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Close association of p38 and JNK with plasminogen-dependent upregulation of PAI-1 in rat astrocytes in vitro

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ABSTRACT

As reported previously, stimulation of astrocytes with plasminogen (PLGn) remarkably enhances their production/release of plasminogen activator inhibitor-1 (PAI-1). In addition, both p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK) are activated in these astrocytes. However, it remains to be determined whether the MAPK activation is associated with the PAI-1 induction in PLGn-stimulated astrocytes. In the present study, we investigated the relationship between MAPK activity and PAI-1 induction in PLGn-stimulated astrocytes. PLGn stimulation led to definitive phosphorylation of three MAPKs: external signal regulated kinase (ERK), JNK and p38. These results suggest that all of these MAPKs, either alone or in combination, are involved in PAI-1 induction. To verify this association, an inhibition experiment was carried out by using inhibitors specific for each MAPK. The results of the immunoblotting analysis indicated that 20 μM SB203580 (the p38 inhibitor) or SP600125 (the JNK inhibitor) suppressed approximately 85% or 40% of PLGn-inducible PAI-1, respectively. Only 20% inhibition was achieved by pretreatment of astrocytes with 20 μM PD98059 (the inhibitor of MEK1/2, an upstream kinase of ERK). In conclusion, p38 and JNK were shown to be the major MAPKs involved in the signaling cascade leading to PAI-1 induction in astrocytes stimulated with PLGn.

The plasmin-generating system, including plasminogen (PLGn) and plasminogen activator, has been shown to be involved in not only extracellular proteolysis but also neuronal migration [8], neurite outgrowth [4] and tissue remodeling [11] in the nervous system. In mammalian tissues, two molecular forms of plasminogen activator are present: urokinase-type plasminogen activator and tissue-type plasminogen activator. These plasminogen activators have been detected in both neurons and glial cells in vitro. The activity of plasminogen activator is regulated through plasminogen activator inhibition by a member of the serpin family, plasminogen activator inhibitor (PAI) [2]. PAI is grouped to PAI-1, PAI-2 and PAI-3. The PAI-1 protein is expressed in both neurons and glial cells, and PAI-2 is known to be present in microglia. If the levels of PAIs increase, the activities of plasminogen activators decrease, which finally results in the downregulation of PLGn activation. The inactive precursor of plasmin PLGn is produced and secreted from microglia [12]. Thus, plasmin activity could be regulated through the cellular interaction between glial cells and/or between glial cells and neurons. In fact, we have previously shown that microglia-producible PLGn has the biological activity to enhance PAI-1 level in astrocytes in vitro, suggesting that plasmin generation in the extracellular space is regulated by microglia and astrocytes. Furthermore, we have suggested that the PLGn-dependent PAI-1 induction may be associated with activation of two mitogen-activated protein kinases (MAPKs) in astrocytes [5]. However, the relationship between the PAI-1 induction and the MAPK activations remains unclear. Therefore, in this study, we investigated the involvement of MAPKs in the PAI-1 induction in PLGn-stimulated astrocytes in vitro.

The antibodies and reagents used in this study were as follows. Anti-glial fibrillary acidic protein antibody was obtained from Shandon Scientific (Pittsburgh, PA). The antibodies against phosphorylated external signal regulated kinase (p-ERK; Thr-183, Tyr-185), phosphorylated c-Jun N-terminal kinase (p-JNK; Thr-183, Tyr-185), phosphorylated p38 (p-p38; Thr-180, Tyr-182), and ERK1/2 were obtained from Promega Corporation. Anti-JNK1/2/3 antibody, anti-JNK3 antibody and anti-p-38 antibody were purchased from Santa Cruz Biotechnology. Anti-rat PAI-1 antibody was supplied by American Diagnostica Inc. (Greenwich, CT). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG were obtained from Nippon BioRad Lab (Tsukuba, Japan). Rat PLGn was purified from rat plasma by using Lysine-Sepharose, Red-Sepharose, and Sephadex 150 column chromatography as described previously [5]. The cell-permeable specific MEK1/2 (an upstream kinase of ERK) inhibitor

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(PD98059) and p38 inhibitor (SB203580) were obtained from Calbiochem-Novabiochem Japan (Tokyo). The cell-permeable specific JNK inhibitor (SP600125) was purchased from Funakoshi Co., Ltd. (Tokyo).

