

Theme Issue Article

Anti-apoptotic roles of plasminogen activator inhibitor-1 as a neurotrophic factor in the central nervous system

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Summary

Plasminogen activator inhibitor-1 (PAI-1), a member of the serpin gene family, is the primary inhibitor of urokinase-type and tissue-type PAs. PAI-1 plays an important role in the process of peripheral tissue remodeling and fibrinolysis through the regulation of PA activity. This serpin is also produced in brain tissues and may regulate the neural protease sequence in the central nervous system (CNS), as it does in peripheral tissues. In fact, PAI-1 mRNA is up-regulated in mouse brain after stroke. The serpin activity of PAI-1 helps to prevent tissue-type PA-induced neuron death. However, we have previously found that PAI-1 has a novel biological function in the CNS: the contribution to survival of neurites on neurons. In neuronally differentiated rat pheochromocytoma (PC-12) cells, a deficiency of PAI-1 *in vitro* caused a significant reduction in Bcl-2 and Bcl-X_L mRNAs and an

increase in Bcl-X_S and Bax mRNAs. The change in the balance between mRNA expressions of the anti- and pro-apoptotic Bcl-2 family proteins promoted the apoptotic sequence: caspase-3 activation, cytochrome c release from mitochondria and DNA fragmentation. Our results indicate that PAI-1 has an anti-apoptotic role in neurons. PAI-1 prevented the disintegration of the formed neuronal networks by maintaining or promoting neuroprotective signaling through the MAPK/ERK pathway, suggesting that the neuroprotective effect of PAI-1 is independent of its action as a protease inhibitor. This review discusses the neuroprotective effects of PAI-1 *in vitro*, together with the relevant data from other laboratories. Special emphasis is placed on its action on PC-12 cells.

Keywords

Plasminogen activator inhibitor-1, neurotrophic factor, ERK-related pathway, astrocytes, PC-12 cells

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Plasminogen activator inhibitor-1 (PAI-1) production and its regulation in peripheral tissues and brain

Elevated concentrations of PAI-1 in blood are risk factors for thrombotic as well as atherosclerotic disease (1, 2). In prospective clinical studies, elevated blood levels of PAI-1 have been implicated with metabolic syndrome. Increased PAI-1 parallels increased body mass index, and PAI-1 gene expression is increased in subcutaneous and visceral fat depots in those with insulin resistance (3, 4). Synthesis of PAI-1 is influenced by cytokines such as tumor necrosis factor- α (TNF- α) and transforming

growth factor- β (TGF- β), typically up-regulated in those with the syndrome of insulin resistance (5–7). In obese subjects, adipose tissue may be the most important site of production of PAI-1. Recently, Zirlik et al. (8) demonstrated that thiazolidinediones suppress TGF- β -induced elevation of PAI-1 release from human adipocytes and suggested that peroxisome proliferator-activated receptor- γ (PPAR- γ) mediates down-regulation of PAI-1 expression. TGF- β also up-regulates PAI-1 gene expression in mast cells and melanocytes (9) and in mesangial cells (10).

In the central nervous system (CNS), TGF- β 1 exerts a neuroprotective activity by mediating upregulation of PAI-1 in astrocytes (11). This underlines the involvement of serine proteases

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(serpins) and extracellular matrix components such as the PAI-1/tissue type-PA axis in the excitotoxic cascade. In fact, PAI-1 mRNA is increased in mouse brain after stroke (12). PAI-1 may protect neuronal cell injury, which has been promoted by tissue-type PA. Additionally, Docagne et al. (13) demonstrated that PAI-1 from astrocytes mediates the neuroprotective activity of TGF- β 1 against N-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity. PAI-1 protected neurons against NMDA-induced neuronal death by modulating the NMDA-evoked calcium influx. They further demonstrated that the activation of the Smad3-dependent transduction pathway mediates TGF- β 1-induced up-regulation of PAI-1 and subsequent neuroprotection (13). Previously, Takahashi et al. (14) demonstrated that nerve growth factor (NGF)-induced expression of PAI-1 mRNA in PC-12 cells is inhibited by genistein and wortmannin. These results suggest that both phosphorylation of the NGF receptor, Trk, and activation of PI-3 kinase-dependent signal transduction pathway are necessary for expression of PAI-1 mRNA in PC-12 cells.

