated with L-arginine and exposed, or not exposed, to a magnetic field. Figure 5A shows $<I_{\text{Exp}}>/<I_{\text{Sham}}>$ values with standard deviation, which were obtained from five independent experiments. Individual differences from unity were small, but they were statistically significant, as indicated by the $p$ values obtained in individual experiments (p values are in the bottom row in figure 5A). These differences were not due to the use of two separate incubators for exposure and sham conditions, because when all coils in the two incubators were operated without current (all sham conditions), the resultant $<I_{\text{Sham1}}>/<I_{\text{Sham2}}>$ values, i.e., the ratio of fluorescence intensities obtained from incubators 1 and 2, exhibited no significant difference from unity, as shown in figure 5B.

We also carried out an experiment to examine the effect of omission of L-arginine, the precursor of NO, under all-sham conditions. The fluorescence intensity observed in the presence of L-arginine $<I_{\text{Arg}}>$ was about 5% higher than that observed in the absence of L-arginine ($<I_{\text{NoArg}}>$; figure 5C). The level of fluorescence intensity in the absence of L-arginine should represent the basal level of DAF-2 fluorescence. Therefore, the 3-5% difference between $<I_{\text{Exp}}>$ and $<I_{\text{Sham}}>$ values obtained in the presence of L-arginine was rather large in comparison to the 5% difference between $<I_{\text{Arg}}>$ and $<I_{\text{NoArg}}>$ values.

The above difference between $<I_{\text{Exp}}>$ and $<I_{\text{Sham}}>$ values is a reflection of the characteristic distribution of individual $I_{\text{Exp}}$ and $I_{\text{Sham}}$ values. Namely, individual $I_{\text{Exp}}$ and $I_{\text{Sham}}$ values in each set of experiment exhibit few overlaps (figure 6A). On the other hand, the data points representing $I_{\text{Sham1}}$ and $I_{\text{Sham2}}$ exhibited considerable overlap (figure 6B).

We next evaluated the effect of a potential difference in temperature in the exposed and sham coils. Temperature in the coils quickly dropped when the door of the incubator was opened in order to place the culture dishes in the coil, and the temperature gradually approached the original level after the door was closed, as shown in figure 3B. It was possible that temporal changes in exposed and
sham coils might be different and could result in differences in NO production by the eNOS as in general, enzyme activity is sensitive to the ambient temperature. Therefore, as a measure of the temperature difference, we calculated the difference in time-averaged temperature values, \( T^*_{\text{Exp}} - T^*_{\text{Sham}} \), as described in Data Analysis. We plotted \( T^*_{\text{Exp}} - T^*_{\text{Sham}} \) against \( <I_{\text{Exp}}> - <I_{\text{Sham}}> \), or \( T^*_{\text{Sham1}} - T^*_{\text{Sham2}} \) against \( <I_{\text{Sham1}}>-<I_{\text{Sham2}}> \) (figure 7). It is apparent that the plots are scattered. Therefore, the differences in temperature values of incubator 1 and 2 were unlikely to be the reason for the difference in fluorescence intensities measured in the two incubators.

We then examined if the vibration of the active Helmholtz coil affected HUVEC function, because HUVECs are known to respond to shear stress of the blood stream by altering NO production [11]. The vibration of the coil would be transmitted to the culture dishes through the dish support placed on the base of the coil. We therefore measured the vibration of the coil base using the motion sensor. As shown in figure 8, the power spectrum of the active coil and that of the inert coil were superimposable; the large peak around 23 Hz probably arose from the rotation of a fan for circulation of air in the incubator, because this peak appeared under both active and inert conditions. We conclude that mechanical vibration did not cause the difference between \( <I_{\text{Exp}}> \) and \( <I_{\text{Sham}}> \) values.

On the basis of the results shown in figure 5, and upon examination of possible confounding factors, i.e., temperature difference and mechanical vibration, we conclude that the magnetic field did affect the level of NO production in HUVECs. Our result is consistent with previous studies that used HUVECs and other types of cells.

