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Induction by staurosporine of hepatocyte growth factor production in human skin fibroblasts independent of protein kinase inhibition

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Abstract

Staurosporine is one of the most potent and well known inhibitors of protein kinases, and it is often used to study the involvement of protein kinases in signal transduction pathways. We now report that staurosporine can induce the production of hepatocyte growth factor (HGF) independently of protein kinase inhibition. Staurosporine markedly stimulated the production of HGF in various cell types, including human skin fibroblasts. Its effect was accompanied by up-regulation of HGF gene expression. The inhibition of protein kinases appears not to be involved in staurosporine-induced HGF production, because other protein kinase inhibitors, K-252a, H-7, GF 109203X and genistein, had no HGF-inducing activity. UCN-01, 7-hydroxystaurosporine, which differs from staurosporine only in its aglycone moiety, also showed HGF-inducing activity, and inactive K-252a differs from staurosporine only in its sugar moiety. These results indicate that the sugar moiety, a six-atom ring structure, is important in the HGF-inducing activity of staurosporine. Experiments were then carried out to determine whether the characteristics of staurosporine-induced HGF production have similarities to those of HGF production stimulated by other HGF inducers. The effect of staurosporine like that of 8-bromo-cAMP and that of cholera toxin was marked in human skin fibroblasts from all four different sources, whereas the effects of epidermal growth factor and phorbol 12-myristate 13-acetate were variable depending on cells. The net increase in HGF production induced by staurosporine was not reduced in protein kinase C-depleted human skin fibroblasts. Moreover, synergistic induction of HGF was detected between staurosporine and interferon-γ as well as between 8-bromo-cAMP and interferon-γ. Staurosporine, however, did not increase intracellular cAMP levels in human skin fibroblasts. These results indicate that staurosporine induced HGF in different cell types via a signaling pathway similar to the cAMP-mediated pathway without increasing cAMP levels.

Keywords: Hepatocyte growth factor; Staurosporine; UCN-01; Cyclic AMP; Induction; Human fibroblast

1. Introduction

HGF, also known as scatter factor, was initially reported and isolated as a mitogenic factor for adult rat hepatocytes in primary culture [1–7], but HGF has multiple functions, including mitogenic, motogenic, morphogenic, and tumor-inhibiting activities, and acts on many kinds of epithelial cells other than hepatocytes and on other types of cells (reviewed in ref. [8,9]). There is mounting evidence that HGF is a vital factor for liver regeneration. HGF is the most potent mitogen for rat hepatocytes reported to date: it markedly stimulates the proliferation of hepatocytes at less than one-tenth the molar concentrations of transforming growth factor-α and EGF [10]. When injected into normal and liver-injured animals, HGF stimulates liver growth and promotes regeneration of the liver [11–13]. Enhancement of liver growth by HGF has also been demonstrated in experiments using HGF gene transgenic mice [14]. Levels of HGF in the plasma and liver of rats that had been

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Abbreviations: C/EBP, CCAAT/enhancer-binding protein; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TK, tyrosine kinase; TNF, tumor necrosis factor.

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exposed to carbon tetrachloride or that had been partially hepatectomized increased markedly prior to liver regeneration [15,16]. These increases were accompanied by elevations of the mRNA levels of this factor in the liver, spleen, and lung [17–19]. In accordance with the increase in HGF levels, rapid down-regulation of the high-affinity HGF receptor in the plasma membranes of those rat livers occurred [20]. Moreover, continuous administration of neutralizing anti-HGF antibody to rats treated with carbon tetrachloride inhibited liver regeneration [21]. Elevated HGF in plasma and liver after hepatic injury may play a role in proliferation of hepatocytes. In addition, incomplete liver formation has been observed in HGF-deficient mice [22].

HGF is mainly produced by mesenchymal cells such as fibroblasts and smooth muscle cells [8,23]. HGF is induced by the activation of PKA- and PKC-mediated pathways [24–26]. Its production is also stimulated by IL-1, TNF-α, IFN-γ, estrogen, ascorbic acid, okadaic acid, norepinephrine, a scatter factor-inducing factor, IFN-inducible protein-10, and growth factors such as EGF and basic fibroblast growth factor whose receptors have TK activities [27–35]. Thus, different protein kinases are involved in the signaling pathways for HGF induction. When we examined the effects of various protein kinase inhibitors on HGF induction, we unexpectedly found that staurosporine, a glycosylated indolo [2,3-*a*] carbazole alkaloid with a potent and broad-spectrum inhibitory activity for protein kinases [36], alone markedly stimulated HGF production in human skin fibroblasts. This effect of staurosporine was not common to other inhibitors of protein kinases. In this report, we describe the characteristics of staurosporine-induced HGF production.

2. Materials and methods

2.1. Materials

Eagle's minimum essential medium, Dulbecco's modified Eagle's medium, and RPMI 1640 medium were purchased from Nissui Pharmaceutical Co. Staurosporine was obtained from Kyowa Medex. K-252a, K-252b, KT5720, GF 109203X, SB 203580, and PD 98059 were from Calbiochem-Novabiochem. H-7 and genistein was from Seikagaku Kogyo Co. and Extrasynthese S. A., respectively. SP 600125 was from BIOMOL Research Laboratories. Natural human IFN-γ and recombinant human IL-1β were from Hayashibara Biochemical Laboratories Inc. and R&D Systems, respectively. Anti-phospho-p38 MAPK and anti-phospho-ERK1/ERK2 (p44/42 MAPK) antibodies were from Cell Signaling Technology. Anti-nonphospho-p38 MAPK and anti-nonphospho-ERK1 (p44 MAPK) antibodies were from Santa Cruz Biotechnology. [α-^{32}P]dCTP (∼110 TBq/mmol) was from Amersham Biosciences, and cAMP radioimmunoassay kits were from Yamasa Shoyu Co. UCN-01 was supplied by Kyowa Hakko Kogyo Co. Human HGF cDNA (*Bam*HI/*Kpn*I fragment, 2.2 kbp) was derived from plasmids originally obtained from Dr. Naomi Kitamura (Tokyo Institute of Technology). Other reagents were obtained from the described sources [25].