Astrocytes were prepared from a newborn rat brain-derived primary cell culture according to the previously described method [14]. The primary cultures were maintained for 3 weeks, then shaken for 24 h to remove microglia and oligodendrocytes. The resultant astrocytic monolayer was treated with 0.05% trypsin and detached. The astrocytes were seeded onto 6-cm dishes at a density of 2×10^6 cells/dish. The purity of the astrocytes was estimated to be at least 98% based on the expression of glial fibrillary acidic protein. Three days after maintaining the astrocytes with Dulbecco's modified essential medium containing 10% PLGn-free calf serum [5], the medium was exchanged for serum-free Dulbecco's modified essential medium and the cells were cultured for an additional 1 day. Then, rat PLGn (100 nM) was added to stimulate the cells. When the effects of MAPK inhibitors were tested, the rat PLGn was added after pretreatment of astrocytes with an MAPK inhibitor for 1 h. At the end of incubation (24 h), the astrocytic conditioned medium was recovered, concentrated using a centrifuging concentrator (Centricut: 10 kDa cut), and freeze-dried.

For the detection of MAPK activation, the astrocytes were treated with phosphatase inhibitor solution (20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM sodium pyrophosphate, 10 mM NaF, 10 mM Na_3VO_4) at the suitable time points (0, 30, 120, 180 min), and collected with a rubber policeman. The recovered cells were immediately freeze-dried.

The freeze-dried astrocytic conditioned medium was solubilized in SDS sample solution (62.5 mM Tris–HCl, 2.5% 2-mercaptoethanol, 2% SDS, 5% glycerol) and subjected to SDS polyacrylamide gel electrophoresis and subsequent transblotting, as described previously [5]. The blotted membrane was incubated with anti-PAI-1 antibody (1:500).

In the analysis of MAPK activation, freeze-dried astrocytes were solubilized in non-reducing SDS sample solution. After protein determination, 2-mercaptoethanol was added to the samples to a final concentration of 5%. These cell samples were subjected to immunoblotting for both phosphorylated MAPKs and MAPKs, as described previously [19]. Transblotted membranes were incubated with primary antibodies such as anti-p-ERK antibody (1:4000), anti-p-JNK (1:2000), anti-p-p38 (1:2000), anti-ERK1/2 (1:400), anti-JNK1/2/3 (1:400), anti-JNK3 (1:400), and anti-p38 (1:400) antibody at 4 °C overnight. HRP-conjugated anti-rabbit IgG (1:1000), or HRP-conjugated anti-mouse IgG (1:1000) as the secondary antibody, was added and the membranes were incubated for 1 h at room temperature.

The densities of the bands of PAI-1 in immunoblotting were measured with a densitometer, and the results are expressed as the means ± S.D. from three independent experiments. Differences between the control and the stimulated groups were assessed via Student's *t*-test. In all cases, *P* values of less than 0.01 were considered significant ($^*P < 0.01$, $^{**}P < 0.005$).

To examine whether the PLGn-induced enhancement of PAI-1 production/release from astrocytes is caused by a specific signal transduction cascade, we determined the PLGn-dependent activation of MAPKs. In our examination of MAPK activation (phosphorylation), we found that three MAPKs (ERK, JNK and p38) were phosphorylated after stimulation with PLGn (Fig. 1). The phosphorylation of ERK was not significant within 60 min (Fig. 1A). However, after 120 min, the phosphorylation of ERK, particularly that of ERK2 (p42), was clearly increased. The second MAPK, JNK, was also phosphorylated by stimulation with PLGn (Fig. 1B). Only JNK (p46), but not JNK (p54), was observed to be phosphorylated with a peak at 120 min after stimulation with PLGn. Whether the JNK (p46) contains JNK3 isoform in addition to JNK1 and JNK2

