Hepatocyte growth factor activates endothelial nitric oxide synthase by Ca^{2+}- and phosphoinositide 3-kinase/Akt-dependent phosphorylation in aortic endothelial cells

Kennedy MAKONDO*, Kazuhiro KIMURA*[1], Naoki KITAMURA†, Takanori KITAMURA*, Daisuke YAMAJI*, Bae Dong JUNG* and Masayuki SAITO*

*Laboratory of Biochemistry, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan, and †Laboratory of Physiology, Department of Biomedical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Hepatocyte growth factor (HGF) causes endothelium-dependent vasodilation, but its relation to endothelial nitric oxide synthase (eNOS) activity remains to be elucidated. Treatment of bovine aortic endothelial cells with HGF increased eNOS activity within minutes, accompanied by an increase of activity-related site-specific phosphorylation of eNOS. The phosphorylation was completely abolished by pretreatment of the cells with a phosphoinositide 3-kinase (PI3K) inhibitor (wortmannin) and by transfection of dominant-negative Akt, and the enzyme activity was inhibited by wortmannin. In addition, eNOS activity and phosphorylation were abolished by pretreatment of the cells with an intracellular Ca^{2+}-chelator, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM), with a suppression of Akt phosphorylation. These results suggest that HGF stimulates eNOS activity by a PI3K/Akt-dependent phosphorylation in a Ca^{2+}-sensitive manner in vascular endothelial cells.

Key words: hepatocyte growth factor (HGF), mitogen-activated protein kinase, nitric oxide, nitric oxide synthase, wortmannin.

INTRODUCTION

Hepatocyte growth factor (HGF), also known as scatter factor, is a mesenchyme-derived multifunctional cytokine with a plethora of biological effects, including mitogenesis, motogenesis, morphogenesis and organogenesis [1] and, in some cases, tumour invasion and metastasis [2]. HGF is also a potent angiogenic molecule and HGF stimulation of endothelial cells promotes cell motility, proliferation and organization into capillary-like tubes [3]. HGF acts on endothelial cells through a high-affinity transmembrane tyrosine kinase receptor, c-Met, and subsequent activation of both phosphoinositide 3-kinase (PI3K) and p42/p44 mitogen-activated protein kinase [(MAPK; also known as extracellular-signal-regulated protein kinase 1/2 (ERK1/2)] pathways [4,5].

Some of these actions, such as activation of endothelial cell motility, are mediated by nitric oxide (NO) produced by inducible NO synthase (iNOS) through a PI3K-dependent up-regulation of the enzyme content, whereas HGF fails to influence the amount of endothelial isoform of NO synthase (eNOS) protein [6]. In addition to these long-term effects, HGF also causes the rapid relaxation of microvessels that is inhibited by a NOS inhibitor [7]. This short-term response, however, cannot be attributed to the induction of iNOS. Under physiological conditions, a significant amount of NO produced in the vascular wall is through the activity of constitutively expressed eNOS [8–10]. In fact, vascular endothelial growth factor (VEGF), another potent angiogenic molecule, produces similar short- and long-term NO-mediated responses that can be explained by increase in activity and protein levels of eNOS [11], rather than iNOS.

eNOS activity is basically regulated by changes in intracellular Ca^{2+} concentration and modulated by phosphorylation of the enzyme [8–10]. Phosphorylation at Ser^{1177} of human eNOS by Akt or AMP-activated protein kinase increases enzyme activity, whereas phosphorylation at Thr^{497} by protein kinase C or phosphorylation at an unidentified residue by p42/p44 MAPK decreases its activity [8–10]. As HGF/Met signalling activates the p42/p44 MAPK and PI3K/Akt signalling pathways in endothelial cells [5,12], we speculated that HGF might directly stimulate eNOS activity and NO production through phosphorylation by Akt and/or p42/p44 MAPK. In the present study, we test this hypothesis by examining HGF effect on eNOS activity and its phosphorylation, and examine further the possible involvement of Ca^{2+} in these effects.

EXPERIMENTAL

Materials

Human recombinant HGF was generously given by Mitsubishi Pharma Co. (Tokyo, Japan). Polyclonal antibodies against phospho-specific p42/p44 MAPK (Thr^{202}/Tyr^{204}) and total p42/p44 MAPK, phospho-specific Akt (Thr^{308} and/or Ser^{473}) and total Akt, and phospho-specific eNOS (Ser^{1177} of human sequence: equivalent to Ser^{1179} of bovine eNOS) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The anti-eNOS antibody and calmodulin (CaM) were bought from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and Wako Pure Chemicals Co. (Osaka, Japan) respectively. p42/p44 MAPK kinase (MEK) inhibitor PD98059 was from Biomol Research Laboratories (Plymouth, PA, U.S.A.), and the PI3K inhibitor wortmannin was from Sigma–Aldrich (St. Louis, MO, U.S.A.). Membrane-permeant Ca^{2+}-chelator bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-

Abbreviations used: BAEC, bovine aortic endothelial cells; BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid tetrakis(acetoxymethyl ester); CaM, calmodulin; HGF, hepatocyte growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; eNOS, endothelial NO synthase; iNOS, inducible NO synthase; PI3K, phosphoinositide 3-kinase; VEGF, vascular endothelial growth factor.

[1] To whom correspondence should be addressed (e-mail k-kimura@vetmed.hokudai.ac.jp).

tetra-acetic acid tetrakis(acetoxymethyl ester) (BAPTA/AM) and BAPTA were from Calbiochem–Novabiochem (San Diego, CA, U.S.A.), and NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) was from Dojindo Co. (Kumamoto, Japan).