2.2. Cell culture

Normal human skin fibroblasts (NB1RGB) isolated from a baby (male, 3 days old) and obtained from the Riken Cell Bank were used between the 5th and 10th passages. In addition, normal human skin fibroblasts isolated from a 4-month-old girl [25], an 8-year-old boy [25], and 200 individual neonatal donors (Cell Systems) were used between the 7th and 12th passages, 8th and 10th passages, and 7th and 10th passages, respectively. MRC-5 human embryonic lung fibroblasts were obtained from the Riken Cell Bank. All fibroblastic cells except skin fibroblasts from neonatal donors were grown as monolayers in Eagle's minimum essential medium supplemented with 10% FBS and 2 mM L-glutamine at 37° in a humidified atmosphere of 5% CO_2 and 95% air, as described previously [26]. Skin fibroblasts from neonatal donors were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. KG-1 human leukemia cell line, provided by Fujisaki Cell Center, Hayashibara Biochemical Laboratories Inc., was cultured in RPMI 1640 supplemented with 5% heat-inactivated FBS and 2 mM L-glutamine [37].

2.3. Determination of HGF levels in the conditioned media

The human skin fibroblasts and MRC-5 cells, trypsinized and suspended in the medium described in the previous section, were seeded in 24- or 96-well plates (Nunc) at a density of 1.8×10^4 cells/cm^2 (1 or 0.17 mL/well, respectively). After reaching confluence, the medium was replaced with the same fresh medium. Staurosporine and other HGF inducers were then added. KG-1 cells (5×10^5 mL^{-1}), suspended in the medium described in the previous section, supplemented with 100 U/mL penicillin and 50 μg/mL streptomycin, were seeded in the 24-well plates, and staurosporine and other HGF inducers were added 1 hr later. The conditioned medium was collected after incubating the cells for 72 hr (human skin fibroblasts and KG-1 cells) or 24 hr (MRC-5 cells), unless stated otherwise, and was frozen at −30° for a human HGF ELISA. The sandwich ELISA for human HGF was performed at room temperature as described previously [38], with a slight modification [28]. The standard curve for human HGF was linear within the range of 0.025–5.0 ng/mL. HGF levels were expressed as ng/mg cellular protein as described previously [25]. Cellular protein was determined

as described previously [25]. All results are expressed as means and SD of several independent experiments.

2.4. Northern blot analysis

The medium of confluent human skin fibroblasts grown in 9-cm dishes (Nunc) was replaced with the same fresh medium and the cells were incubated for 24 hr. Staurosporine was then added without a medium change. After incubating for an appropriate period, total RNA was isolated from the cells using RNA-Bee (TEL-TEST). Northern blotting was performed as described previously [25]. Briefly, total RNA (10 μg) was denatured with 2.2 M formaldehyde and 50% formamide, fractionated on 1% agarose gels containing 2.2 M formaldehyde, and transferred to a Biodyne nylon membrane. The membrane-bound RNA was hybridized to a ^{32}P-labeled human HGF cDNA probe. After being washed, the membrane was exposed to an imaging plate at room temperature, and the plate was analyzed using a Bio-imaging analyzer, BAS-2000 (Fuji Photo Film Co.). The membranes were rehybridized with a GAPDH cDNA probe as an internal control. The signal intensity of the 6.8-kb HGF mRNA band was normalized to the reference gene GAPDH and expressed as fold-change relative to the control cultures, which were not incubated. The human HGF and GAPDH cDNA fragments were labeled with [α-^{32}P]dCTP by the megaprime DNA labeling system (Amersham Biosciences) according to the manufacturer's instructions.

2.5. Western blotting

The medium of confluent human skin fibroblasts grown in 6-well plates (Nunc) was replaced with the same fresh medium and the cells were incubated for 15 hr. Staurosporine or IL-1β was then added without a medium change. After incubating for an appropriate period, cells were washed once with ice-cold PBS, scraped into PBS, and washed three times more with PBS. Cells were then lysed by adding 100 μL of SDS sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% Bromophenol Blue). Lysates were boiled for 10 min, briefly sonicated, and centrifuged. Protein in extracts was determined by a modification [39] of the method of Lowry *et al.* [40]. Equivalent protein aliquots were separated by 10% SDS–PAGE, transfered electrophoretically to Immobilon-P transfer membranes (Millipore), and probed with anti-phospho-p38 MAPK antibody or anti-phospho-ERK1/ERK2 antibody. Blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences) and detected with the ECL Plus Western blotting detection reagents (Amersham Biosciences). The blots were then reprobed with anti-nonphospho-p38 MAPK antibody or anti-nonphospho-ERK1 antibody. Densitometric analysis of the bands was performed using UMAX PowerLook II flat-bed scanner (UMAX Data Systems) and Intelligent Quantifier software (Bio Image Systems). The signal intensity of the phosphorylated p38 MAPK and ERK bands was normalized to the total p38 MAPK and ERK, respectively, and expressed as fold-change relative to the control cultures, which were not treated with either staurosporine or IL-1β.

2.6. cAMP determination

Confluent human skin fibroblasts, cultured and exposed to staurosporine and cholera toxin for an appropriate period in 24-well plates (Nunc) as described above, were washed twice with cold PBS, and 0.2 mL of cold 0.1 N HCl was added. After 20 min on ice, HCl extracts of cells were collected and cAMP was assayed using Yamasa cAMP kits. Cellular protein was determined as described previously [25].

2.7. Data analysis

The data was analyzed by Dunnett's multiple comparison test or unpaired Student's *t*-test. *P* values less than 5% were regarded as significant. The IC_{50} values were calculated by linear regression analysis.

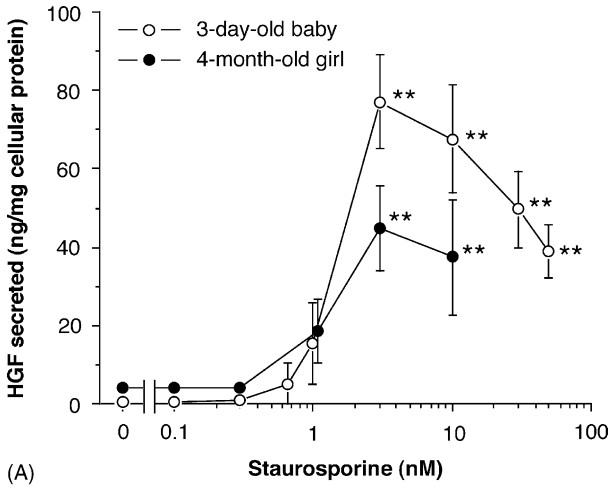
3. Results

3.1. Induction of HGF in human skin fibroblasts and other cells by staurosporine

Human skin fibroblasts from three different sources were incubated for 72 hr with various doses of staurosporine, and the amounts of HGF secreted into the media were measured by an HGF ELISA. Staurosporine increased HGF production in all these types of fibroblasts in a dose-dependent manner (Fig. 1A and B). Its effect was half-maximal at concentrations of 1.0–1.5 nM and maximal at 3–10 nM, showing more than 9-fold stimulation. Staurosporine induced higher levels of HGF production in cells from neonatal donors than those in cells from other origins. The stimulating effect of staurosporine was minimal at 24 hr of incubation, but marked at 48 and 72 hr (Fig. 2). This time-course was similar to that of fibroblasts treated with 8-bromo-cAMP.

