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ELEVATION OF EXTRACELLULAR MAGNESIUM RAPIDLY RAISES INTRACELLULAR FREE MG^{2+} IN HUMAN AORTIC ENDOTHELIAL CELLS: IS EXTRACELLULAR MG^{2+} A REGULATORY CATION?

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1. ABSTRACT.

Extracellular magnesium ions $[Mg^{2+}]o$ are known to regulate functions of endothelial cells, but whether $[Mg^{2+}]_o$ can alter intracellular free ionized magnesium $[Mg^{2+}]_i$ in these cells remains unknown. The present studies, using digital imaging microscopy and the Mg^{2+} fluorescent probe, mag-fura-2, determined effects of elevation of $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$ in cultured human aortic endothelial cells. With normal Mg^{2+}(1.2 mM)-containing incubation media, $[Mg^{2+}]_i$ was 0.51±0.04 mM with a heterogeneous distribution. The ratio of $[Mg^{2+}]_i/[Mg^{2+}]_o$ was 0.52±0.07. Elevation of $[Mg^{2+}]_o$ up to 4.8 mM increased $[Mg^{2+}]_i$ to 0.80±0.07 mM in 2-10 min and lowered the ratio of $[Mg^{2+}]_i/[Mg^{2+}]_o$ to 0.16±0.02. Irrespective of the observed increments of $[Mg^{2+}]_i$, a subcellular heterogeneous distribution of $[Mg^{2+}]_i$ was always evident in all cells tested. Our results suggest that $[Mg^{2+}]_o$ can regulate $[Mg^{2+}]_i$ more rapidly than heretofore believed, supporting the hypothesis that extracellular Mg^{2+} can exert regulatory effects on endothelial cell functions and probably act as extracellular regulatory cations.

2. INTRODUCTION

Although calcium ions (Ca^{2+}) act as important intracellular "second messengers" in regulation of endothelial cell functions (1), the possible role of ionized magnesium $[Mg^{2+}]$ as a physiological regulator in these cells has not received experimental attention until very recently. Evidence has accumulated to suggest that extracellular Mg^{2+} concentrations ($[Mg^{2+}]_o$) can also play critical roles in various activities of endothelial cells. Changes of $[Mg^{2+}]_o$ have been found to influence synthesis/release of endothelial-derived relaxation factors (EDRFs) (2,3), intracellar Ca^{2+} concentrations ($(Ca^{2+})_i$) and intracellular Ca^{2+} release (4), activities of cation channels (5,6), Ca^{2+}-Na^{+} exchange (7), membrane potential (8), and uptake and metabolism of low density lipoprotein (9) and permeability to water and albumin (10), as well as proliferation and migration of endothelial cells (11). In addition, experimental deficiency in $[Mg^{2+}]_o$ has been reported to enhance free radical-induced cytotoxicity in endothelial cells (12).

Despite these important known cellular attributes and functions, underlying mechanism(s) of the actions of $[Mg^{2+}]_o$ in endothelial cells are not fully understood. One possibilty is that regulatory effects of $[Mg^{2+}]_o$ may be mediated via rapid changes in intracellular Mg^{2+} concentration $[Mg^{2+}]_i$. Recently, evidence has been provided that $[Mg^{2+}]_o$ may be regulatory cations in several types of eukaryotic cells, including vascular cells (13-16). $[Mg^{2+}]_i$ is known to be a cofactor for more than 325 enzymes and signal-transduction proteins, and it regulates bioenergetics and ion transport (for reviews, see 17-19). It would, thus, be of importance to know whether or not changes in $[Mg^{2+}]_o$ can alter rapidly $[Mg^{2+}]_i$ in endothelial cells. The present communication reports for the first time the basal level of $[Mg^{2+}]_i$ and effects of $[Mg^{2+}]_o$ on regulation of $[Mg^{2+}]_i$ in human vascular endothelial cells.

3. MATERIALS AND METHODS

Experiments were carried out using a human aortic endothelial cell line (No. AG09799A, 20 passages) obtained from the NIA Aging Cell

