Modulation of the erythropoietin-induced proliferative pathway by cAMP in vascular smooth muscle cells

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Received 29 April 2002; accepted in final form 6 August 2002

ERYTHROPOIETIN (Epo) is the main glycoprotein growth factor that regulates the survival, proliferation, and differentiation of erythroid progenitor cells (18). Its receptor is expressed not only in these cells but also in vascular smooth muscle cells (VSMC) and endothelial cells (3, 4).

Recombinant human erythropoietin (rHuEpo) is used to treat renal anemia. In some cases, introduction of rHuEpo leads to a rise in blood pressure. The mechanism of Epo-induced rise in blood pressure is multifactorial, including direct action on cells in vessel walls and elevation of cytosolic free calcium concentration ([Ca2+]i) or inhibition of nitric oxide synthesis in VSMC (2, 21, 28). We have recently shown that rHuEpo increases [Ca2+]i, augments mitogen-activated protein kinase (MAPK) activity, and, subsequently, promotes DNA synthesis in VSMC (1). Several studies have shown that the activation of MAPK seems to be a critical mediator in the growth-signal transduction pathway (23).

cAMP is a multipotential second messenger that regulates cell proliferation and apoptosis (16, 32). Many studies have shown that an increase in intracellular cAMP results in suppression of growth factor-mediated DNA synthesis and reduction of the number of VSMC or other kinds of cells (30, 32). Although the precise mechanisms of these inhibitory actions by cAMP are still not clear, these studies have shown that cAMP modulates the MAPK pathway. Therefore, in the present study, we investigated the possible involvement of cAMP in the Epo-induced MAPK pathway.

MATERIALS AND METHODS

Materials. All materials were obtained from Sigma Chemical (St. Louis, MO) except for the following. rHuEpo (erythropoietin-β) was a generous gift from Chugai Pharmacological (Tokyo, Japan). Cilostazol (Cil) was a generous gift from Otsuoka Pharmaceutical (Tokushima, Japan) and was dissolved in dimethyl sulfoxide (DMSO). The p44/42 MAPK assay kit, anti-p44/p42 MAPK and anti-phospho-p44/p42 MAPK (Thr202/Tyr204) antibodies, anti-MEK1/2 and anti-phospho-MEK1/2 (Ser217/Ser221) antibodies, and anti-phospho-Raf-1 (Ser259) were purchased from Cell Signaling Technology (Beverly, MA). Anti-Raf-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D1 antibody was purchased from Transduction Laboratories (Lexington, KY).

Cell culture. VSMC were prepared from aortas of male Sprague-Dawley rats (150–200 g) as described previously (15). In brief, cells were grown in DMEM supplemented with 10% fetal bovine serum (ICN Biomedicals, Osaka, Japan), 100 U/ml penicillin, and 100 mg/ml streptomycin (Life Technology, Rockville, MD). The cells were used between passages 3 and 8 to prevent change in cell phenotype. The cells were

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were plated at 1–2 × 10^5 cells/ml in 100 × 20-mm cell culture dishes (Falcon) in DMEM supplemented as described above and were grown subconfluently for 48–72 h. They were then made “quiescent” by incubation for 24 h in serum-free DMEM.

Measurement of DNA synthesis. To determine the effect of rHuEpo on DNA synthesis, we measured incorporation of the thymidine analog 5-bromo-2′-deoxyuridine (BrdU) using a commercial kit for detection of BrdU incorporation by ELISA (Cell Proliferation ELISA BrdU calorimetric; Roche Diagnostics, Tokyo, Japan). Briefly, cells were seeded at 5 × 10^4 cells on flat-bottomed 96-well plates (Falcon) in DMEM overnight for adequate attachment. DMSO was added to the control as a vehicle of Fsk. After treatment with medium containing Fsk for 36 h, 0.5 mg/ml MTT was added to all wells. The plates were then further incubated at 37°C for 4 h. The crystalline by-product was dissolved in acid-isopropanol (0.04 N HCl-isopropanol). Samples were read at a test wavelength of 570 nm and a reference wavelength of 650 nm on a multiwell plate reader (Behring ELISA Processor II). Background absorbance of the medium without the cells was subtracted. Cell viability is expressed as a percentage of that of the control.

Preparation of cell lysate and Western blotting. Cells were rinsed with ice-cold PBS and resuspended in 400 μl of lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 10% glycerol, 1 μM leupeptin, 1 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 2 mM sodium orthovanadate]. The protein concentration of the cell lysate was normalized by using the Bradford assay (5). The supernatants were then subjected to an immune complex protein kinase assay or total cell lysate immunoblotting.

After incubation with the primary antibody, the membranes were probed with a secondary antibody followed by detection with an enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Tokyo, Japan). The intensities of the bands were quantified by using a GT-9000 scanner (Epson, Tokyo, Japan), and densitometric analysis was performed using NIH Image 1.56 software. The results are expressed as relative activity of phosphorylated Elk-1, MEK, p44/p42 MAPK, and Raf-1 per total activity, divided by unstimulated (control) activity.