Cell culture and treatment

Bovine aortic endothelial cells (BAEC) from Cell Systems (Kirkland, WA, U.S.A.) were maintained in CS-C medium [Dulbecco's modified Eagle's medium and Ham's F-12 medium (1:1, v/v) containing 10 % (v/v) fetal bovine serum, 15 mM Hepes, 10 ng/ml acidic fibroblast growth factor and 50 units/ml heparin; Cell Systems] on Type I-collagen-coated plates (Asahi Techno Glass Co., Tokyo, Japan) at 37 °C and 5 % CO_2 under humidified conditions. After BAEC were grown to confluence, the cells were serum-starved in Phenol-Red-free M199 (Sigma–Aldrich) containing 2 mM L-glutamine and 0.2 % BSA overnight prior to use.

For transient transfections of cDNA, BAEC were seeded at $(2.5–3.0) \times 10^5$ cells/well in 6-well collagen-coated plates and maintained in CS-C medium for 18–24 h until approx. 60–80 % confluent. Transfections of the dominant-negative Akt–Myc cDNA and empty vector (Upstate Biotechnology, Lake Placid, NY, U.S.A.) were carried out using Fugene 6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, transfection complexes were prepared with 6 μl of Fugene 6 reagent, 2 μg of cDNA and Opti-MEM I to a final volume of 100 μl (Gibco, Grand Island, NY, U.S.A.). After incubation at room temperature for 30–45 min, the complexes were flooded on to the cells in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 % (v/v) fetal calf serum and incubated for 24 h. Cells were serum-starved for 6 h in M199 before use. The tranfection efficiency was confirmed by Western-blot analysis with anti-Myc (Upstate Biotechnology) and anti-Akt antibodies.

Measurement of eNOS activity

eNOS activity was quantified as the conversion of L-[U-^{14}C]arginine into L-[U-^{14}C]citrulline as described previously [13] with minor modifications. Briefly, confluent BAEC were serum-starved and the medium was replaced before treatment. Following the experimental treatments, cells were harvested in ice-cold homogenization buffer [50 mM Tris/HCl (pH 7.4), 250 mM sucrose, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol and Complete protease inhibitor cocktail (1 tablet/50 ml; Roche, Indianapolis, IN, U.S.A.)]. For each sample, 10 μl of homogenate was incubated in duplicate at 37 °C for 10 min in a 100 μl of reaction mixture {50 mM Hepes (pH 7.9), 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.1 mM tetrahydro-L-biopterin (BH_4), 1 mM NADPH, 10 μM FAD, 10 μg/ml CaM and 1.43 μM L-[U-^{14}C]arginine (Amersham Biosciences, Little Chalfont, Bucks., U.K.)}. The reaction was terminated by addition of 200 μl of stop solution [100 mM Hepes and 10 mM EDTA (pH 5.2)]. The reaction mixture was then applied on to a 0.5 ml neutralized AG 50W-X4 resin (Na^+ form, 200–400 dry mesh; Bio-Rad, Hercules, CA, U.S.A.) column to separate L-[U-^{14}C]citrulline. The flow through was analysed by liquid-scintillation counting and enzyme activity expressed as fmol of L-[U-^{14}C]citrulline produced/mg of homogenate protein per 10 min.

In some experiments, eNOS activity was determined by measuring the intracellular conversion of arginine into citrulline as described previously [14]. Briefly, confluent serum-starved BAEC in 6-well plates were incubated in buffer A [25 mM Hepes (pH 7.3), 109 mM NaCl, 5.4 mM KCl, 0.9 mM $CaCl_2$, 1 mM $MgSO_4$ and 25 mM glucose] for 1 h at 37 °C, and inhibitors were added during the last 30 min. The buffer was replaced with 1 ml of prewarmed buffer A containing a mixture of 10 μM L-arginine and 10 μCi/ml L-[2,3,4,5-^{3}H]arginine (Amersham Biosciences) in the absence or presence of inhibitors and immediately stimulated with HGF. Each treatment was performed in duplicate. Following incubation for a further 20 min at 37 °C, cells were rinsed with ice-cold PBS and scraped into 1 ml of stop solution [20 mM sodium acetate (pH 5.5), 1 mM L-citrulline, 2 mM EDTA and 2 mM EGTA] and sonicated for 5 min. An aliquot for protein determination and total cellular ^{3}H incorporation was collected. The remaining sample was applied on to a 1 ml neutralized AG 50W-X4 resin column to separate L-citrulline, and the flow-through was analysed as described above. Protein concentration was determined by the Lowry method using BSA as a standard [15].

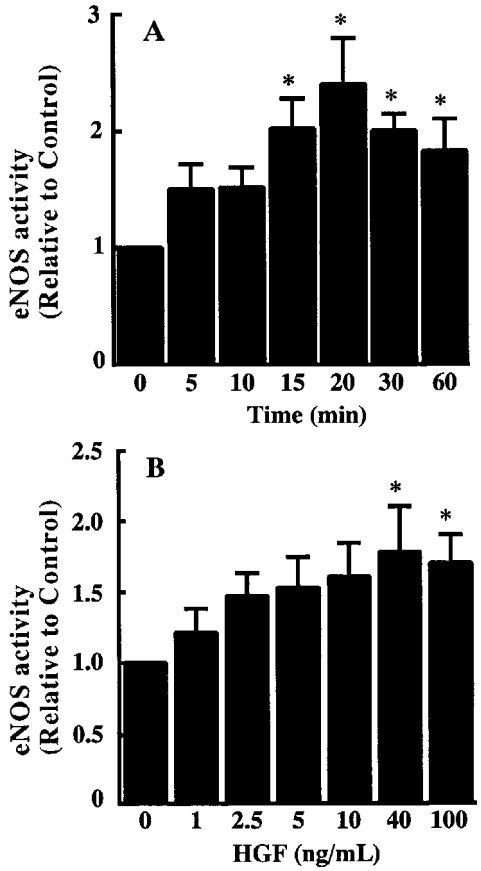

Figure 1 HGF induces eNOS activation in BAEC

Confluent BAEC were serum-starved overnight and stimulated with HGF (40 ng/ml) for the indicated time periods (**A**) or with different doses of HGF for 20 min (**B**). eNOS activity in the cell homogenates was determined as the conversion of L-[U-^{14}C]arginine into L-[U-^{14}C]citrulline and expressed as fold increases relative to the controls (untreated). Results represent the means ± S.E.M. of five (time course) and seven (dose–response) independent experiments. *$P < 0.05$ compared with cells without HGF addition (time 0 and dose 0).