Fig. 3 shows the HGF mRNA levels in human skin fibroblasts incubated for various periods with staurosporine. The levels increased as early as 10 hr after the addition of staurosporine. Staurosporine caused about 10-fold increase at 24 and 36 hr.

The effect of staurosporine was compared with the effects of other known HGF inducers in human skin fibroblasts from four different sources (Fig. 4). The dose of each inducer employed was the optimal one. The effect of staurosporine was comparable to or more potent than



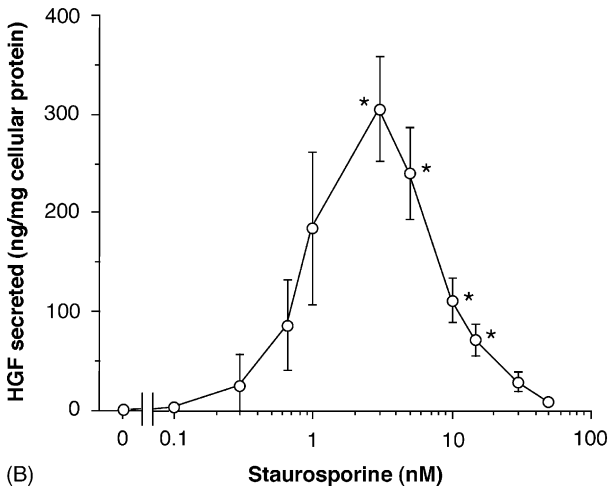


Fig. 1. Dose–response of staurosporine-induced HGF production in human skin fibroblasts. Confluent cells isolated from a 3-day-old baby and from a 4-month-old girl (A) and neonatal donors (B) were incubated for 72 hr with the indicated concentrations of staurosporine. The HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three–four independent experiments. Where SD bars are not shown, the SD was smaller than the symbol. Values that are significantly different from those of the control are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

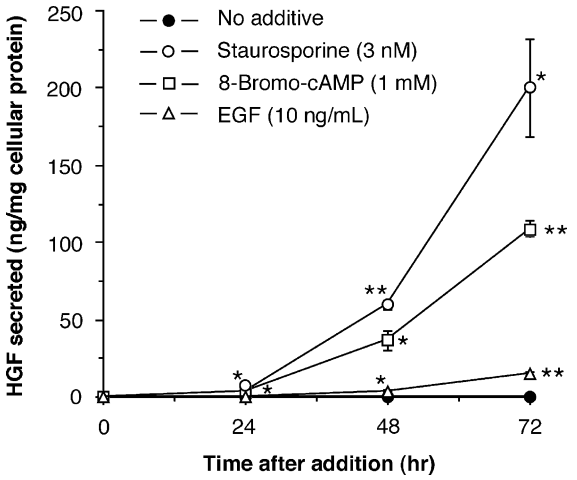


Fig. 2. Time-course of staurosporine-induced HGF production in human skin fibroblasts. Confluent cells isolated from neonatal donors were incubated for the indicated periods with various additives. The HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three independent experiments. Where SD bars are not shown, the SD was smaller than the symbol. Values that are significantly different from those of the control are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

that of cholera toxin in fibroblasts from all four sources and was comparable to that of 8-bromo-cAMP in cells from three sources. The HGF production-inducing effects of EGF and PMA were variable among fibroblasts from four sources. Staurosporine induced HGF more or less markedly than did EGF and PMA depending on cells. The levels of HGF production induced by staurosporine and 8-bromo-cAMP decreased with the age of donors: the amount of HGF produced by the two inducers in cells from neonatal donors was largest (Fig. 4D) and that in cells from an 8-year-old donor was smallest (Fig. 4C). The effect of staurosporine on HGF production in other types of cells was next examined. Staurosporine also stimulated HGF production in MRC-5 cells (data not shown) and in the KG-1 leukemia cell line (Fig. 5).

3.2. HGF induction by staurosporine in a manner independent of protein kinase inhibition

Since staurosporine is known to inhibit a broad range of protein kinases, including PKC, PKA, and TKs [36], the effects of various protein kinase inhibitors on HGF production in human skin fibroblasts were examined. Neither K-252a (1–300 nM), a broad-spectrum inhibitor of protein kinases, H-7 (1–30 μM), an inhibitor of PKC and PKA, GF 109203X (10–1000 nM), a PKC inhibitor, nor genistein (1–30 μM), a TK inhibitor, induced production of HGF (data not shown). We confirmed that these protein kinase inhibitors actually inhibited activities of protein kinases in the cells (Table 1). In these experiments, we employed PMA-induced HGF production,

Table 1
IC_{50} values for the inhibition of protein kinases in the cells by protein kinase inhibitors

Protein kinase inhibitor	IC_{50} (μM)		
	PKC	PKA	TK
Staurosporine	0.017	–	–
K-252a	0.017	0.018	0.0045
H-7	24	17	26
GF 109203X	0.20	–	–
Genistein	–	–	4.5

For estimation of IC_{50} values for the inhibition of protein kinases, confluent cells from neonatal donors were pre-incubated for 2 hr with or without various doses of the indicated protein kinase inhibitors and then incubated for a further 24 hr (PKC) or 72 hr (PKA and TK) with or without 30 nM PMA (PKC), 1 mM 8-bromo-cAMP (PKA), or 10 ng/mL of EGF (TK) in the presence or absence of the protein kinase inhibitors. The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments.