TNF- α also enhances the release of PAI-1 from cultured human astrocytes after an increase in intracellular ceramide (15). TNF- α has been shown to activate the sphingomyelin (SM) pathway located in plasma membrane, where the activated sphingomyelinase (SMase) hydrolyzes SM to generate ceramide (16). The anthracycline antibiotic daunorubicin induces apoptosis in cells by triggering ceramide generation through *de novo* synthesis or SM hydrolysis. The stimulation effect of TNF- α on PAI-1 synthesis was attenuated by the pretreatment of astrocytes (15) or human umbilical vein endothelial cells (17) with daunorubicin. Interestingly, the daunorubicin-induced increase in ceramide content was blocked by adding the ceramide synthase inhibitor, fumonisin B1, while TNF- α -induced ceramide increase was not affected by this drug (15, 17). Fumonisin B1 treatment restored the daunorubicin-induced decrease in PAI-1 release, but did not affect the TNF- α -induced increase in PAI-1 release. These data imply the possibility that the subcellular topology of ceramide production determines its lipid mediator function in the regulation of PAI-1 synthesis, because both TNF- α and daunorubicin could increase ceramide levels in cells (15, 17). The suppression of PAI-1 release with daunorubicin accelerated the death of neuronally differentiated PC-12 cells. This result suggests an anti-apoptotic role of PAI-1 in the CNS (15).

Anti-apoptotic roles of PAI-1 in the CNS

Tissue-type PA is the primary PA in the brain (18, 19). In the adult nervous system, tissue-type PA is induced in the hippocampus after seizures, kindling and long-term potentiation (20). Moreover, tissue-type PA and plasminogen are involved in the regulation of neuronal survival in response to excitotoxin (21, 22). Like the peripheral PA/plasmin system, the specific inhibitor(s) may exist to regulate this neural protease cascade. Previously, neuroserpin was identified in the brain as a novel serpin family member (23, 24). It is secreted axonally during embryogenesis and in adult nervous systems and inhibits PAs and plasmin, but not thrombin (25). Neuroserpin may play a regulatory role in motor learning and neuronal survival (26). PAI-1 is also found in the CNS. However, the neural PAI-1 is not co-express-

ed with PAs in a pattern suggestive of the role of physiological PA regulator (27).

As described above, insufficient release of PAI-1 from cultured astrocytes affected the survival of neuronally differentiated PC-12 cells (15). To further investigate the role of PAI-1 in the nervous system, PC-12 cells were maintained in a PAI-1-deficient culture medium derived from daunorubicin-pretreated astrocytes. Although a trace amount of PAI-1 was released from the PC-12 cells, but, there was no influence on these experiments. The deficiency of PAI-1 in the medium caused a significant reduction in Bcl-2 and Bcl-XL mRNAs and an increase in Bcl-Xs and Bax mRNAs in PC-12 cells. The changes in balance between mRNA expressions of the anti- and pro-apoptotic Bcl-2 family proteins caused caspase-3 activation following the release of cytochrome c from mitochondria (28). Apoptotic morphological change and DNA fragmentation were also observed in the neuronal cells. Addition of exogenous PAI-1 protein to the inhibitor-deficient medium blocked the apoptotic changes in PC-12 cells. Later, we demonstrated that the neurites of PC-12 cells formed by differentiation with NGF disappeared by exposing them to serum-free medium (the retraction of neurites seemed not to be due to the cells undergoing apoptosis), while the addition of PAI-1 prevented the disintegration of the neuronal networks in a concentration-dependent manner (29). Addition of other serpins, aprotinin or antipain, which can inhibit plasmin or PA activity, did not prevent the disintegration. This suggests that PAI-1's neuroprotective effect does not involve its function as PA inhibitor. PAI-1 promoted the release of the survival factors, interleukin (IL)-6 and vascular endothelial growth factor (VEGF) from the PC-12 cells, but only in amounts on the order of pg/ml. However, studies *in vitro* indicate that to achieve their neuroprotective effects, both cytokines must be present in amounts on the order of ng/ml (30, 31). Therefore, in our results, PAI-1 may maintain the morphology of the neuronal networks of PC-12 cells directly without the aids of neuroprotective effects of IL-6 and VEGF.