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Repository (Camden, NJ). Measurements of $[Mg^{2+}]_i$ in single cells were done according to previously established methods (16). Briefly, endothelial cells were cultured in 199 media (Sigma Chem. Co., St Louis, MO) with 15% fetal serum albumin (FSA) at 37°C in an humidified atmosphere composed of 95% air-5% CO_2. The cells were loaded with mag-fura-2 (Molecular Probes, Eugene, OR) by incubating them with 5 micromolar mag-fura-2/AM in the culture media for 60 min under 95% air-5% CO_2. Then, the coverslips were placed in a chamber on a thermostat-regulated stage of a Nikon fluorescence microscope and superfused with 1.2 mM Mg^{2+} HEPES buffer solutions (pH 7.4, 37°C) followed by 4.8 mM Mg^{2+} solutions (pH 7.4, 37°C). The ionic activities of Mg^{2+} in HEPES buffer solutions were monitored by unique Mg^{2+} ion selective electrodes (NOVA Biomedical Corp, Waltham, MA) and adjusted with $MgSO_4$ (21). The HEPES buffer solutions also contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.2, KH_2PO_4 1.2, HEPES 5, and glucose 10. Measurement of $[Mg^{2+}]_i$ was performed using a TN8500 FluorPlex III Image Analyzer (Tracor Northern, Madison , WN). Images of mag-fura-2 fluorescence at 510 nm emission were obtained with 335 and 370 nm excitation wavelengths using a silicon intensified target (SIT) camera. The time interval of switching between these two wavelengths was 2 seconds. Background fluorescence for both excitation wavelengths were acquired from blanks for each experiment and subtracted from each pair of images separately before ratioing. Fluorescence intensity ratios ($R_{335/370}$) were obtained by dividing the fluorescence intensity at 335 nm by the intensity at 370 nm. No image misalignments occurred when these two ratiometric images were superimposed.

To obtain absolute values of $[Mg^{2+}]_i$ in single cells, a final concentration of 5 micromolar mag-fura-2 pentapotassium salt containing either 10.0 mM (max) or 0 mM $MgSO_4$ (min) was used for an in-vitro calibration. The calibration solutions also contained: KCl 115 mM, NaCl 20 mM, and HEPES 5 mM, buffered with NaOH to pH 7.1 under air, at 37°C. From these Mg-standard solutions, the maximum and minimum intensities of fluorescence were obtained at the 335 nm and 370 nm wavelengths and a ratio of ($R_{335/370}$) was generated. $[Mg^{2+}]_i$ was calculated according to the following equation (22):

$$[Mg^{2+}]_i = K_d \times B \times (R-R_{min})/(R_{max}-R)$$

and a Kd of 1.5 mM (22) was used for the mag-fura-2/ Mg^{2+} complex. B is the ratio of fluorescence intensity of free mag-fura-2 to Mg-bound mag-fura-2 at 370 nm.

Where appropriate, mean values ± S.E.M. were calculated and compared for statistical significance using paired t tests. A p-value less than 0.05 was considered significant.

Table 1. Effects of Alteration of $[Mg^{2+}]_o$ on $[Mg^{2+}]_i$ in Single Human Aortic Endothelial Cells[a].

$[Mg^{2+}]_o$ (mM)	$[Mg^{2+}]_i$[b] (mM)	$[Mg^{2+}]_i/[Mg^{2+}]_o$
1.2	0.51±0.04	0.43±0.01
4.8	0.80±0.07*	0.16±0.01*

[a] Total of 10 different cells in each of 4 different cultures were used in these studies;
[b] Means ± S.E.M.;
* The significance of difference between the two groups was determined by Student's t-test, $P < 0.001$.

4. RESULTS

With 1.2 mM extracellular Mg^{2+} activity, the basal level of $[Mg^{2+}]_i$ in human aortic endothelial cells, estimated from the ratio (F340/ F380), is 0.51±0.04 mM, ranging between 0.45 mM and 0.57 mM (Table 1). The distribution of $[Mg^{2+}]_i$ was heterogeneous, i.e., there were "hot" areas of $[Mg^{2+}]_i$, referred to as intensive brightness spots which were scattered among the cells, and around these were weak brightness spots, areas with very low $[Mg^{2+}]_i$. Increasing $[Mg^{2+}]_o$ to 4.8 mM Mg^{2+} caused about a 60% significant increase of $[Mg^{2+}]_i$ in 2-10 min, from a basal value of 0.51 mM to a mean value of 0.80±0.07 mM (ranging between 0.67 mM to 0.88 mM), while the $[Mg^{2+}]_i/[Mg^{2+}]_o$ ratio decreased from 0.43±0.01 to 0.16±0.01 (Table 1). The rises of $[Mg^{2+}]_i$ were maintained for at least 30 min. Concomitant with the $[Mg^{2+}]_i$ elevation, the number and intensity of brightness spots were increased within each endothelial cell.

5. DISCUSSION

Based on digitized video-image analysis techniques, utilizing a fluorescent probe (mag-fura-2) and a superfusion system, we were able to: 1. measure both the mean values of $[Mg^{2+}]_i$ and their spatial distributions within single endothelial cells, and 2. continually define $[Mg^{2+}]_i$ responses to alterations of $[Mg^{2+}]_o$ in the same cells. The present study shows that the mean basal level of $[Mg^{2+}]_i$ of human aortic endothelial cells was in the sub-millimolar range. This value is in keeping with the levels of $[Mg^{2+}]_i$ observed in other cells and tissues studied by the same indicator (16,19,20,22-26). Overall, most recent studies, including those using ^{31}P-nuclear magnetic resonance (NMR) spectroscopy

on heart and brain, suggest that $[Mg^{2+}]_i$ in most tissue types is on the order of submillimolar (14,15,19,27-30).