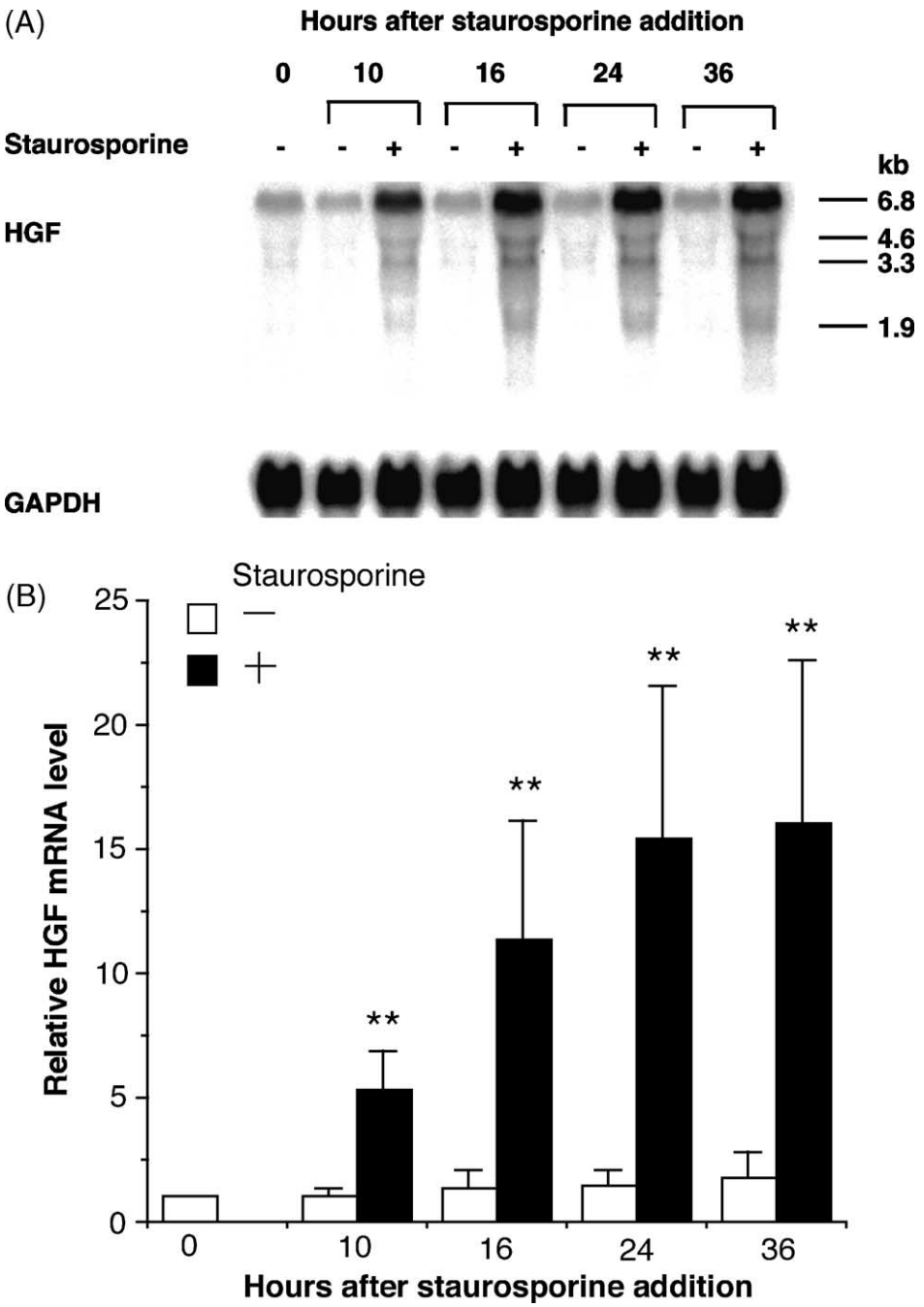


Fig. 3. Up-regulation of HGF gene expression by staurosporine in human skin fibroblasts. Confluent cells isolated from neonatal donors were incubated with or without 3 nM staurosporine for the indicated periods. Total RNA was isolated and Northern blotted using ^{32}P-labeled cDNA probes for human HGF and then for human GAPDH. Autoradiographs (A) are representative of five independent experiments. The data (B) are means of five independent experiments. Bars indicates SD. Values that are significantly different from those of cultures incubated with the medium alone are indicated by $^{**}P < 0.01$ (Student's *t*-test).

8-bromo-cAMP-induced HGF production, and EGF-induced HGF production as PKC-, PKA-, and TK-mediated responses, respectively. In addition, neither K-252b (1–300 nM), a broad-spectrum protein kinase inhibitor, nor KT5720 (10–400 nM), a PKA inhibitor, was effective (data not shown). These results suggest that inhibition of protein kinases is not the reason for staurosporine-induced HGF production.

3.3. Structural requirement of staurosporine for HGF induction

The chemical structural region(s) of staurosporine required for HGF induction was next determined. Staurosporine has a pyranose-like ring (sugar moiety) linked to two nitrogen atoms belonging to an indolo [2,3-*a*] carbazole skeleton (aglycone moiety). Inactive K-252a,