Both PI-3 kinase/Akt and MAPK/ERK pathways play central roles in the survival of neurons (32, 33), although other undiscovered pathways may exist. Of the two survival pathways, PAI-1 specifically promoted the signal transduction of the MAPK/ERK pathway in neuronally differentiated PC-12 cells (29). This pathway is important in mediating NGF-induced neurite outgrowth response in PC-12 cells (34, 35), but is not required for the NGF-induced proliferation of the cells (34). We tested whether PAI-1 alone could influence the neurite outgrowth of PC-12 cells, but did not find that it had any direct effect on neuronal differentiation.

Experiments with MCF-7 breast cancer cells have shown that PAI-1, after complex formation with urokinase-type PA, can sustain the phosphorylation of ERKs in the cells *via* a urokinase-type PA receptor-related mechanism of action (36). Farias-Eisner et al. (37) have demonstrated that, in NGF-induced differentiation of PC-12 cells, the expression and function of urokinase-type PA's receptor is required transiently only during the early stages of their differentiation. Thus, the urokinase type PA-related mechanism has no connection with the survival mechanism involving PAI-1. Identification of the PAI-1's receptor or receptor-like protein(s) that leads to the activation of MAPK/ERK

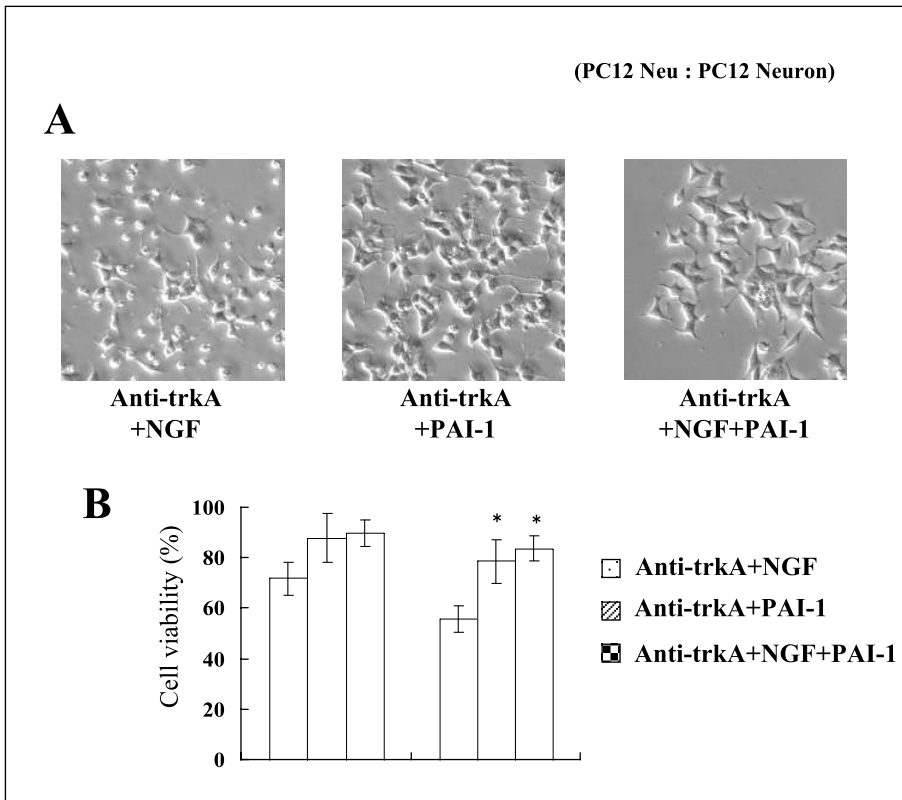


Figure 1: The effect of PAI-1 on the morphology (A) and cell viability (B) of NGF-differentiated and anti-Trk A antibody-treated PC-12 cells. Cells were treated with anti-Trk A antibodies for 30 min after differentiation with NGF. The antibody-treated neurons were incubated for 24 or 72 hours in serum-free medium with NGF (50 ng/ml) alone, PAI-1 (75 ng/ml) alone, NGF plus PAI-1 or none. The viability of antibody-untreated cells in the presence of NGF alone was defined as control. * $P < 0.05$ vs. the antibody-treated cells plus NGF.

pathway is needed. An immunohistochemical study on the localization of PAI-1 in human brain tissues (38) indicated that it is present in neurons and in astrocytes and that in Alzheimer's diseases (AD), weak PAI-1 labeling is seen in association with senile plaques and ghost tangles. This finding suggests that PAI-1 is involved in a variety of physiological and pathological processes in the brain.

Patho-physiological roles of PAI-1 in Alzheimer's disease (AD) brain