Statistics. The results are expressed as means ± SE. Data were analyzed by analysis of variance combined with Fisher’s protected least significant difference test. Differences with P < 0.05 are considered to be significant. Figures 1–8 are each representative of three experiments.

RESULTS

Effects of cAMP on rHuEpo-induced DNA synthesis. As shown in Fig. 1A, incubation of rHuEpo for 36 h
resulted in an increase in the amount of DNA synthesis. Pretreatment with Fsk (10^{-6} or 10^{-5} M) for 15 min suppressed the increase in the amount of DNA synthesis by rHuEpo. Coincubation of Fsk with Rp-cAMP triethylamine, a cAMP antagonist, canceled this suppression of DNA synthesis. Single administration of Fsk also reduced the amount of DNA synthesis compared with that of the control (Fig. 1 A). When we incubated VSMC with 1 μM Rp-cAMPS for 36 h alone, the reading at 450 nm/650 nm was 0.50 ± 0.01 (n = 8), and that of the control was 0.43 ± 0.01 (n = 8) (P < 0.01, 1 μM Rp-cAMPS vs. control). Another cAMP-producible agent, Cil, which is a type 3 phosphodiesterase inhibitor, had the same effect as Fsk (Fig. 1 B).

Viability of VSMC. Figure 2 shows the dependency of cell viability on the concentration of Fsk. No significant decrease in cell viability was caused by treatment with Fsk up to a concentration of 10^{-4} M. However, at a concentration of 10^{-3} M, the percentage of viable cells decreased significantly (78 ± 7.5%; P < 0.05) compared with that of the control. Because these results indicated that Fsk at a concentration of 10^{-3} M is not...
lethal for VSMC, we used Fsk at a concentration of $10^{-5}$ M in the subsequent experiments.

**Modulation of the activity and phosphorylation of MAPK and phosphorylation of MEK in VSMC.** Experiments were carried out to determine whether rHuEpo induces activation of p44/42 MAPK. As shown in Fig. 3A, rHuEpo stimulated phosphorylation of Elk-1, which is one of the substrates of p44/42 MAPK, in a time-dependent course. Peak activation was observed after 5 min of exposure to rHuEpo. On the other hand, its activation was inhibited by pretreatment with $10^{-5}$ M Fsk for 15 min, and this inhibition was canceled by Rp-cAMPS triethylamine (1 µM) (Fig. 3B and C).

We further examined p44/42 MAPK phosphorylation in VSMC. As shown in Fig. 4A, rHuEpo stimulated MAPK phosphorylation in a dose-dependent manner. Pretreatment with Fsk for 15 min inhibited rHuEpo-induced MAPK phosphorylation in a dose-dependent manner (Fig. 4B). This inhibitory effect continued for at least 30 min (Fig. 4C). Next, we investigated whether cAMP can also attenuate rHuEpo-induced MEK phosphorylation. Treatment with Fsk resulted in suppression of this phosphorylation in a dose-dependent manner (Fig. 5, A and B). Fsk had no effect on Raf-1 phosphorylation, but

We also investigated whether PKC inhibitors decrease rHuEpo-induced MEK or MAPK phosphorylation. Both staurosporine and calphostin C inhibit rHuEpo-stimulated MEK phosphorylation (Fig. 7, A and B) and MAPK phosphorylation (Fig. 7, C and D).

**Raf-1 phosphorylation at Ser259 by cAMP.** Mutation of Ser259 in Raf-1 has been reported (26) to result in a constitutively active kinase, suggesting that phosphorylation of Ser259 is inhibitory. Incubation with Fsk for 30 min resulted in augmentation of Raf-1 phosphorylation in a dose-dependent manner (Fig. 8, A and B). Fsk had no effect on Raf-1 phosphorylation, but

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**Fig. 5. Effect of Fsk on rHuEpo-induced MEK phosphorylation.** A: representative dose dependency of Fsk. After incubation with Fsk for 15 min, rHuEpo was added to the medium. Incubation time with rHuEpo is 5 min. B: densitometry for MEK phosphorylation. Conditions 1–5 in A correspond to data in B: n = 3 experiments for each condition. *P < 0.05 vs. condition 1. #P < 0.05 vs. condition 2. C: time course of incubation with or without Fsk.

**Fig. 6. Effect of Fsk on PMA-induced MAPK phosphorylation.** A: time course of PMA-stimulated MAPK phosphorylation. VSMC were starved for 24 h and stimulated with PMA for various periods. B: after VSMC were preincubated with or without Fsk, PMA was added. Incubation time with PMA was 5 min.
Fsk with rHuEpo caused a slight increase in Raf-1 phosphorylation (Fig. 8, C and D).

**DISCUSSION**

This study clearly demonstrates that cAMP inhibits the rHuEpo-induced Ras-Raf-MEK-MAPK pathway, suppressing the PKC-induced Raf-1 activation at least. It had previously been shown that Epo stimulates the MAPK pathway not only in erythroid progenitor cells and a human leukemia cell line (9, 37) but also in VSMC (1) and PC-12 cells (7).