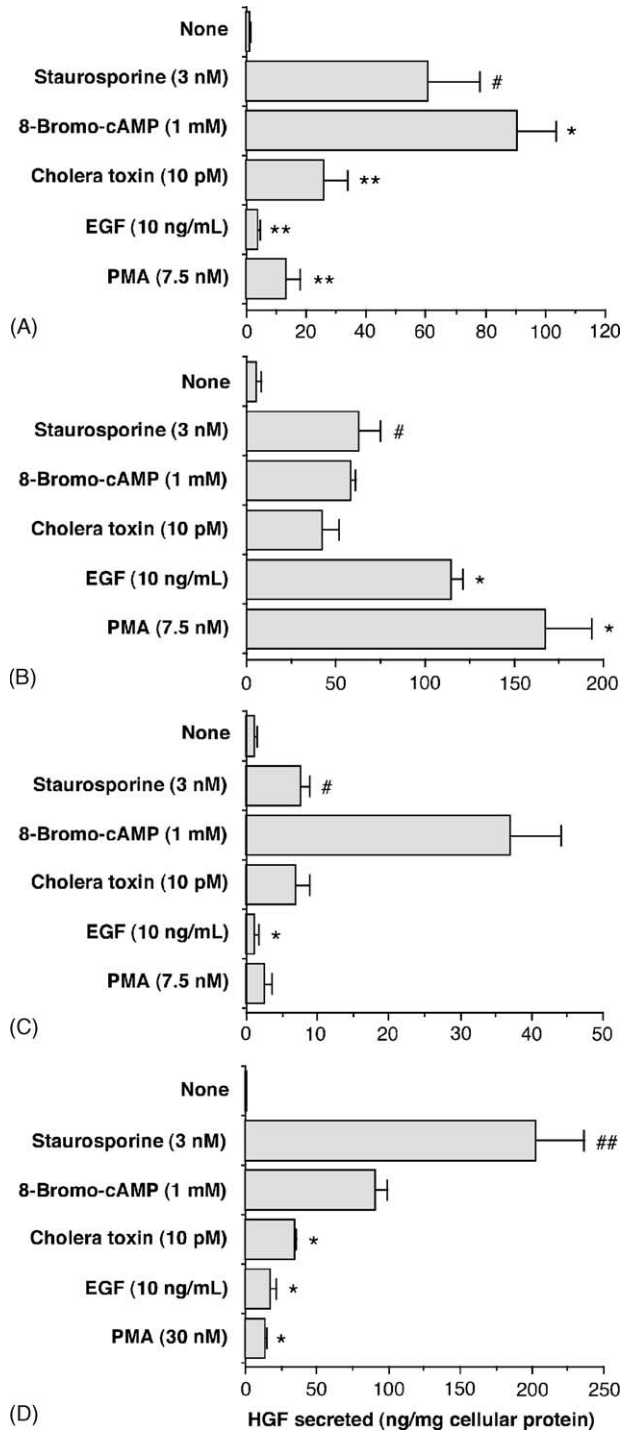


Fig. 4. Effects of staurosporine and other HGF inducers on HGF production in human skin fibroblasts from four different sources. Confluent cells isolated from a 3-day-old baby (A), a 4-month-old girl (B), an 8-year-old boy (C), and neonatal donors (D) were incubated for 72 hr with or without staurosporine and other HGF inducers. The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Where SD bars are not shown, the SD was too small to draw. Values that are significantly different from those of the cells treated with staurosporine are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, as compared with the control (Student's *t*-test).

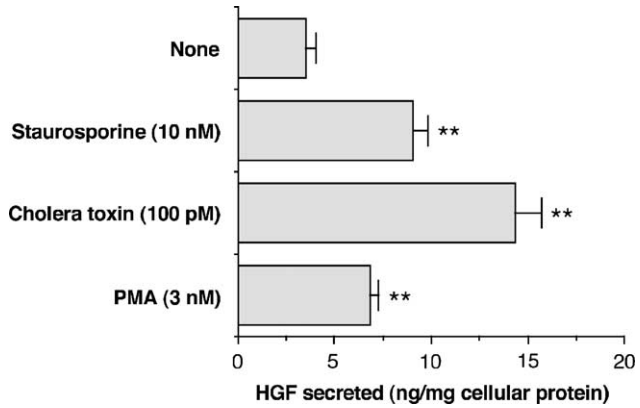


Fig. 5. Effects of staurosporine and other HGF inducers on HGF production in KG-1 human leukemia cells. KG-1 cells were incubated for 72 hr with or without staurosporine and other HGF inducers. The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of the control are indicated by $^{**}P < 0.01$ (Dunnett's test).

K-252b, and KT5720, all have structures very similar to that of staurosporine, the only difference being in their sugar moiety: the former three have a five-atom ring instead of a pyranose-like one. On the other hand, UCN-01 is 7-hydroxystaurosporine which has a sugar moiety identical to that of staurosporine but differs from staurosporine in its aglycone moiety. UCN-01 stimulated HGF production at concentrations 10 times higher than that of staurosporine, and its maximal effect was comparable to that of staurosporine (Fig. 6). These results indicate that a six-atom ring structure is important for staurosporine- and UCN-01-induced HGF production.

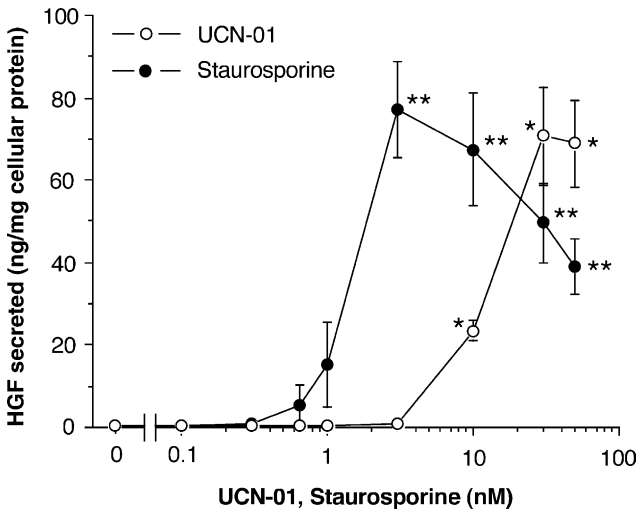


Fig. 6. Effect of UCN-01 on HGF production in human skin fibroblasts. Confluent cells isolated from a 3-day-old baby were incubated for 72 hr with the indicated concentrations of UCN-01 and staurosporine. The HGF secreted into the medium was measured by an ELISA. The data are means ± SD of three independent experiments. Where SD bars are not shown, the SD was smaller than the symbol. Values that are significantly different from those of the control are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

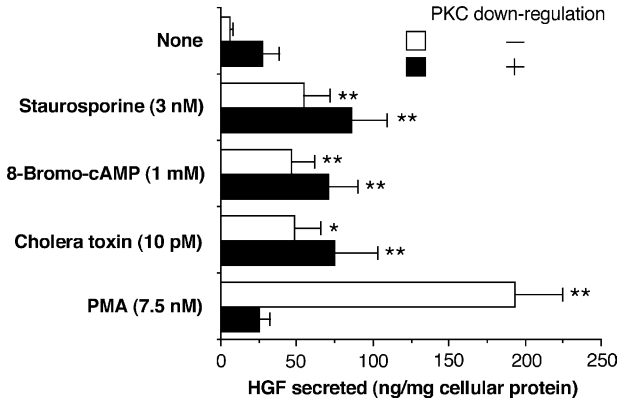


Fig. 7. Effect of PKC down-regulation with PMA on HGF production induced by staurosporine and other HGF inducers in human skin fibroblasts. Confluent cells from a 4-month-old girl were pretreated for 24 hr with or without 0.5 μM PMA. The cells were then washed and incubated with the indicated additives for an additional 72 hr in the presence or absence of 0.5 μM PMA. The HGF secreted into the medium was measured by an ELISA. The data are means of five independent experiments. Bars indicate SD. Values that are significantly different from those of control cultures with no inducer are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