AD patients exhibit characteristic pathologies including extracellular senile plaques composed mainly of amyloid- β ($A\beta$) peptide, intracellular neurofibrillary tangles of hyperphosphorylated tau protein, and localized deposition of amyloid in the blood vessels of the brain. The $A\beta$ comes from proteolytic processing of a larger molecule, $A\beta$ precursor protein. Excessive production or reduced degradation of the $A\beta$ can lead to its accumulation and the formation of senile plaques in AD. Various proteases have been implicated in $A\beta$ degradation, including neprilysin, insulin degrading enzyme, and plasmin (39). For example, studies *in vitro* have linked the tissue-type PA/plasmin system with $A\beta$ turnover (40, 41). $A\beta$ can stimulate tissue-type PA mRNA expression in cell culture, resulting in the production of plasmin and subsequent $A\beta$ degradation (41). Melchor et al. (42) demonstrated that in two different mouse models of AD, chronically elevated $A\beta$ in the brain correlated with the up-regulation of PAI-1 and inhibition of the tissue-type PA/plasmin sys-

tem. In addition, $A\beta$ injected into the hippocampus of mice lacking either tissue-type PA or plasminogen persists, including PAI-1 expression and causing activation of microglial cells and neuronal damage. Conversely, $A\beta$ injected into wild-type mice is rapidly cleared and does not cause neuronal degradation (42). Thus, the inhibition of the tissue-type PA/plasmin system by PAI-1 may facilitate $A\beta$ deposition by slowing down the clearance of the peptide. This mechanism could start a vicious cycle of accumulating $A\beta$, leading to increased PAI-1 expression, further tissue-type PA inhibition, and therefore, even more $A\beta$ deposition (42).

Neurotrophic roles of PAI-1 released from astrocytes and its survival signaling exerted in PC-12 cells

As have been reviewed above, PAI-1 may have at least two functions, one as a protease inhibitor, another as a neurotrophic factor in the brain. Although PAI-1 is suggested to promote the deposition of $A\beta$ and progression of AD by suppressing tissue-type PA/plasmin system-related $A\beta$ clearance (42), this serpin conversely plays a neuroprotective role toward the nervous injury induced by the proteolytic cascade in the excitotoxic brain lesions (22). Thus, the significance of PAI-1 accumulation in AD awaits further clarification. In this final section, we describe recent data from our laboratories on the PAI-1's role in NGF-induced differentiation and survival of PC-12 cells in a serum-free medium (43).