Western-blot analysis

Following the experimental treatments, BAEC were washed with ice-cold PBS and scraped in ice-cold lysis buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 5 mM EDTA, 20 mM sodium fluoride,

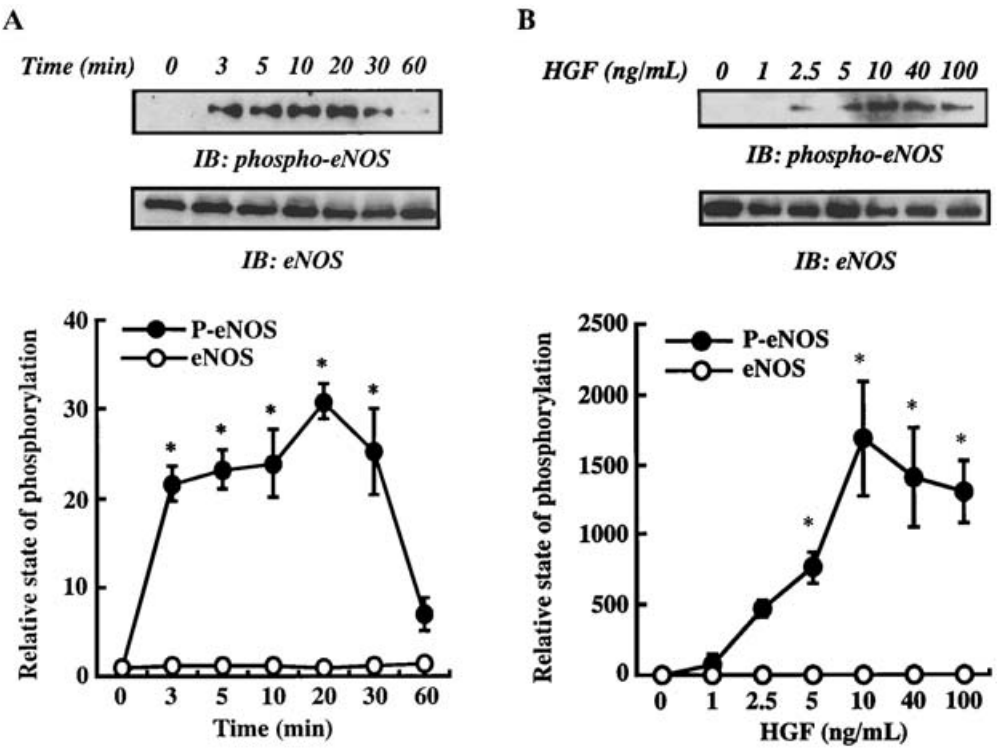

Figure 2 HGF activates eNOS phosphorylation in BAEC

Serum-starved BAEC were treated with HGF (40 ng/ml) for the indicated time periods (**A**) or with increasing concentrations of HGF for 5 min (**B**). The cell lysates (30 μg/lane) were resolved by SDS/PAGE [7.5 % (w/v) gel] and examined for the phosphorylation state of the eNOS protein at Ser^{1179} by immunoblotting (IB). Representative blots are shown. Lower panels, densitometric analyses of phospho- (P-eNOS) and total eNOS protein are expressed as fold increases relative to controls (untreated). Results are the means $\pm$ S.E.M. of three independent experiments. $^*P < 0.05$ compared with cells without HGF addition (time 0 and dose 0).

10 mM sodium pyrophosphate, 2 mM sodium vanadate, 1 % Nonidet 40 and Complete protease inhibitor cocktail]. Harvested cells were incubated on ice for 30 min followed by centrifugation at 12000 ***g*** for 20 min at 4 °C to obtain cell lysates. Portions of the cell lysate (30 μg of each sample) were resolved by SDS/PAGE under reducing conditions and proteins were electroblotted on to a PVDF membrane (Immobilon™; Millipore, Bedford, MA, U.S.A.). The membrane was blocked overnight at 4 °C in 5 % (w/v) skimmed milk in 20 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.01 % Tween 20, followed by incubation with a primary antibody overnight at 4 °C. Following incubation, the membrane was exposed to a horseradish peroxidase-conjugated secondary goat anti-(rabbit IgG) antibody (Zymed Lab. Inc., San Francisco, CA, U.S.A.) for 1 h at room temperature. Visualization was performed using either enhanced chemiluminescence (ECL® Amersham Biosciences) or Super Signal (Pierce Chemical Co., Rockford, IL, U.S.A.) detection systems according to the manufacturers' instructions. Intensities of immunoreactive bands in the Western blots were analysed densitometrically on a Macintosh computer using the public domain National Institutes of Health (NIH) Image program (http://rsb.info.nih.gov/nih-image/).

Statistical analysis

Results are expressed as means $\pm$ S.E.M. of measurements in different cell cultures (independent experiments). Statistical analysis was done using ANOVA and Fischer's test, with $P < 0.05$ being considered as significant.

RESULTS

To test the hypothesis that HGF stimulates eNOS activity in vascular endothelial cells, BAEC were treated with HGF followed by measuring eNOS activity as the conversion of arginine into citrulline. The treatment with HGF significantly increased eNOS activity within 5 min, peaked by 20 min and remained sustained for the whole experimental duration (60 min) (Figure 1A). This increased enzyme activity was evident at the lowest dose (1 ng/ml) used in the experiments and was significant at concentration of 40 ng/ml HGF and above (Figure 1B).