3.4. Similarities between staurosporine-stimulated HGF production and HGF production stimulated by other inducers

HGF is induced by different intracellular signaling pathways: its production in human skin fibroblasts is stimulated by cAMP- and phorbol ester-mediated pathways, IL-1, TNF-α, IFN-γ, and various growth factors. To better understand the molecular mechanism(s) underlying staurosporine-induced HGF production, we compared the characteristics of staurosporine-induced HGF production with those of HGF production induced by phorbol ester, cAMP, IL-1 and other cytokines. As stated above, the effect of staurosporine as well as the effects of cholera toxin and 8-bromo-cAMP were marked in the fibroblasts from all four sources, whereas the effects of EGF and PMA were variable depending on cells: the effects of EGF and PMA were more potent in cells from one source but less potent in cells from the other three sources than those of staurosporine (Fig. 4). The net increase in HGF production induced by staurosporine was not reduced in the cells pretreated with a high dose (0.5 μM) of PMA in order to deplete PKC (Fig. 7). We reported previously that IFN-γ synergistically enhanced the 8-bromo-cAMP- and cholera toxin-induced HGF production in human skin fibroblasts in contrast to its inhibiting effect on EGF- and PMA-induced HGF production [41]. Synergy was also observed with staurosporine- and IFN-γ-mediated induction of HGF (Fig. 8). The stimulation of HGF production by IL-1 [28] and IFN-γ [28] and TNF-α (data not shown) in human skin fibroblasts was much less potent than that by cholera toxin. Thus, the characteristics of staurosporine-induced HGF production resemble those of HGF production stimulated by cAMP agonists but not those of HGF production stimulated by other HGF inducers. Fig. 9 shows intracellular cAMP levels in cells exposed to staurosporine. Although cAMP levels were markedly elevated after treatment of cells with 10 pM cholera toxin, they did not significantly change after staurosporine treatment.

Lindroos *et al.* [42] recently reported that staurosporine-induced up-regulation of platelet-derived growth factor receptor (PDGFR)-α gene in rat pulmonary myofibroblasts is due, at least in part, to activation of p38 MAPK. A recent study of Xiao *et al.* [43] showed that staurosporine-induced production of macrophage inflammatory protein-2 in rat

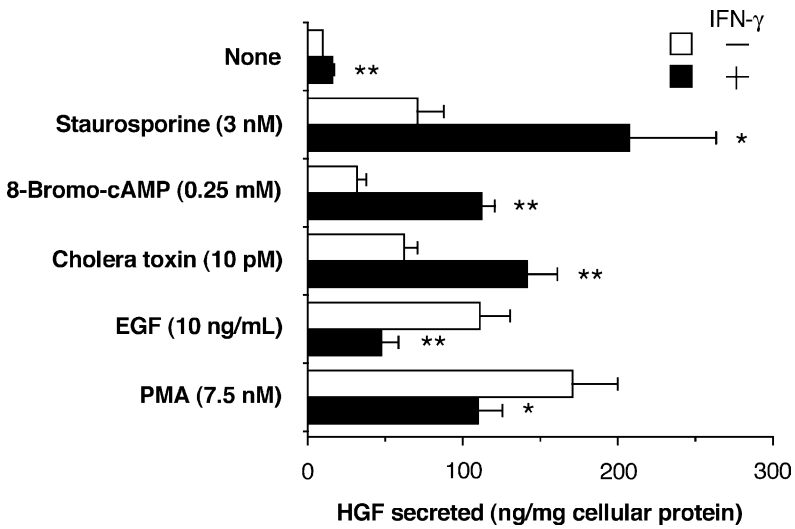


Fig. 8. Effect of IFN-γ on HGF production stimulated by staurosporine and other HGF inducers in human skin fibroblasts. Confluent cells from a 4-month-old girl were incubated for 72 hr with the indicated concentrations of staurosporine and other HGF inducers in the presence and absence of 100 U/mL of IFN-γ. The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Where SD bars are not shown, the SD was too small to draw. Values that are significantly different from those of the HGF inducers alone are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

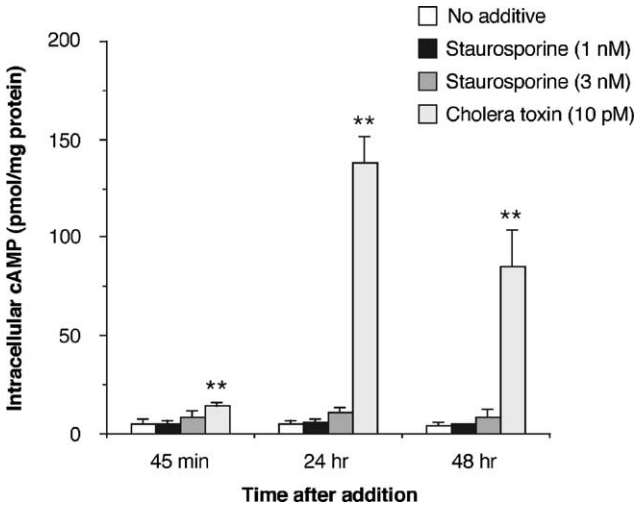


Fig. 9. Effect of staurosporine on intracellular cAMP levels in human skin fibroblasts. Confluent cells from a 4-month-old girl were incubated for the indicated periods with various additives. The intracellular cAMP extracted was measured by a radioimmunoassay. The data are means of four independent experiments. Bars indicate SD. Where SD bars are not shown, the SD was too small to draw. Values that are significantly different from those of the control are indicated by $^{**}P < 0.01$ (Dunnett's test).

peritoneal neutrophils is dependent on the activation of both p38 MAPK and ERK. In order to test an involvement of p38 MAPK and ERK pathways in staurosporine-induced HGF production, we used specific MAPK inhibitors. The inhibitor of p38 MAPK SB 203580 inhibited staurosporine (3 nM)-induced HGF production by 49 and 58% at 1 and 5 μM, respectively, whereas neither the ERK kinase inhibitor PD 98059 (12.5 and 25 μM) nor the c-Jun NH_2-terminal kinase (JNK) inhibitor SP 600125 (5 and 10 μM) inhibited it (Fig. 10). However, phosphorylation of