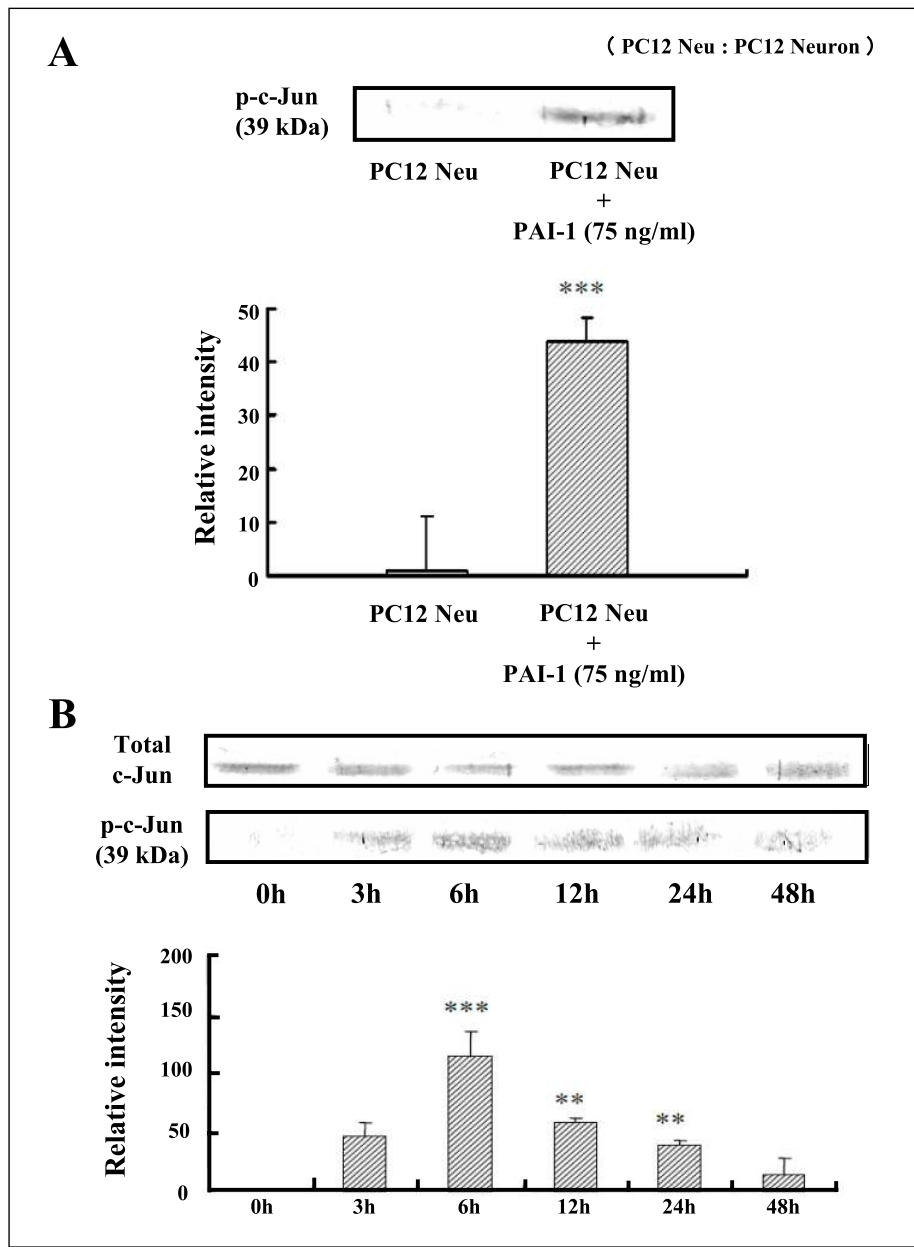


Figure 2: Effect of PAI-I on the phosphorylation of c-Jun. A) The effect of PAI-I on the phosphorylation of c-Jun in NGF-differentiated and anti-Trk A antibody-treated PC-12 cells. Cells were incubated for 6 hours in serum-free medium with or without PAI-I (75 ng/ml). The phosphorylated (p) or total c-Jun was made visible by Western blotting and the bands were quantified using an NIH Image. The bar represents the mean \pm SD (n=3). ***P<0.001 vs. medium alone. B) Time-dependent PAI-I phosphorylation of c-Jun in the antibody-treated neurons. The cells were incubated for ~48 hours in a serum-free medium with PAI-I (75 ng/ml). The bar represents the mean \pm SD (n=3). **P<0.01, ***P<0.001 vs. 0 hours.

The effect of PAI-I on the NGF-induced neurite outgrowth and survival signaling in PC-12 cells

NGF-induced neurite formation of PC-12 cells is promoted by adding PAI-1 in serum-free medium. The total length of neuronal networks formed by NGF plus PAI-1 was about 3.6 times greater than that which was formed by NGF alone (28). In neuronal cells, NGF, following its binding to high affinity Trk A receptor, promotes neurite outgrowth and cell survival *via* at least two pathways: the MAPK/ERK pathway and PI-3 kinase/Akt pathway (32, 33). In PC-12 cells, the MAPK/ERK pathway is known to be important in mediating NGF-induced neurite outgrowth response (35). We therefore examined the effect of exogenous PAI-1 on the NGF-induced phosphorylation of Trk A receptor and ERKs (43). The phosphorylated state of Trk A in PC-12 cells treated with NGF plus PAI-1 was time-dependent

and greater than that in the cells treated with NGF alone. Additionally, PAI-1 alone caused phosphorylation of the NGF receptor. PAI-1 also increased the phosphorylated state of ERKs. Therefore, PAI-1 may help to increase the NGF-induced levels of phosphorylated ERKs. The enhanced activation of ERKs may result from the cooperation of NGF and PAI-1 for direct phosphorylation of Trk A (43).

The effect of PAI-I on the survival of PC-12 cells treated with anti-Trk A antibodies

To better understand the role of PAI-1 in the survival of neurons under a serum deprivation stress, the NGF receptors of PC-12 cells were blocked with anti-Trk A antibodies. As shown in Figure 1A, after exposure to serum-free medium, the neurons treated with antibodies lost the neurites and showed the char-

