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Molecules and Cells

Basic Fibroblast Growth Factor Increases Intracellular Magnesium Concentration through the Specific Signaling Pathways

Bing-Zhe Hong[1,4], Sun-Ah Park[1], Han-Na Kim[1], Tian-Ze Ma[1], Han-Gyu Kim[1], Hyung-Sub Kang[2], Hwan-Gyu Kim[3], and Yong-Geun Kwak[1,*]

Basic fibroblast growth factor (bFGF) plays an important role in angiogenesis. However, the underlying mechanisms are not clear. Mg^{2+} is the most abundant intracellular divalent cation in the body and plays critical roles in many cell functions. We investigated the effect of bFGF on the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) in human umbilical vein endothelial cells (HUVECs). bFGF increased $[Mg^{2+}]_i$ in a dose-dependent manner, independent of extracellular Mg^{2+}. This bFGF-induced $[Mg^{2+}]_i$ increase was blocked by tyrosine kinase inhibitors (tyrphostin A-23 and genistein), phosphatidylinositol 3-kinase (PI3K) inhibitors (wortmannin and LY294002) and a phospholipase Cγ (PLCγ) inhibitor (U73122). In contrast, mitogen-activated protein kinase inhibitors (SB202190 and PD98059) did not affect the bFGF-induced $[Mg^{2+}]_i$ increase. These results suggest that bFGF increases the $[Mg^{2+}]_i$ from the intracellular Mg^{2+} stores through the tyrosine kinase/PI3K/PLCγ-dependent signaling pathways.

INTRODUCTION

The family of fibroblast growth factors (FGFs) currently consists of 22 structurally related polypeptides, including the two prototypes, FGF-1 (acidic FGF) and -2 (basic FGF, bFGF) (Ornitz and Itoh, 2001; Powers et al., 2000). Basic fibroblast growth factor (bFGF) is a potent mitogen that regulates angiogenesis during tumor angiogenesis, growth and development (Folkman and Shing, 1992; Giavazzi et al., 2001). bFGF also has the potential to improve collateral angiogenesis in human myocardial and lower leg ischemia (Laham et al., 2000; Lazarous et al., 2000).

However, the underlying mechanisms are not clear. The binding of bFGF to its receptors induces dimerization and subsequent phosphorylation of the receptors through the activation of tyrosine kinase receptors (Klint and Claesson-Welsh, 1999; Schlessinger, 2000), which leads to the activation of several intracellular signaling molecules such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and phospholipase Cγ (PLCγ) (Klint and Claesson-Welsh, 1999; Webber et al., 2005).

Magnesium is the most abundant intracellular divalent cation, and plays essential roles in a wide range of fundamental cellular reactions (Wolf and Cittadini, 2003). Since magnesium functions as an allosteric modulator of several enzymes or bridges structurally distinct molecules, magnesium coordinates DNA duplication with an adequate metabolic response. Considering the various functions of magnesium, it is not surprising that many pathological symptoms and diseases are attributed to alterations of Mg^{2+} homeostasis. A low magnesium status has been found to be important in the pathogenesis of cardiovascular diseases such as hypertension and thrombosis (Liao et al., 1998; Mussoni et al., 2001; Peacock et al., 1999). All these clinical features are known to be associated with endothelial dysfunction as a common pathogenic event (Cines et al., 1998; Maier et al., 2004b). Additionally, Lapidos et al. (2001) reported that Mg^{2+} promoted the spreading and migration of endothelial cells. However, the regulation of intracellular Mg^{2+} concentrations ($[Mg^{2+}]_i$) has not been well characterized compared to intracellular Ca^{2+} concentrations. Furthermore, there is no report on the effect of bFGF on $[Mg^{2+}]_i$.

In the present study, we investigated the effects of bFGF on the $[Mg^{2+}]_i$ in human umbilical vein endothelial cells (HUVECs) and clarified its underlying mechanisms. We found that bFGF increased $[Mg^{2+}]_i$ from the intracellular stores through the activation of the tyrosine kinase/PI3K/PLCγ-dependent signaling pathways in HUVECs.