As shown in figure 6A, the plots of \( I_{\text{Exp}} \) and those of \( I_{\text{Sham}} \) were almost completely separated. It could be argued that this separation is a chance phenomenon. However, we suggest that this is unlikely based on the following consideration: the chance that 4 plots from exposed samples and 4 plots from sham samples will be separated is 0.023 (= 2 \times 4! \times 4! / 8!). Hence, the probability of the occurrence of such a phenomenon in several independent experiments is very small. We consider that the separation did not occur by chance and that it was a result of interaction between the magnetic field and HUVECs.

Our result shows that \( <I_{\text{sham}}> \) was larger than \( <I_{\text{exp}}> \) in some experiments, whereas it was smaller in other experiments (figure 5A). In previous studies, application of a magnetic field was found to increase the level of endothelial NOS in human keratinocytes [7], whereas in another study it reduced expression of NOS in human monocytes [8]. These disparate results are often attributed to differences in the experimental conditions (e.g., cell type or the pattern of the applied magnetic field). However, in our case the disparate results were obtained under the same experimental conditions. We consider that the cellular response to the magnetic field may be dependent on the stage of the cell cycle (e.g., cell growth and division, DNA replication), as was suggested in previous studies [12]. The level of NO is determined by the balance between production and conversion into other chemical species. In general, this homeostasis involves negative feedback, which is essential in biological systems [13]. Negative feedback results from signal transduction pathways that usually form interconnected networks and "cross talk" with each other [14]. Complex signal transduction pathways can show quite divergent responses, including directionally opposite responses, even when an identical stimulus is applied [15]. In our case, the magnetic field may alter NO production rate depending on the stage of the cell cycle. However, the magnetic field may also alter the conversion rate. This hypothesis would explain our results because cells are likely to be distributed in multiple stages in the cell cycle and this distribution is probably not identical in individual experiments. Alternatively, the magnetic field might have affected the feedback mechanism, but this effect may be different at different stages and, in some stages, the feedback may "over-compensate" for the increased NO production. If so, the different distribution of stages again determine the final NO level. At present, we cannot evaluate these possibilities. Our future experiments aim to evaluate these hypotheses.
Figure 7. Analysis of temperature differences in exposed and sham coils. Closed circles: plots of difference in the ensemble-averaged fluorescence intensity of exposed and sham ($<I_{\text{Exp}}>-<I_{\text{Sham}}>$) against the time-averaged difference in temperature of exposed and sham ($T^*_{\text{Sham}} - T^*_{\text{Exp}}$). Open circles: similar plots, but the differences in fluorescence intensities ($<I_{\text{Sham1}}> - <I_{\text{Sham2}}>$) and the temperature differences, $T^*_{\text{Sham2}} - T^*_{\text{Sham1}}$, were calculated between sham samples in incubators 1 and 2.

Figure 8. Power spectra of mechanical vibration. Power spectra were calculated based on the data collected using the motion sensor; the output was in volts. Dotted line, measured spectrum of the inert coil; solid line, spectrum of the active coil.

Acknowledgements:

We thank Professor Shun Murabayashi from Hokkaido University, Dr. Mikio Miyata from Kitasato Institute Hospital and Mitsugu Sakabe from Tokai University for their helpful comments and discussions.

M. I., T. H. and M. M. would like to acknowledge the Yukawa Institute for Theoretical Physics for valuable discussions at the three successive international symposiums entitled “What is Life?” (NY2007) in 2007, “What is Creativity?” (YITP-W-08-11) in 2008, and “What is Evolution?” (YITP-W-09-14) in 2009.

All the authors further thank the Yukawa Institute for Theoretical Physics at Kyoto University. Discussions during the YITP workshop YITP-W-10-12 on "International and
Interdisciplinary Workshop on Novel Phenomena in Integrated Complex Sciences: from Non-living to Living Systems" were useful to complete this work.

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan to T. H. (No. 21200025) and H. M. (No. 20200024).

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