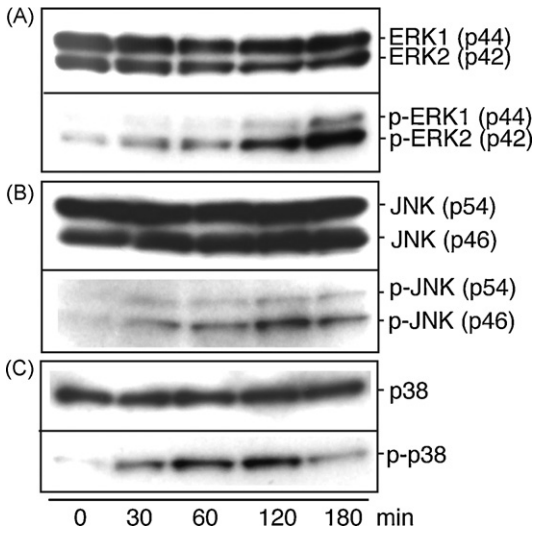

Fig. 1. Plasminogen-dependent phosphorylation of MAPKs. Astrocytes (2×10^6 cells/dish) maintained with serum-free Dulbecco's modified essential medium for 1 day were stimulated with 100 nM PLGn, and the cells were recovered at 0, 30, 60, 120, and 180 min, as described in the text. The cells were homogenized and aliquots (20 μg proteins) were subjected to immunoblotting for ERK1/2 and phosphorylated ERK (A), for JNK1/2/3 and phosphorylated JNK (B), and for p38 and phosphorylated p38 (C). Typical results are shown.

was investigated in the PLGn-stimulated astrocytes. Immunoblotting using a JNK3-specific antibody revealed that JNK3 was clearly present at the position of JNK (p46), but not at JNK (p54) (data not shown). Thus, JNK (p46) comprising JNK1-3 appears to serve in PAI-1 induction through the activation (phosphorylation). The third MAPK, p38, began to be activated from 30 min after PLGn stimulation, peaked at 120 min, and thereafter declined (Fig. 1C). These results suggest that all of these MAPKs, either alone or in combination, are associated with upregulation of the PAI-1 level in PLGn-stimulated astrocytes.

To investigate the association between MAPK activity and PAI-1 production/release, a specific inhibitor for each MAPK was tested (Fig. 2). PD98059, SP600125 and SB203580 were selected as an MEK1/2 (an upstream kinase of ERK) inhibitor, a JNK inhibitor and a p38 inhibitor, respectively, and the cells were pretreated with these inhibitors for 1 h prior to PLGn stimulation. The MEK1/2 inhibitor PD98059 appeared to show weak inhibition at any dose (Fig. 2A). The JNK inhibitor SP600125 inhibited the PAI-1 induction to a certain extent when added at a higher dose (20 μM) (Fig. 2B). The p38 inhibitor SB203580 strongly suppressed the PAI-I induction in a dose-dependent fashion (0-20 μM) (Fig. 2C). Quantitative analysis revealed that the levels of PLGn-dependent PAI-1 decreased to approximately 15% in 20 μM SB203580 (the p38 inhibitor)-pretreated astrocytes and decreased to approximately 60% in 20 μM SP600125 (the JNK inhibitor)-pretreated astrocytes (Fig. 2D). On the other hand, pretreatment with 20 μM PD98059 (the MEK1/2 inhibitor) downregulated PLGn-induced PAI-1 by approximately 20% (Fig. 2D). These results indicate that p38 and JNK are mainly involved in PAI-1 induction in PLGn-stimulated astrocytes.

In this study, we attempted to clarify the association between MAPK and PAI-1 induction in PLGn-stimulated astrocytes, and verified that p38 and JNK play major roles in the PAI-1 induction.

As reported previously, astrocytes respond to PLGn by markedly enhancing PAI-1 production/release [5]. The enhanced amounts of PAI-1 are believed to serve primarily as plasminogen activator inhibitors. Interestingly, a new role of PAI-1 as a neurotrophic factor has also been proposed recently [16]. The responsiveness of astrocytes to PLGn suggests the occurrence of a specific signal