Next, we examined whether HGF induced eNOS phosphorylation at Ser^{1179}, the residue linked to eNOS activation. Treatment of BAEC with HGF robustly induced eNOS phosphorylation in a time- and dose-dependent manner (Figure 2). eNOS phosphorylation was rapid, being observed within 3 min of HGF addition, and reached a maximum between 20–30 min (Figure 2A). Cells treated with increasing concentrations of HGF showed a dose-dependent increase in the phosphorylation of eNOS, which peaked at 10 ng/ml and with no further increase at higher doses (Figure 2B). We also examined HGF stimulation of p42/p44 MAPK and Akt phosphorylation in BAEC. As expected, HGF potently stimulated phosphorylation of p42/p44 MAPK (Thr^{202}/Tyr^{204}) and Akt (Thr^{308}) (Figure 3), indicating activation of both protein kinases. Both p42/p44 MAPK and Akt phosphorylation were rapid, being evident within 3 min, and peaked by 5 min of HGF treatment. The phosphorylation levels remained significantly elevated throughout the 60 min incubation period (Figures 3A and 3C). In the dose–response experiments,

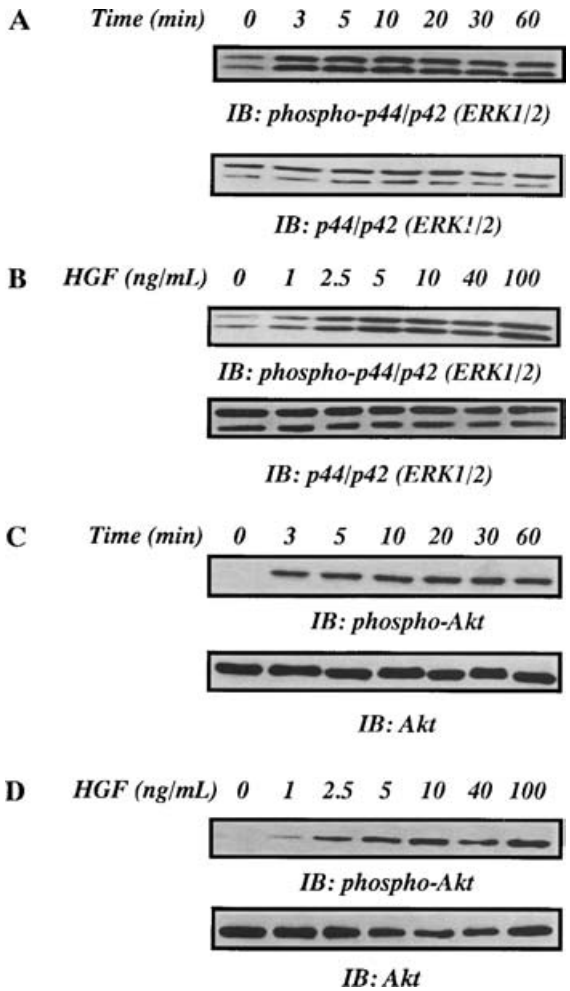

Figure 3 HGF induces p42/p44 MAPK and Akt phosphorylation in BAEC

BAEC were treated as in the legend to Figure 2. The cell lysates (30 μg/lane) were resolved by SDS/PAGE [10 % (w/v) gels], and examined for phosphorylation of p42/p44 MAPK and Akt with anti-phospho-[p44/p42 MAPK(Thr^{202}/Tyr^{204})] and anti-[phospho-Akt (Thr^{308})] antibodies. Representative blots of three independent experiments for p42/p44 MAPK (**A**, **B**) and Akt (**C**, **D**) respectively, are shown.

phosphorylation of both protein kinases was significant at 2.5 ng/ml and reached a maximum response at 10 ng/ml HGF, but no further increment was obtained with higher doses of HGF (Figures 3B and 3D).

To evaluate the role of p42/p44 MAPK and PI3K/Akt signalling pathways in the HGF-induced eNOS phosphorylation, we tested the effect of PD98059, a MEK inhibitor, and wortmannin, a PI3K inhibitor, on eNOS phosphorylation. Treatment of BAEC with PD98059 had minimal, or no, effect on the HGF-induced eNOS phosphorylation (Figure 4A). In contrast, treatment with wortmannin completely abrogated the phosphorylation (Figure 4A). Parallel analysis of p42/p44 MAPK and Akt phosphorylation revealed that the MEK inhibitor completely abrogated the HGF-induced phosphorylation of p42/p44 MAPK, but without any noticeable effect on Akt phosphorylation (Figures 4B and 4C). On the other hand, treatment with wortmannin obliterated the HGF-induced phosphorylation of Akt, whereas p42/p44 MAPK phosphorylation remained intact (Figures 4B and 4C).

To confirm the role of Akt activity in the HGF-induced eNOS phosphorylation, the effect of transient expression of a dominant-negative Akt was examined. Following transfection, the exogenous dominant-negative Akt containing a Myc-epitope was found in the cell lysate in relatively lower amounts than those of endogenous Akt (Figure 5). HGF stimulation of cells transfected with control vector increased Akt and eNOS phosphorylation to the same extent as in naïve control cells (Figure 5). However, cells transfected with the dominant-negative Akt not only showed blunted HGF-induced Akt phosphorylation, but also eNOS phosphorylation without noticeable changes in the total eNOS protein content (Figure 5).

To evaluate the relationship between HGF-modulated eNOS activity and its phosphorylation at Ser^{1179}, we examined the effect of wortmannin on the activity in cell homogenates and intact cells. In both assay systems, treatment of cells with wortmannin had no effect on the basal eNOS activity. However, wortmannin significantly attenuated the HGF-stimulated eNOS activation, more so in the cell homogenate, and completely inhibited enzyme activity in the intact cell assay system (Figure 6). On the other hand, treatment of cells with the MEK inhibitor was without any noticeable effect on basal and HGF-induced eNOS activity (Figure 6).