MATERIALS AND METHODS

Reagents

Mag-fura-2, acetoxymethyl (AM) was purchased from Molecular Probes (USA). Medium M-199, SU1498, genistein, and Matrigel matrix were purchased from Sigma Chemical (USA).

[1]Department of Pharmacology, Chonbuk National University Medical School, Jeonju 560-182, Korea, [2]Department of Pharmacology, Chonbuk National University College of Veterinary Medicine, Jeonju 561-180, Korea, [3]Division of Biological Sciences, Chonbuk National University, Jeonju 561-756, Korea, [4]Present address: Department of Cardiology, Xinhua Hospital, Dalian University Medical School, Dalian 116021, China
*Correspondence: ygkwak@chonbuk.ac.kr

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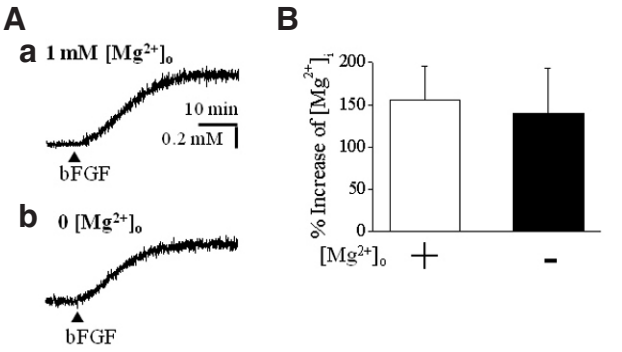

Fig. 1. The effects of bFGF (10 ng/ml) on $[Mg^{2+}]_i$ in the absence (Ab) or presence (Aa) of extracellular Mg^{2+} (1 mM $MgCl_2$) in HUVECs loaded with mag-fura-2 AM. (A) Representative tracings. The arrows indicate the starting point of the perfusion with bFGF. (B) Average values of the percentage increases of $[Mg^{2+}]_i$ in each group. The percentage increases of $[Mg^{2+}]_i$ were normalized to the basal level immediately before the perfusion with bFGF. Each column with a vertical bar denotes the means ± SEM.

bFGF, tyrphostin A-23, wortmannin, LY294002, U73122, U73343, SB202190, and PD98059 were from Merck Biosciences (Germany). Collagenase type II was obtained from Worthington (USA). The remaining reagents were purchased from Sigma Chemical (USA).

Cell culture

HUVECs were prepared from human umbilical cords by collagenase digestion, as described previously (Hong et al., 2006). HUVECs were maintained in M-199 medium supplemented with 20% (v/v) fetal bovine serum (FBS). The primary cultured cells used in this study were between passages 2 and 4. The ethics committee of Chonbuk National University Medical School approved this study, the subjects were fully informed, and we obtained a written consent from them. This study conformed with the principles outlined in the Declaration of Helsinki.

Fluorimetric determination of the $[Mg^{2+}]_i$

HUVECs were grown on collagen-coated glass coverslips and incubated with 5 μM mag-fura-2/AM for 60 min at 37°C in a 5% CO_2 incubator. After washing the cells with a extracellular solution, the coverslip was transferred into the measuring chamber on the stage of an inverted fluorescence microscope (Nikon, Japan), and the cells were continuously perfused with the extracellular solution at 37°C. The fluorescence ratios were obtained by exciting the dye at wavelengths of 340 and 380 nm and collecting at wavelength of 475 nm emission, and these values were converted to $[Mg^{2+}]_i$ by *in situ* calibration (Raju et al., 1989). The extracellular solution contained 140 mM NaCl, 5 mM KCl, 1 or 0 mM $MgCl_2$, 11 mM glucose and 10 mM HEPES (pH 7.35 with NaOH).