It is evident from our new findings that $[Mg^{2+}]_i$ in human aortic endothelial cells is rapidly responsive to $[Mg^{2+}]_o$, unlike that observed for several other cell types (17,18). Increasing $[Mg^{2+}]_o$ to 4.8 mM Mg^{2+} caused an increase of $[Mg^{2+}]_i$ within 2-10 min, from a basal value of 0.51 mM to a mean value of 0.80mM, i.e., about a 1.6-fold increase (Table 1). Recently, possible interference of $(Ca^{2+})_i$ with measurements of $[Mg^{2+}]_i$, using mag-fura-2, has been suggested (31). However, an overestimation of $[Mg^{2+}]_i$ caused by interference with excess $(Ca^{2+})_i$ seems unlikely in the present stusdies, because: 1. the basal value of $(Ca^{2+})_i$ in these human aortic endothelial cells was found to be 74±22 nM and is not affected by elevation of $[Mg^{2+}]_o$ (4); 2) the dissociation constant of the mag-fura-2•Ca^{2+} complex is about 65 µM; and 3) the fluorescence intensity of the latter is not altered by changes in Ca^{2+} until it exceeds 5 µM (22).

Little is known about the cellular regulation of $[Mg^{2+}]_i$ homeostasis in endothelial cells. Although we do not show here the kinetic changes of $[Mg^{2+}]_i$ in response to elevation of $[Mg^{2+}]_o$, our data clearly indicate that rises in $[Mg^{2+}]_i$ in human aortic endothelial cells occur within a short time (2-10 min), much faster than previously recognized (17,18,32). Somewhat similar findings have been noted recently in several other types of cells and tissues (16,20,28-30,33-35). In contrast to a slow process of transport for many other cell types (31), the plasma membranes of vascular endothelial cells may be highly permeable to $[Mg^{2+}]_o$. The rise in $[Mg^{2+}]_i$ could be a direct result of increasing Mg^{2+} influx via changes of the transmembrane Mg^{2+} gradient. However, at both 1.2 and 4.8 mM $[Mg^{2+}]_o$, the levels of $[Mg^{2+}]_i$ were regulated at levels well below the equilibrium potential given by the Nernst equation. This suggests that Mg^{2+} may, at least partially, be actively-transported out. Several carrier-mediated magnesium extrusion systems have been proposed, such as Mg^{2+}/Ca^{2+} exchange (36), or Na^{+}/Mg^{2+} exchange (37). In addition, intracellular Mg^{2+} buffering may also be involved. For instance, alteration of $[Mg^{2+}]_o$ could influence proton (H^{+}) and Ca^{2+} transport as well as the status of cellular bioenergetic metabolism (28,30,35,38). All these intracellular events could modify Mg^{2+} binding at intracellular sites. Undoubtedly, these direct or indirect mechanisms, which remain to be unmasked, may play a role in the regulation of Mg^{2+} homeostasis.

Similar to that observed for subcellular $(Ca^{2+})_i$ distributions, $[Mg^{2+}]_i$ also appears to be segregated in various eukaryotic cell types, including liver, skeletal and vascular muscle (15,16,28,39-41). However, to our knowledge, the present studies are the first to show a heterogeneous distribution of the concentration of $[Mg^{2+}]_i$ in endothelial cells; the latter being seen, irrespective of $[Mg^{2+}]_o$. The brightness spots, observed herein, most likely represent Mg^{2+} release from extensive Mg^{2+} binding and uptake elements of internal storage sites which limit Mg^{2+} diffusion. However, the role of such heterogeneous distribution of $[Mg^{2+}]_i$ in the regulation of cellular functions is not clear, and further studies will be needed to define, precisely, the intracellular compartments for $[Mg^{2+}]_i$ in human endothelial cells, particularly with respect to Mg^{2+} permeability and transport characteristics.

Recently, agonist-induced changes of $[Mg^{2+}]_i$ have been reported in the intact brain (29,42), vascular smooth muscle cells (43-45), fibroblasts (46), renal epithelial cells (26), and pancreatic acinar and beta-cells (25,34). The elevations in $[Mg^{2+}]_i$ by increases of $[Mg^{2+}]_o$ in endothelial cells, observed herein, could be of significant physiological relevance, because $[Mg^{2+}]_i$ at this submillimolar range is known to fit the Michaelis-Menton constant (K_m) values for Mg^{2+} activation or inhibition of many enzymes (32), regulatory functions of G-proteins (17,18,47) and cation channel activity (5,6).

We propose that modulation of endothelial cell functions by $[Mg^{2+}]_o$ may be mediated by numerous Mg^{2+}-sensitive regulatory systems through changes in plasma ionized Mg^{2+}. Through the use of a new ion selective electrode for $[Mg^{2+}]_o$, it has been shown that despite no alteration in total plasma Mg, the ionized $[Mg^{2+}]_o$ significantly varies in a number of pathophysiological states in humans, including ischemic heart disease, angina, hypertension, diabetes mellitus, migraine headache, renal disorders, and stroke (14,15,19,48).

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