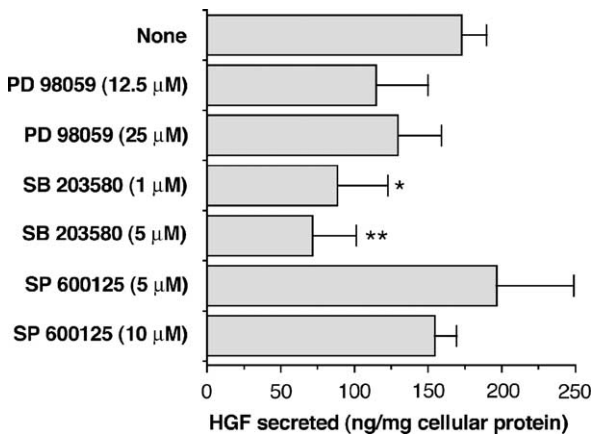


Fig. 10. Effects of MAPK inhibitors on staurosporine-induced HGF production in human skin fibroblasts. Confluent cells from neonatal donors were pre-incubated for 2 hr with the indicated dose of MAPK inhibitors and then incubated for 72 hr with 3 nM staurosporine in the presence or absence of the MAPK inhibitors. The HGF secreted into the medium was measured by an ELISA. The data are means of three independent experiments. Bars indicate SD. Values that are significantly different from those of staurosporine alone are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ (Dunnett's test).

p38 MAPK was not increased by staurosporine (3 nM) in human skin fibroblasts during 24-hr incubation, although under the same conditions IL-1β markedly induced phosphorylation of p38 MAPK at 15 min of incubation (Fig. 11A and B). Staurosporine did not induce phosphorylation of either ERK1 or ERK2 in human skin fibroblasts (data not shown). In contrast, EGF-induced HGF production was potently inhibited by PD 98059 [31] and SP 600125 (data not shown).

4. Discussion

We have demonstrated that staurosporine is a potent inducer of HGF production in different cell types, including human skin fibroblasts and KG-1 human leukemia cells. Staurosporine is a highly potent but nonselective inhibitor of protein kinases [36], inhibiting PKC, PKA, myosin light-chain kinase, several receptor-specific TKs, cdc2 kinase, and others. Amongst them, PKC is the most sensitive. The inhibition of PKC, however, appears not to be involved in the staurosporine-induced HGF production, because the more selective PKC inhibitor GF 109203X did not induce production of HGF. Other inhibitors of serine/threonine protein kinases and TKs tested, including H-7, K-252a and genistein, were all ineffective in inducing HGF production, although they actually inhibited activities of protein kinases in the cells. Therefore, it seems likely that the stimulation of HGF production by staurosporine is not due to its inhibitory effects on protein kinases. The ineffectiveness of K-252a, whose structure differs from that of staurosporine only in a sugar moiety, and the less potent but significant induction of HGF by 7-hydroxystaurosporine, UCN-01, indicate that the six-atom ring structure of the sugar moiety is important for induction of HGF. It is noteworthy that the half-maximal dose of UCN-01 required for HGF induction was 10-fold higher than that of staurosporine, whereas the IC_{50} values of the former for inhibition of PKC-α, -β, and -γ are lower than those of the latter [44].

The characteristics of staurosporine-induced HGF production are similar to those of cAMP agonist-induced HGF production. Staurosporine and the cAMP agonists 8-bromo-cAMP and cholera toxin showed a potent and constant HGF production-inducing effect in skin fibroblasts from all four different sources, while the effects of EGF and PMA varied depending on cells. Staurosporine is an effective inhibitor of the EGF-stimulated receptor TK [45]. Staurosporine and the cAMP agonists showed synergistic HGF production-inducing effects with IFN-γ, whereas IFN-γ inhibited EGF- and PMA-induced HGF production. The intracellular cAMP levels, however, did not change in the cells after the addition of staurosporine. There are conflicting reports regarding the effect of staurosporine on intracellular cAMP levels: it does not alter the intracellular cAMP levels in the JAR human placental