Statistical analysis

All results are presented as means ± SEM. Comparisons were made between the different treatments using the Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

The effects of bFGF on $[Mg^{2+}]_i$ in HUVECs

We first tested whether bFGF alters $[Mg^{2+}]_i$ in the HUVECs. Figure 1A shows the effect of bFGF on $[Mg^{2+}]_i$ in the HUVECs

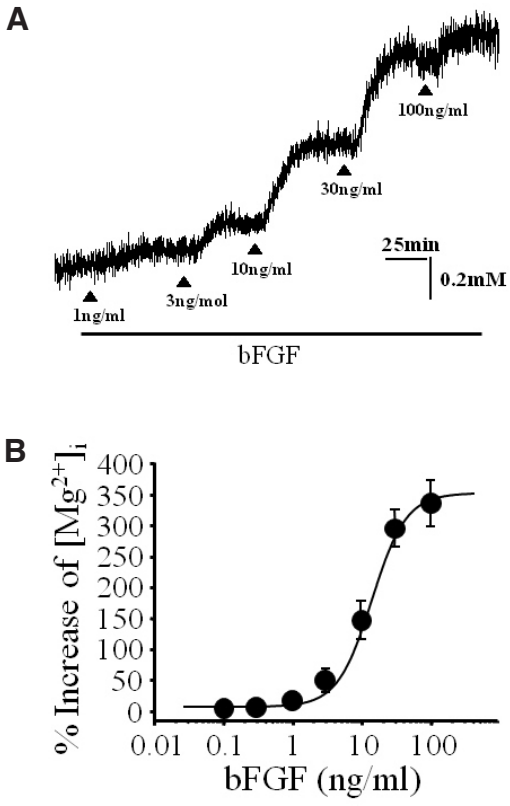

Fig. 2. The dose-dependency of the bFGF-induced $[Mg^{2+}]_i$ increase in the absence of extracellular Mg^{2+} (1 mM). (A) Representative tracings. The arrows indicate the starting points of the perfusion with various doses of bFGF. (B) Dose-response curve fitted with a Hill equation. The data are expressed as means ± SEM.

in the presence or absence of extracellular Mg^{2+} (1 mM $MgCl_2$). The mean basal level of $[Mg^{2+}]_i$ in the HUVECs was 0.51 ± 0.04 mM in the presence of extracellular Mg^{2+} (n = 48). The level of $[Mg^{2+}]_i$ started to increase in HUVECs at approximately 5 min after bFGF (10 ng/ml) treatment, and reached a maximum level approximately 25 min after bFGF treatment. The bFGF-induced increase of $[Mg^{2+}]_i$ was 155.2 ± 40.2% (n = 6) or 140.3 ± 52.2% (n = 6) of the basal $[Mg^{2+}]_i$ level in the presence or absence of extracellular Mg^{2+}, respectively. These results indicate that the bFGF-induced increase of $[Mg^{2+}]_i$ was mainly due to releasing Mg^{2+} from the intracellular stores. Therefore, the subsequent experiments in the study were performed with bFGF in the absence of extracellular Mg^{2+}. bFGF also increased $[Mg^{2+}]_i$ in a dose-depen-dent manner with an EC_{50} of 12.01 ± 1.03 ng/ml and the Hill coefficient of 1.63 ± 0.21 (n = 6) (Fig. 2).

To rule out the Ca^{2+}-induced fluorescence in our experimental system, we examined the effect of bFGF on the $[Mg^{2+}]_i$ level in the presence or absence of a Ca^{2+} chelator BAPTA-AM (Fig. 3A). In the presence or absence of a Ca^{2+} chelator BAPTA-AM, the bFGF-induced increase of $[Mg^{2+}]_i$ was 147.3 ± 32.3% (n = 6) and 142.5 ± 24.1% (n = 6) of the basal $[Mg^{2+}]_i$ level, respectively. This result indicates that a fluorescence change in HUVECs loaded with mag-fura 2-AM by bFGF reflects the change in $[Mg^{2+}]_i$ only. It is well known that Mg^{2+} movement across the cell membrane is dependent on the concentration of Na^+ (Schweigel et al., 2006; Tashiro et al., 2005; Zhang and Melvin, 1996). Therefore, we tested whether the bFGF-induced $[Mg^{2+}]_i$ increase was Na^+-dependent (Fig. 3B). bFGF increased $[Mg^{2+}]_i$