We next examined the possible involvement of Ca^{2+} in the HGF-induced eNOS activity. Treatment of cells with an intracellular Ca^{2+} chelator, BAPTA/AM, not only reduced basal enzyme activity, but also completely abolished HGF stimulation of eNOS activity (Figure 7); these effects were comparable with those of a well known NOS inhibitor L-NAME (Figure 7A). Furthermore, intracellular Ca^{2+} chelation completely abrogated the HGF-induced eNOS and Akt phosphorylation, but with no effect on p42/p44 MAPK phosphorylation (Figure 7B), whereas treatment of cells with a non-cell-permeant form of BAPTA and use of nominally Ca^{2+}-free Hepes buffer had no effect on the HGF-induced activation of the cellular signalling pathways (results not shown).

DISCUSSION

In the present study, we have demonstrated in BAEC that HGF/c-Met signalling stimulates eNOS activity in a dose-dependent manner, obviously indicating enhancement of NO production. To the best of our knowledge, this report represents the first direct evidence of acute regulation of eNOS activity by HGF in endothelial cells and the results explain the mechanism of the acute HGF effect on microvessels [7].

The eNOS activity is dependent on the presence of Ca^{2+} and CaM and, therefore, regulated by changes in cytosolic Ca^{2+}, but also controlled at post-translational levels such as through palmitoylation, protein–protein interactions and phosphorylation by various protein kinases [8–10]. In the present study, we demonstrated that HGF robustly induced eNOS phosphorylation at Ser^{1179}, an activity-related residue, which was completely abolished by a PI3K inhibitor and activation-deficient Akt, but not by a MEK inhibitor. These results indicate that HGF activates Akt through the PI3K pathway, leading to eNOS phosphorylation and thereby enzyme activation. In addition, the blockade of the eNOS activity by the PI3K inhibitor offers evidence that the HGF-induced eNOS phosphorylation is responsible for the enzyme activation, and this is supported further by the fact that the HGF-induced eNOS activity correlated well with the phosphorylation dynamics for both the time-course and dose–response experiments.

In the present study, pretreatment of BAEC with an intracellular Ca^{2+}-chelator, BAPTA/AM, not only reduced the basal activity, but also completely abrogated the HGF-induced eNOS

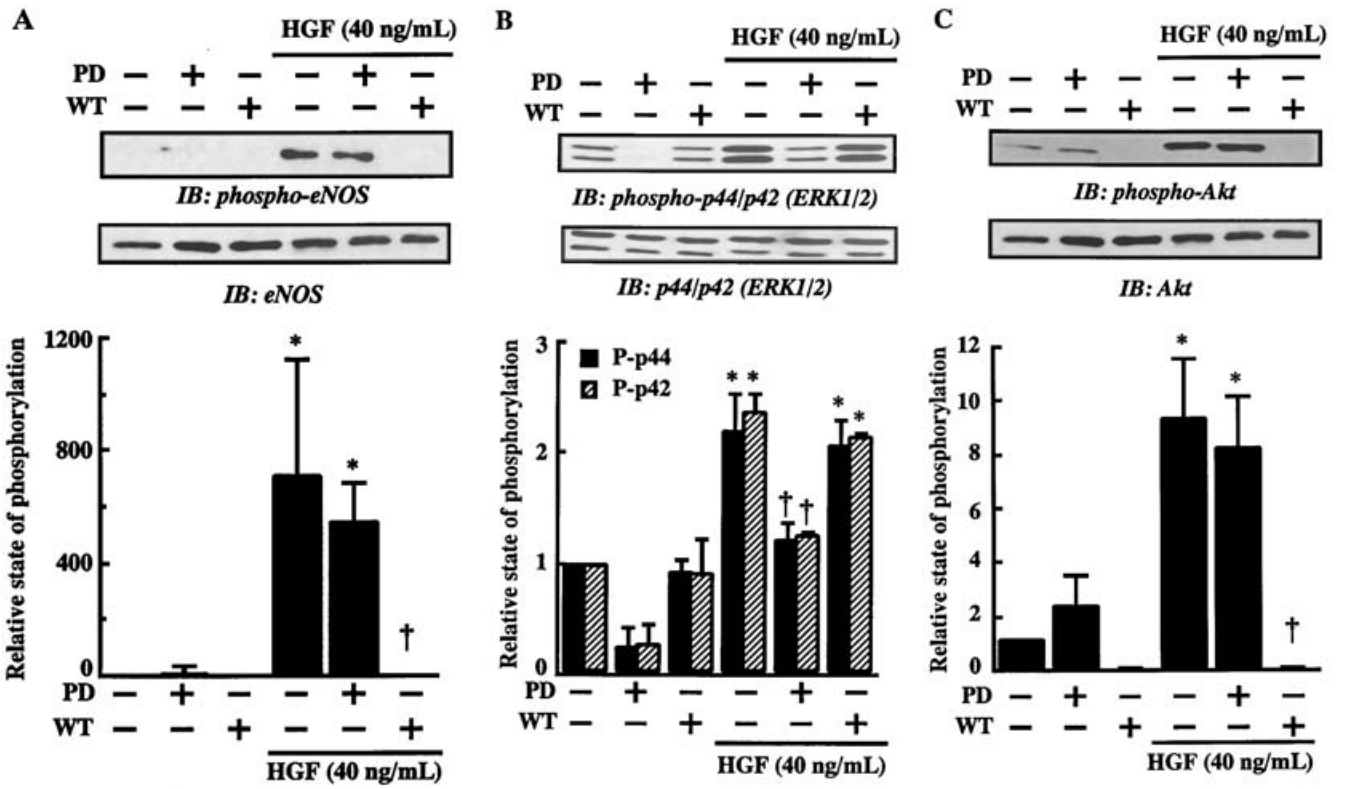

Figure 4 Effect of inhibitors of p42/p44 MAPK and Akt activation on the HGF-induced eNOS phosphorylation

Confluent BAEC were pretreated with PD98059 (10 μM), a MEK inhibitor, or wortmannin (WT; 500 nM), a PI3K inhibitor, for 30 min prior to stimulation with HGF (40 ng/ml) for 5 min. Following treatments, cell lysates (30 μg/lane) were analysed with (**A**) anti-(phospho-eNOS) and anti-eNOS antibodies, (**B**) anti-[phospho-p44/p42 MAPK(Thr^{202}/Tyr^{204})] and anti-(total p44/p42MAPK) antibodies, and (**C**) anti-[phospho-Akt (Thr^{308})] and anti-(total Akt) antibodies. Representative blots and densitometric analyses of three independent experiments are shown. *$P < 0.05$ compared with cells without HGF addition. †$P < 0.05$ compared with HGF treatment alone.