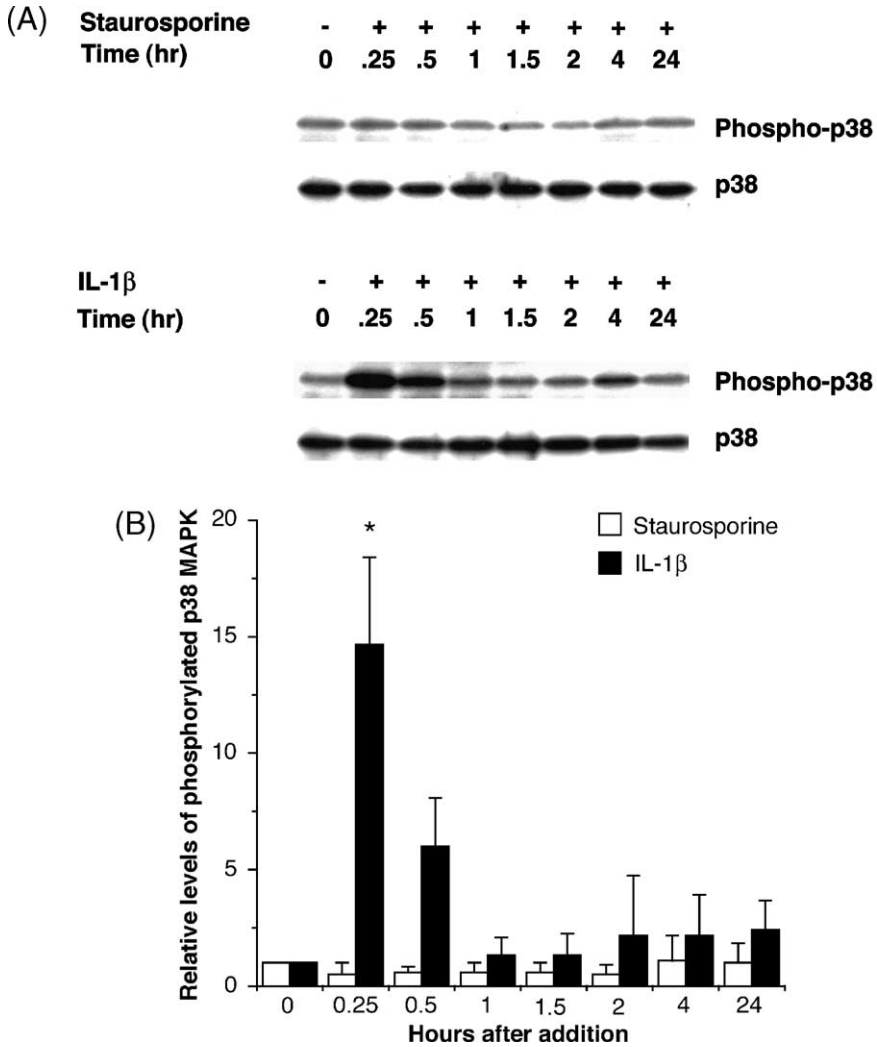


Fig. 11. Effect of staurosporine on the levels of phosphorylated p38 MAPK in human skin fibroblasts. Confluent cells isolated from neonatal donors were incubated with or without staurosporine (3 nM) and IL-1β (0.1 ng/mL) for the indicated periods. Equal amounts of cell extracts (10–20 μg) were subjected to SDS–PAGE and immunoblotting with anti-phospho-p38 MAPK antibody or anti-nonphospho-p38 MAPK antibody. Immunoblots (A) are representative of four independent experiments. The data (B) are means of four independent experiments. Bars indicates SD. Values that are significantly different from those of untreated cultures (0 hr) are indicated by $^{*}P < 0.05$ (Dunnett's test).

choriocarcinoma cell line [46], but agonist-induced elevation of the cellular cAMP content in the human leukemic T-cell line Jurkat is augmented by staurosporine [47]. Therefore, induction of HGF by staurosporine is mediated by a cAMP-independent signaling pathway. However, this does not rule out the possibility that the cAMP-independent signaling pathway for the effect of staurosporine and the cAMP-dependent signaling pathway converge at some subsequent point, leading to induction of HGF production, for example at the step of activation of a transcription factor important for HGF gene expression or a *cis*-acting element in the promoter of HGF gene.

There have been other examples whose expressions or activations are induced by both staurosporine and cAMP agonists. Those include CD14, cyclooxygenase-2, and C/EBPβ [48–52]. The induction of CD14 expression on bone marrow granulocytes by cAMP analogues, however, is independent of intracellular cAMP, because the adenylate cyclase activator forskolin does not induce CD14 expression despite increasing intracellular concentrations of cAMP. Involvement of a trypsin-sensitive unconventional purinoreceptor is suggested as a mechanism of its induction. On the other hand, HGF production is induced by forskolin as well as cAMP analogues and cholea toxin, and the induction is dependent on intracellular cAMP [25].

The precise nature of the signaling pathway involved in staurosporine-induced HGF production remains to be elucidated. Jiang and Zarnegar [53] have shown that the mouse HGF gene is up-regulated by induction of DNA-binding activities of C/EBPβ and C/EBPδ and by increases in their protein levels in NIH 3T3 fibroblasts exposed to some cytokines such as TNF-α, IL-6, and EGF. It has been reported that both staurosporine and forskolin increase the activity of C/EBPβ in vascular smooth muscle cells as described above [52]. Recent studies have also shown that both the expressions of C/EBPβ and C/EBPδ and their

DNA-binding activities are intensified by cAMP signaling in several cells, including neurons and osteoblasts [54,55]. Thus, it is conceivable that the activation and/or induction of C/EBPβ and C/EBPδ are involved in the staurosporine-induced HGF production. Functional studies of the human HGF gene promoter should facilitate the elucidation of the molecular mechanism by which staurosporine stimulates HGF production.

Recent study showed an involvement of p38 MAPK and both p38 MAPK and ERK in staurosporine-induced production of PDGFR-α and macrophage inflammatory protein-2, respectively [42,43]. Staurosporine-induced HGF production was inhibited more than 50% by an inhibitor of p38 MAPK, SB 203580, but we could not detect any increase in the levels of phosphorylated p38 MAPK and phosphorylated ERK in staurosporine-treated human skin fibroblasts. Moreover, IL-1β and TNF-α which cause the activation of p38 MAPK had minimal effect on HGF production in human skin fibroblasts [28]. We do not know the exact reason for this discrepancy at present. However, one possible explanation is that SB 203580 might inhibit a protein kinase(s) or enzyme(s) other than p38 MAPK which is involved in staurosporine-induced HGF production, although the high degree of specificity of SB 203580 is indicated by its failure to affect the activities of a number of other protein kinases [56]. In fact, it has recently been reported that SB 203580 inhibits cyclooxygenase-1 and -2 with approximate IC_{50} values of 2 μM [57]. Further work is needed to determine the precise role of p38 MAPK in staurosporine-induced HGF production.

The levels of HGF production induced by staurosporine and 8-bromo-cAMP in human skin fibroblasts decreased with the age (from neonatal to 8 years old) of donors. A recent study of Miyazaki *et al*. [58] showed that 8-bromo-cAMP-treated human skin fibroblasts from aged donors (over 80 years) produced more HGF than those from young (8 months old and 12 years old) and middle-aged donors. Thus, it seems likely that human skin fibroblasts from neonatal and aged donors are capable of producing higher amount of HGF than human skin fibroblasts from donors of other ages when appropriately stimulated. Human embryonic lung fibroblast strains such as MRC-5 and IMR-90 produce a high amount of HGF without any inducers [23].

Injection of HGF is effective for treating animal models of chronic hepatic and renal diseases such as hepatic and renal fibrosis and liver cirrhosis [59,60]. HGF inducers, especially low-molecular weight ones, may also be useful as therapeutic agents for these diseases. Although staurosporine has a potent stimulating effect on HGF production, it also blocks transition of the cell cycle and inhibits cell proliferation [36]. This may be due to the inhibition of cdc2 kinase and other cell cycle-related kinases [36]. Nevertheless, staurosporine provides a structural basis for the development of more selective HGF inducers that are capable of discriminating both activities. Staurosporine derivatives that have a potency similar to that of staurosporine for the induction of HGF but not for the inhibition of protein kinases would be useful as therapeutic agents for hepatic and renal diseases.

In conclusion, this study demonstrated that the protein kinase inhibitor staurosporine markedly stimulated HGF production in different cell types. Our results suggest that this effect of staurosporine is not due to its inhibitory effects on protein kinases but is mediated by a signaling pathway similar to the cAMP-mediated pathway without an increase in cAMP levels.

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