The rHuEpo concentration used in the present study and our previous studies (2, 3, 21) is much higher than...
the plasma concentration with a therapeutic dose in renal anemia patients. The reasons why we should use such a relatively high concentration of rHuEpo are mentioned in our previous report (2).

One of the biological effects of the MAPK pathway is cell-proliferating action, but the precise mechanism is unclear in VSMC. Upon activation in a fibroblast cell line, MAPK translocates to the nucleus, where it phosphorylates transcription factors and induces early gene translocation (6). Activation of the MAPK pathway at the Go/G1 transition has been shown to induce expression of cyclin D (39). We also found that upregulation of cyclin D1 protein, which is an essential D-type cyclin in VSMC (11), is upregulated by rHuEpo in a time-dependent manner (data not shown).

The relation between inadequate upregulation of vascular MAPK activity and physiological vascular disturbance such as exacerbating hypertension or forming of atherosclerosis needs to be debated. The mechanisms underlying the regulation of activity of MAPK believed to be involved in the pathway for cell proliferation are altered in a hypertensive animal model (19, 35). Involvement of vascular MAPK activation in Epo-induced blood pressure rise has not been observed. However, because the hypertensive effects of rHuEpo are augmented in a genetic model of hypertension but not in Wistar-Kyoto rats (33), it is substantially beneficial to explore the relationship between the MAPK pathway and mechanisms of Epo-induced blood pressure rise by using an in vivo model.

The mechanisms of inhibition of MAPK by cAMP include three different pathways. Phosphorylation of Raf-1 directly by cAMP-activated protein kinase A (PKA) at two serine residues results in suppression of both Ras binding and Raf-1 activity. This mechanism is most suspected (12, 25). The other two pathways are 1) phosphorylation of Rap1 by PKA, which induces Rap1 GTP binding by an unknown mechanism, resulting in competition with Ras for the binding of Raf-1 and thereby blocking Ras-induced Raf-1 activation (38); and 2) inhibition of MAPK by cAMP via suppression of B-Raf activity by an as-yet-unknown mechanism (31). The latter two pathways have not been confirmed by using VSMC.

Epo mediates phosphorylation of Raf-1 (8), and this increase in enzymatic activity coincides with tyrosine phosphorylation of Raf-1 on residue Tyr341 (34). In VSMC, the characteristics of Raf-1 phosphorylation residues, which are phosphorylated by Epo, are not known. In our experiments, Fsk phosphorylated Raf-1 at Ser259 (Fig. 8), the phosphorylation of which is inhibitory (26). However, recent works have not revealed direct Ser259 phosphorylation by PKA. It is thought that another mechanism underlies this regulation.

Raf-1 activity is regulated by 14-3-3 protein (13), which is expressed in all eukaryotic organisms and is known to be a key regulator of cell division, signaling, and apoptosis. Its biological significance in VSMC or erythroid cells is still unclear. Both Ser3259 and Ser621 in Raf-1 protein are potential 14-3-3-binding sites (27).

Furthermore, Ser621 phosphorylation by PKA in the catalytic domain can inhibit catalytic activity (25). The coordination between Raf-1 and 14-3-3 protein through these serine residues is a regulatory point.

Raf-1 and MAPK are activated by Epo PKC-dependent, and PKCα controls Epo receptor signaling in erythroid progenitor cells (9, 22). PKCα is a novel (Ca2+ dependent) PKC, and its existence has already been confirmed (17). We therefore investigated the effects of Fsk on Epo-induced [Ca2+]i elevation, but a significant change in [Ca2+]i was not observed in VSMC (data not shown), suggesting that Raf-1 activation by Epo is regulated by Ca2+-independent PKC in VSMC.

The intracellular cAMP level was increased by addition of Epo in mouse erythroleukemia SKT6 cells (20) and BFU-E-derived erythroblasts (24) but not in Epo-dependent clone of DA-1 (DA-1ER) (36) and murine erythrocytoma cells (TSAS) (10). Epo may also have the ability to increase the intracellular cAMP level in other kinds of cells. We explored whether rHuEpo can increase the intracellular cAMP level in VSMC, and we could not induce alteration of cAMP (data not shown). Even if Epo can increase the cAMP level in other kinds of cells, the biological effect of cAMP in proliferation or differentiation stimulated by Epo in nonerythroid cells is still unknown. Regarding erythropoiesis, these discrepant outcomes imply that it is questionable whether the increase in intracellular cAMP by Epo is necessary in erythropoiesis or not.

In conclusion, cAMP inhibits the Epo-induced Raf-1-MEK-MAPK pathway by inhibiting PKC, resulting in suppression of Epo-stimulated DNA synthesis in rat VSMC.

We thank Yuko Watanabe for technical assistance. Part of the work presented in the original manuscript was presented in abstract form at the 34th Annual Meeting of the American Society of Nephrology (J Am Soc Nephrol 12: T1-273, 2001).

This study was supported in part by Grant-in-Aid 13671125 for scientific research from the Ministry of Education, Science and Culture, Japan, and by grants from Jinsihinketsu-kenkyuuukai and Jin-kenkyuuukai.

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