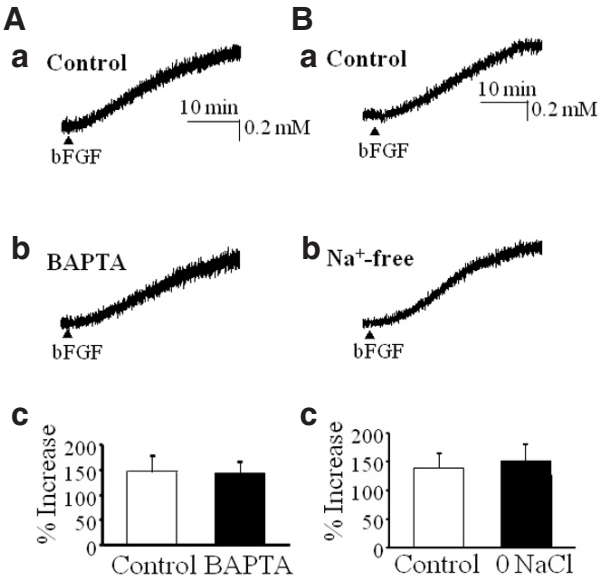

Fig. 3. The effects of extracellular BAPTA-AM (100 μM, (A)) and the removal of extracellular Na^+ (B) on the bFGF-induced $[Mg^{2+}]_i$ increase in the absence of extracellular Mg^{2+}. To remove the extracellular Na^+, the extracellular Na^+ (NaCl 140 mM) was replaced with choline chloride (140 mM). (a, b) Representative tracings. The arrows indicate the starting point of the perfusion with BFGF. (c) Average values of the percentage increases of $[Mg^{2+}]_i$ in each group. Each column with a vertical bar denotes the means ± SEM.

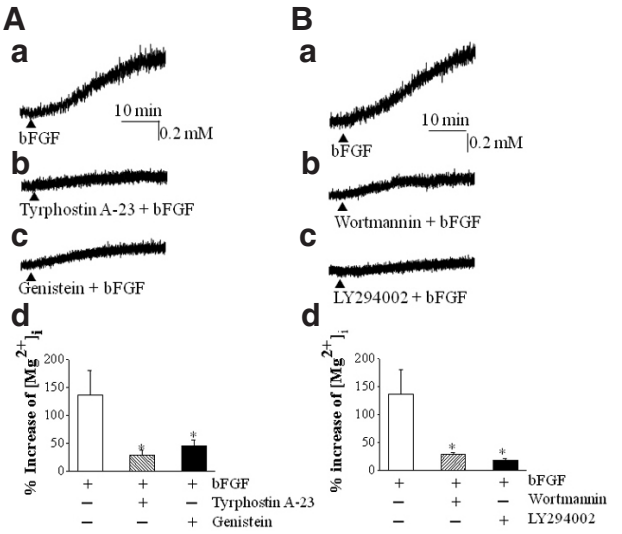

Fig. 4. The effects of tyrosine kinase inhibitors (A) and PI3K inhibitors (B) on the bFGF (10 ng/ml)-induced increase of $[Mg^{2+}]_i$ in the Mg^{2+}-free extracellular solution. (a, b, and c) Representative tracings. The mag-fura-2-loaded cells were incubated with tyrphostin A-23 (10 μM), genistein (30 μM), wortmannin (30 nM) or LY294002 (25 μM) for 20 min before the perfusion with bFGF. The arrows indicate the starting points of the perfusion with bFGF. (d) Average values of the percentage increases of $[Mg^{2+}]_i$ in each group. Each column with a vertical bar denotes the means ± SEM. *, Significantly different from the control (bFGF alone; $P < 0.05$).