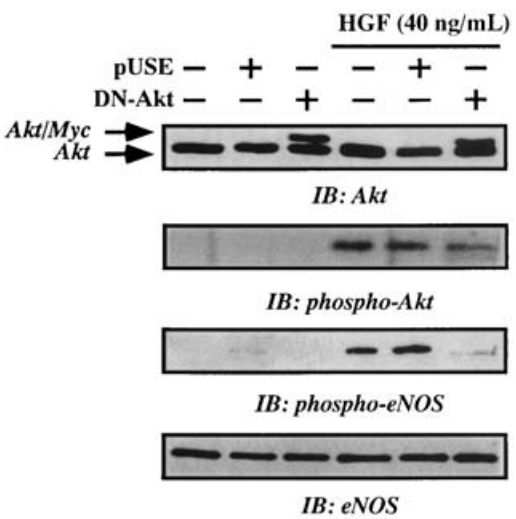

Figure 5 Effect of dominant-negative Akt cDNA on HGF-induced eNOS phosphorylation

BAEC were transfected with empty vector (pUSE) or dominant-negative Akt cDNA (DN-Akt) and starved for 6 h prior use. Following treatment with HGF (40 ng/ml) for 5 min, cell lysates were prepared and analysed with anti-[phospho-Akt (Thr^{308})], anti-(phospho-eNOS), anti-(total Akt) and anti-eNOS antibodies. Representative blots of three independent experiments are shown.

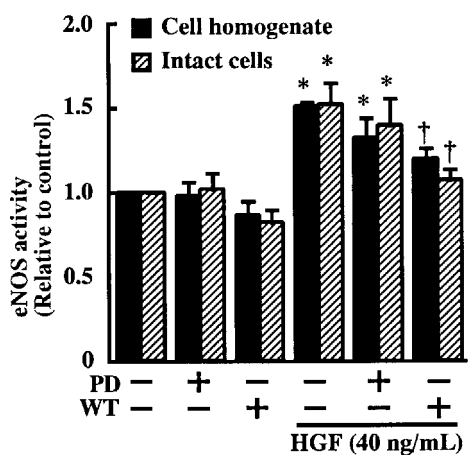

Figure 6 Effect of inhibitors of p42/p44 MAPK and Akt activation on the HGF-stimulated eNOS activity

BAEC were treated with PD98059 (10 μM) and wortmannin (WT; 500 nM) for 30 min prior to treatment with HGF (40 ng/ml) for 20 min. Then eNOS activity in the cell homogenate and intact cells were assayed and expressed as fold increases relative to controls (untreated). Results are means $\pm$ S.E.M. of four independent experiments. *$P < 0.05$ compared with cells without HGF addition. †$P < 0.05$ compared with HGF treatment alone.

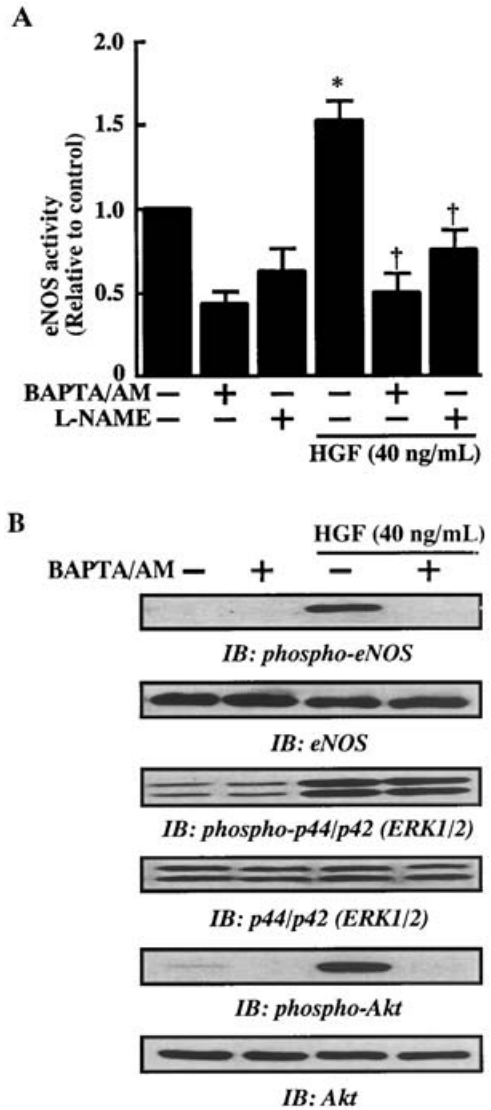

Figure 7 Effect of an intracellular Ca^{2+} chelator on HGF-induced eNOS activation and phosphorylation

Confluent BAEC were pretreated with BAPTA/AM (20 μM), an intracellular Ca^{2+} chelator, or L-NAME (1 mM), a NOS inhibitor, for 30 min prior to treatment with HGF (40 ng/ml) for 20 min to determine eNOS activity in the intact cells (**A**) and for 5 min to analyse the HGF-induced phosphorylation of eNOS, p44/p42 MAPK and Akt (**B**). Results in (**A**) are means $\pm$ S.E.M. of four independent experiments. $^*P < 0.05$ compared with cells without HGF addition. $\dagger P < 0.05$ compared with HGF treatment alone. (**B**) Representative blots of three independent experiments are shown.