by 139.3 ± 23.6% (n = 6) and 149.9 ± 29.5% (n = 6) of the basal $[Mg^{2+}]_i$ level in the presence or absence of the extracellular Na^+, respectively. These results indicate that the bFGF-induced increase of $[Mg^{2+}]_i$ was independent of the extracellular Na^+.

bFGF increases $[Mg^{2+}]_i$ through tyrosine kinase/PI3K/PLCγ-dependent pathways

The main intracellular signaling pathways of bFGF include the tyrosine receptor kinase, the mitogen-activated protein kinase (MAPK), the PI3K-dependent Akt pathway, and PLCγ (Klint and Claesson-Welsh, 1999; Schlessinger, 2000; Webber et al., 2005). To clarify the molecular mechanisms of the bFGF signaling pathway involving the regulation of $[Mg^{2+}]_i$, various inhibitors of these proteins were used. The bFGF receptor is known to be a kind of receptor tyrosine kinases (Schlessinger, 2000) and $[Mg^{2+}]_i$ could be regulated by tyrosine kinase-dependent mechanism (Ishijima and Tatibana, 1994). Therefore, we first examined whether tyrosine kinase was involved in the bFGF-induced $[Mg^{2+}]_i$ increase (Fig. 4A). Pretreatment with tyrosine kinase inhibitors, tyrphostin A-23 and genistein, resulted in a marked inhibition of the bFGF-induced increase of $[Mg^{2+}]_i$ from 136.0 ± 42.9% (n = 6) to 28.6 ± 6.3% (n = 6) and 44.6 ± 10.3% (n = 6) compared to the basal level, respectively.

Next, PI3K inhibitors wortmannin and LY294002 also inhibited the bFGF-induced increase of $[Mg^{2+}]_i$ from 136.2 ± 44.6% (n = 6) to 28.6 ± 4.6% (n = 6) and 17.1 ± 4.6% (n = 6) of the basal level, respectively (Fig. 4B). It has been recently reported that the intracellular signaling pathways of bFGF include the activation of PLCγ (Klint and Claesson-Welsh, 1999; Webber et al., 2005). We thus examined whether PLCγ was involved in the bFGF-induced $[Mg^{2+}]_i$ increase (Fig. 5A). A PLC inhibitor, U73122, blocked the bFGF-induced increase of $[Mg^{2+}]_i$ from 144.6 ± 46.9% (n = 6) to 40.0 ± 18.3% (n = 6) of the basal level. However, an inactive analogue of U73122, U73343, did not affect the bFGF-induced increase of $[Mg^{2+}]_i$ (128.6 ± 17.1%, n = 6). MAPK inhibitors, SB202190 and PD98059, did not significantly affect the bFGF-induced increase of $[Mg^{2+}]_i$ (from 155.4 ± 40.0% to 125.7 ± 40.2% and 120.1 ± 14.3% of the basal level, respectively; Fig. 5B). Interestingly, there was neither additional effect nor synergistic effect between PI3K and PLC inhibitors in inhibiting the bFGF-induced increase of $[Mg^{2+}]_i$ (Fig. 6). These results suggest that bFGF regulates $[Mg^{2+}]_i$ through the signaling pathway involving receptor tyrosine kinase, PI3K, PLCγ, but not MAPK.

DISCUSSION

The present study examined the effects of bFGF on the $[Mg^{2+}]_i$ in HUVECs and clarified the underlying mechanism behind it. Our results indicate that bFGF induced an increase of $[Mg^{2+}]_i$ from the intracellular Mg^{2+} stores through tyrosine kinase/PI3K/PLCγ signaling pathways.

The mechanism by which the $[Mg^{2+}]_i$ levels are regulated is still poorly understood. Mg^{2+} movement across the cell membrane is dependent on the concentration of Na^+. Although it is known that the cell membrane exhibits very low Mg^{2+} permeability (Quamme and Rabkin, 1990), a cell membrane Na^+/Mg^{2+} exchanger has been known to induce an Na^+-dependent Mg^{2+} efflux at a stoichiometric ratio of $2Na^+$:$1Mg^{2+}$ (Zhang and Melvin, 1996). However, in the present study, the bFGF-induced increase of $[Mg^{2+}]_i$ was not affected by the removal of extracellular Na^+ or Mg^{2+}. These results indicate that bFGF increased the $[Mg^{2+}]_i$ from the intracellular Mg^{2+} stores, and the increase of $[Mg^{2+}]_i$ was independent of the extracellular Na^+ and Mg^{2+}.