activity, suggesting the involvement of Ca^{2+}-dependent mechanisms in the HGF-induced eNOS activation. It has been reported [9,10] that eNOS phosphorylation at Ser^{1179} by Akt decreases dependence of the enzyme for Ca^{2+}/CaM and allows enzyme activation at basal levels of Ca^{2+}, especially in the case of fluid-shear stress [9,10]. However, as stated above, the formation of Ca^{2+}/CaM complex and its eventual binding to eNOS is an important prerequisite in the enzyme activation. It is, therefore, generally thought, albeit controversially, that Ca^{2+} does not directly influence eNOS activity if phosphorylated, but our present results, showing that Ca^{2+} sequestration abolished the HGF-induced Akt and eNOS phosphorylation, suggest that Ca^{2+} is required in the HGF-induced signalling leading to eNOS phosphorylation. Similarly, oestrogen- and sphingosine 1-phosphate-induced Akt phosphorylation and/or activation are Ca^{2+}-dependent [13,14], whereas those elicited by VEGF and insulin are insensitive to Ca^{2+} depletion [16,17]. Furthermore, it is of note that the exact mechanisms linking Ca^{2+} and PI3K/Akt activation and consequent eNOS activation after HGF stimulation still remain elusive.

Following HGF activation, c-Met signalling is elicited through its recruitment and activation of several signalling transducers such as Src tyrosine kinase, Grb2 and Gab1, and the subsequent interaction with additional Src-homology-2-containing signal transducers, including PI3K and phospholipase C-γ [18,19]. It is interesting to note that, during the preparation of this manuscript, a recent report [20] has implicated a Src-dependent signalling mechanism in HGF-induced NO production and endothelial cell growth, probably through the activation of phospholipase C-γ and consequent formation of Ins(1,4,5)P_3, as reported previously [21] for cGMP generation in BAEC after stimulation with VEGF. In addition, it has been reported [22,23] that the p85 regulatory and p110 catalytic subunits of PI3K potentially interact with Ca^{2+}/CaM through their Src-homology-2 domains. Thus it is reasonable to speculate that Src-mediated intracellular Ca^{2+} movement might be indispensable and possibly represent a co-ordinated pathway involving Ca^{2+} and PI3K/Akt signalling in HGF-induced eNOS activity, and that the effects of BAPTA/AM observed in the present study cannot be attributable to non-specific effects, since p42/p44 MAPK phosphorylation remained intact, and a non-cell-permeant form of BAPTA did not affect the HGF-induced activation of PI3K/Akt/eNOS.

NO has emerged to be a key signalling molecule in the maintenance of cardiovascular homoeostasis such as the control of vascular tone and maintenance of endothelial cell integrity [24]. In addition, both *in vitro* and *in vivo* studies have provided compelling evidence that NO directly regulates angiogenesis in vascular cells [11,24,25]. Therefore the acute regulation of eNOS activity through its phosphorylation by HGF may be of importance not only in vasodilation [7], but also in the other HGF-induced functions, including angiogenesis [3], limiting of neointimal proliferation [26] and tissue regeneration after myocardial ischaemic damage [27]. In addition to these direct effects, HGF is able to trigger smooth muscle cells to release a potent angiogenic molecule, VEGF [28], and may promote angiogenesis in synergy [29] through increased NO production [11,25,30].

In conclusion, we have clearly demonstrated that HGF enhances eNOS activity and its phosphorylation at Ser^{1179} in endothelial cells. The phosphorylation was mediated by PI3K/Akt-dependent signalling pathway via a Ca^{2+}-dependent mechanism(s) and linked to eNOS activity. These observations are important in understanding the role of HGF in the vasculature and also in the therapeutic use of recombinant HGF or its gene for peripheral arterial diseases [31].

This work was supported by grants from the Ministry of Education, Science and Culture of Japan and Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) from the Bio-oriented Technology Research Advancement Institution, Japan. The work was also supported by a Research Fellowship from the Ministry of Education, Science and Culture of Japan (Monbusho) for Foreign Students to K.M., and a Research Fellowship from the Japan Society for the Promotion of Science (JSPS) for Young Scientists to T.K.

REFERENCES

1 Grierson, I., Heathcote, L., Hiscott, P., Hogg, P., Briggs, M. and Hagan, S. (2000) Hepatocyte growth factor/scatter factor in the eye. Prog. Ret. Eye Res. **19**, 779–802