Several signaling pathways including cAMP (Romani et al.,

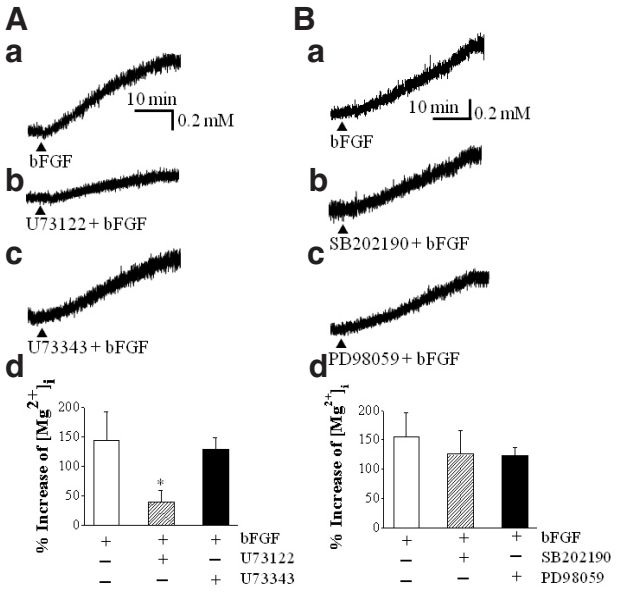

Fig. 5. The effects of inhibitors for PLC (A) or MAP kinase (B) on the bFGF (10 ng/ml)-induced increase of $[Mg^{2+}]_i$ in the Mg^{2+}-free extracellular solution. (a, b, and c) Representative tracings. The mag-fura-2-loaded cells were incubated with U73122 (Ab, 1 μM), its inactive analogue U73343 (Ac, 1 μM), SB202190 (Bb, 10 μM) or PD98059 (Bc, 30 μM) for 20 min before the perfusion with bFGF. The arrows indicate the starting points of the perfusion with VEGF. (d) Average values of the percentage increases of $[Mg^{2+}]_i$ in each group. Each column with a vertical bar denotes the means ± SEM. *, Significantly different from the control (bFGF alone; $P < 0.05$).

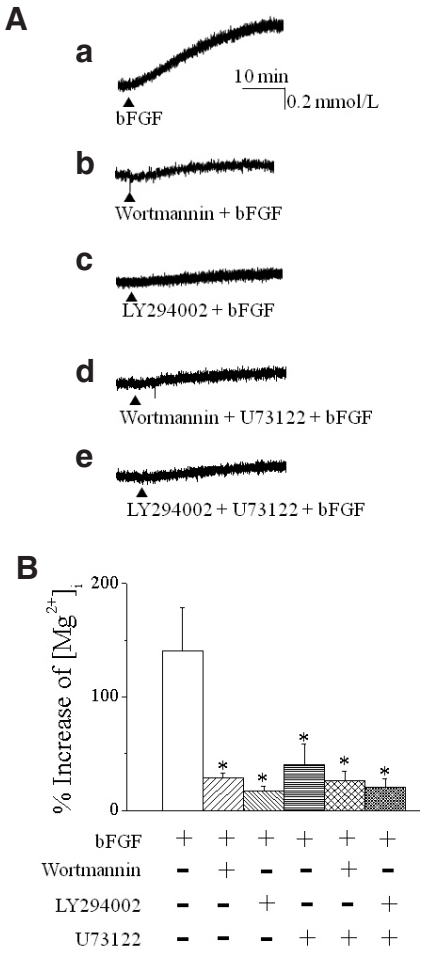

Fig. 6. The effects of PI3K inhibitors and PLC inhibitor on the bFGF (10 ng/ml)-induced increase of $[Mg^{2+}]_i$ in the Mg^{2+}-free extracellular solution. (A) Representative tracings. The mag-fura-2-loaded cells were incubated with wortmannin (30 nM) or LY294002 (25 μM), or/and U73122 (Ab, 1 μM) for 20 min before the perfusion with bFGF. The arrows indicate the starting points of the perfusion with bFGF. (B) Average values of the percentage increases of $[Mg^{2+}]_i$ in each group. Each column with a vertical bar denotes the means ± SEM. *, Significantly different from the control (bFGF alone; $P < 0.05$).

1993; Watanabe et al., 1998), G-proteins (Shi et al., 1995), PKC (Romani et al., 1992), MAP kinase (Kim et al., 2005), tyrosine-kinase (Ishijima and Tatibana, 1994) or PI3K (Kurosawa et al., 1993), have been reported to be involved in the regulation of $[Mg^{2+}]_i$. Additionally, the binding of bFGF to its receptor leads to the activation of several intracellular signaling molecules such as MAPKs, PI3K and PLCγ (Klint and Claesson-Welsh, 1999; Webber et al., 2005). In the present study, the bFGF-induced increase of $[Mg^{2+}]_i$ was blocked by the inhibitor for tyrosine kinase, PI3K, and PLC, but not by inhibitors for MAP kinase. These results strongly indicate that bFGF induces the release of $[Mg^{2+}]_i$ from the intracellular Mg^{2+} stores through the tyrosine kinase/PI3K/PLCγ signaling pathways.

The mitochondria, endo-sarco-plasmic reticulum and nucleus are known to be the three main intracellular Mg^{2+} pools, with concentrations ranging between 16 and 20 mM in each of these compartments (Gunther, 1986; Wolf et al., 2003). Additionally, the cytoplasm also contains a sizable amount of Mg^{2+} (4-5 mM), mostly in a complex with ATP, phosphocreatine and other phosphonucleotides (Romani and Scarpa, 2000; Scarpa and Brinley, 1981). However, very little information is currently available about how Mg^{2+} is regulated, especially about which intracellular Mg^{2+} store(s) could be dynamically regulated. bFGF might release Mg^{2+} from the mitochondria, endoplasmic reticulum (ER), nucleus or cytoplasm. In this study, we found that bFGF released Mg^{2+} from the intracellular Mg^{2+} stores through the tyrosine kinase/PI3K/PLCγ signaling pathways independent of extracellular Mg^{2+}. To clarify the underlying mechanisms for the bFGF-induced effect, it isnecessary to find out which intracellular Mg^{2+} store(s) might be regulated by bFGF.

The process of angiogenesis involves several discrete steps, including proliferation, migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (Bussolino et al., 1997). It is interesting that magnesium is shown to stimulate angiogenesis and enhance the motogenic response to angiogenic factors (Lapidos et al., 2001; Maier et al., 2004a; 2004b). Indeed, the stimulation of endothelial growth by magnesium and the enhancement of endothelial migration are partially mediated by the regulation of energy production and the stimulation of the activity of enzymes which require magnesium (Hackett et al., 1987; Wolf and Cittadini, 1999; 2003). A key function magnesium is required for is the assembly of actin polymers and myosin ATPase activity, which are two key components responsible for cell migration (Wolf and Cittadini, 2003). Magnesium also exerts a stimulatory effect on chemotaxis and cell spreading in HUVECs (Lapidos et al., 2001). In the present study, we found that bFGF increased $[Mg^{2+}]_i$. Considering all

these factors, the bFGF-induced increase of $[Mg^{2+}]_i$ might contribute to the bFGF-induced angiogenesis.

In conclusion, bFGF induced the release of Mg^{2+} from intracellular Mg^{2+} stores through the tyrosine kinase/PI3K/PLCγ-signaling pathways in HUVECs. This is the first report that has clarified bFGF-induced regulation of $[Mg^{2+}]_i$.

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