2 Maulik, G., Shrikhande, A., Kijima, T., Ma, P. C., Morrison, P. T. and Salgia, R. (2002) Role of the hepatocyte growth factor receptor, c-Met, in oncogenesis and potential for therapeutic inhibition. Cytokine Growth Factor Rev. **13**, 41–59
3 Rosen, E. M., Lamszus, K., Laterra, J., Polverini, P. J., Rubin, J. S. and Goldberg, I. D. (1997) HGF/SF in angiogenesis. Ciba Found. Symp. **212**, 215–226
4 Derman, M. P., Cunha, M. J., Barros, E. J., Nigam, S. K. and Cantley, L. G. (1995) HGF-mediated chemotaxis and tubulogenesis require activation of the phosphatidylinositol 3 kinase. Am. J. Physiol. **268**, F1211–F1217
5 Nakagami, H., Morishita, R., Yamamoto, K., Taniyama, Y., Aoki, M., Matsumoto, K., Nakamura, T., Kaneda, Y., Horiuchi, M. and Ogihara, T. (2001) Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and Akt in endothelial cells. Hypertension **37**, 581–586
6 Purdie, K. J., Whitley, G. S. J., Johnstone, A. P. and Cartwright, J. E. (2002) Hepatocyte growth factor-induced endothelial cell motility is mediated by the upregulation of inducible nitric oxide synthase expression. Cardiovasc. Res. **54**, 659–668
7 Metais, C., Li, J., Li, J., Simons, M. and Sellke, F. W. (1998) Effects of coronary artery disease on expression and microvascular response to VEGF. Am. J. Physiol. **275**, H1411–H1418
8 Govers, R. and Rabelink, T. J. (2001) Cellular regulation of endothelial nitric oxide synthase. Am. J. Physiol. Renal Physiol. **280**, F193–F206
9 Fulton, D., Gratton, J.-P. and Sessa, W. C. (2001) Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? J. Pharmacol. Exp. Ther. **299**, 818–824
10 Fleming, I. and Busse, R. (2003) Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase. Am. J. Physiol. **284**, R1–R12
11 Papapetropoulos, A., Garcia-Cardena, G., Madri, J. A. and Sessa, W. C. (1997) Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J. Clin. Invest. **100**, 3131–3139
12 Stuart, K. A., Riordan, S. M., Lidder, S., Crostella, L., Williams, R. and Skouteris, G. G. (2001) Hepatocyte growth factor/scatter factor-induced intracellular signaling. Int. J. Exp. Pathol. **81**, 17–30
13 Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N. et al. (2001) Estrogen induced the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. J. Biol. Chem. **276**, 3459–3467
14 Igarashi, J., Bernier, S. G. and Michel, T. (2001) Sphingosine 1-phosphate and activation of endothelial nitric-oxide synthase: differential regulation of Akt and MAP kinase pathways by EDG and bradykinin receptors in vascular endothelial cells. J. Biol. Chem. **276**, 12420–12426
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. **193**, 265–275
16 Brouet, A., Sonveaux, P., Dessy, C., Balligand, J. L. and Feron, O. (2001) Hsp90 ensures the transition from the early Ca^{2+}-dependent to the late phosphorylation-dependent activation of the endothelial nitric-oxide synthase in vascular endothelial growth factor-exposed endothelial cells. J. Biol. Chem. **276**, 32663–32669
17 Montagnani, M., Chen, H., Barr, V. A. and Quon, M. J. (2001) Insulin-stimulated activation of eNOS is independent of Ca^{2+} but requires phosphorylation by Akt at Ser^{1179}. J. Biol. Chem. **276**, 30392–30398
18 Okano, Y., Mizuno, K., Osada, S., Nakamura, T. and Nozawa, Y. (1993) Tyrosine phosphorylation of phospholipase $C\gamma$ in c-Met/HGF receptor-stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. Biochem. Biophys. Res. Commun. **190**, 842–848
19 Maina, F., Pante, G., Helmbacher, F., Andres, R., Porthin, A., Davies, A. M., Ponzetto, C. and Klein, R. (2001) Coupling Met to specific pathways results in distinct developmental outcomes. Mol. Cell **7**, 1293–1306
20 Maejima, Y., Ueba, H., Kuroki, M., Yasu, T., Hashimoto, S., Nabata, A., Kobayashi, N., Ikeda, N., Saito, M. and Kawakami, M. (2003) Src family kinases and nitric oxide production are required for hepatocyte growth factor-stimulated endothelial cell growth. Atherosclerosis **167**, 89–95
21 He, H., Venema, V. J., Gu, X., Venema, R. C., Marrero, M. B. and Caldwell, R. B. (1999) Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through Flk-1/KDR activation of c-Src. J. Biol. Chem. **274**, 25130–25135
22 Joyal, J. L., Burks, D. J., Pons, S., Matter, W. F., Vlahos, C. J., White, M. F. and Sacks, D. B. (1997) Calmodulin activates phosphatidylinositol 3-kinase. J. Biol. Chem. **272**, 28183–28186
23 Fischer, R., Julsgart, J. and Berchtold, M. W. (1998) High affinity calmodulin target sequence in the signaling molecule PI 3-kinase. FEBS Lett. **425**, 175–177
24 Williams, I. L., Wheatcroft, S. B., Shah, A. M. and Kearney, M. T. (2002) Obesity, atherosclerosis and the vascular endothelium: mechanism of reduced nitric oxide bioavailability in obese humans. Int. J. Obesity **26**, 754–764
25 Murohara, T., Asahara, T., Silver, M., Bauters, C., Masuda, H., Kalka, C., Kearney, M., Chen, D., Chen, D., Symes, J. F. et al. (1998) Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. J. Clin. Invest. **101**, 2567–2578
26 Yasuda, S., Noguchi, T., Gohda, M., Arai, T., Tsutsui, N., Matsuda, T. and Nonogi, H. (2000) Single low-dose administration of human recombinant hepatocyte growth factor attenuates intimal hyperplasia in a balloon-injured rabbit iliac artery model. Circulation **101**, 2546–2549
27 Miyagawa, S., Sawa, Y., Taketani, S., Kawaguchi, N., Nakamura, T., Matsuura, N. and Matsuda, H. (2002) Myocardial regeneration therapy for heart failure: hepatocyte growth factor enhances the effect of cellular cardiomyoplasty. Circulation **105**, 2556–2561
28 Van Belle, E., Witzenbichler, B., Chen, D., Silver, M., Chang, L., Schwall, R. and Isner, J. M. (1998) Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation **97**, 381–390
29 Xin, X., Yang, S., Ingle, G., Zlot, C., Rangell, L., Kowalski, J., Schwall, R., Ferrara, N. and Gerritsen, M. E. (2001) Hepatocyte growth factor enhances vascular endothelial growth factor-induced angiogenesis *in vitro* and *in vivo*. Am. J. Pathol. **158**, 1111–1120
30 Zachary, I. (2001) Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. Am. J. Physiol. Cell Physiol. **280**, C1375–C1386
31 Morishita, R., Aoki, M., Nakamura, S., Higaki, J., Kaneda, Y. and Ogihara, T. (2000) Gene therapy for cardiovascular disease using hepatocyte growth factor. Ann. N.Y. Acad. Sci. **902**, 369–376

Received 27 February 2003/19 May 2003; accepted 21 May 2003
Published as BJ Immediate Publication 21 May 2003, DOI 10.1042/BJ20030326