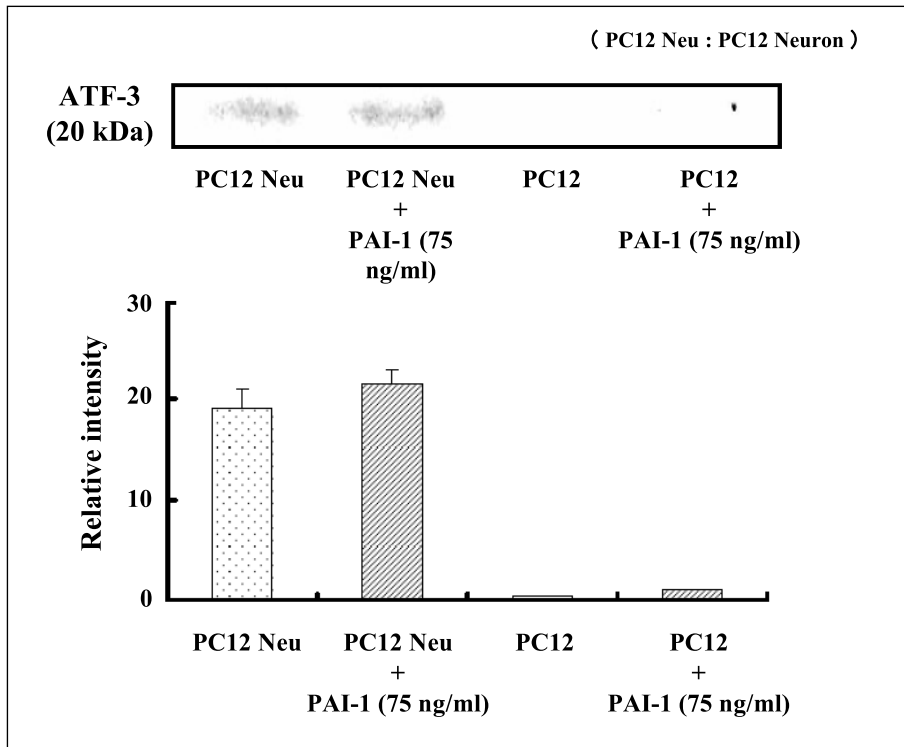


Figure 3: The effect of PAI-1 on the ATF-3 levels in anti-TrkA antibody-treated PC-12 neurons and undifferentiated PC-12 cells. The cells were incubated for 6 hours in serum-free medium with or without PAI-1 (75 ng/ml). The ATF-3 was made visible by Western blotting, and the bands were quantified using a NIH Image.

acteristic morphology of necrotic and/or apoptotic cells. In the presence of PAI-1 alone or NGF plus PAI-1, however, the neurons maintained their morphology and survival (Fig. 1A and B). This result suggests that another survival signaling independent of NGF receptor also acts in conjunction with PAI-1 (43).

The transcription factor c-Jun is activated after NGF withdrawal and is thought to mediate neuronal apoptosis (44). In contrast, Dragounow et al. (45) have demonstrated that c-Jun plays an important role in the promotion of neurite outgrowth and survival in PC-12 cells. Therefore, c-Jun may have a key role in "turning on" the neuron's regeneration program after injury (46). We hypothesized that when NGF signaling is blocked, PAI-1 might contribute to the survival of neuronally differentiated PC-12 cells by activating c-Jun. To test our hypothesis, we examined the effect of PAI-1 on the phosphorylation of c-Jun in the cells (43). As shown in Figure 2A, exposing the neurons treated with antibodies to serum-free medium did not induce the phosphorylation of c-Jun. However, the presence of PAI-1 promoted c-Jun's phosphorylation in the cells. Figure 2B shows the time-dependent effect of PAI-1 on the state of c-Jun in the cells: the levels of phosphorylated c-Jun peaked and then gradually decreased.

ATF3 is also a member of the cAMP response element-binding protein (CREB)/ATF family transcription factors and is strongly expressed in response to nerve injury (47). ATF3 can form a heterodimer with c-Jun and therefore may determine whether c-Jun activation leads to cell death or to survival. Figure 3 shows the ATF3 levels in the antibody-treated neurons and undifferentiated PC-12 cells after exposure to PAI-1. Our result indicates that ATF3 is produced in response to c-Jun activation.

However, PAI-1's ability to promote ATF3 expression in the cells was insignificant. These findings suggest that PAI-1 contributes to differentiation and survival of PC-12 cells as a neurotrophic factor not only by activating the MAPK/ERK pathway through Trk A phosphorylation, but also by activating the c-Jun/AP-1 pathway through an as yet unknown receptor or mechanism (43).

Conclusions

In PC-12 cells, PAI-1 has a positive influence on neurite elongation and neuron survival, especially, under serum deprivation stress (15, 28). c-Jun/ATF3 heterodimer may work with PAI-1 to enhance the survival of PC-12 cells (43). ATF3 is thought to be expressed only in injured neurons (47), and its transcriptional activity differs according to its counterpart. Recent studies suggest that the expressed ATF3, together with activated c-Jun as the partner, can prevent neuronal death (48) or enhance neurite sprouting (49). In our experiments, PC-12 cells were differentiated and maintained by the presence of exogenous PAI-1 in serum-free medium (43). The serum deficiency might put the neuronal cells under stress and lead to the expression of ATF3. PAI-1 promoted the activation of c-Jun in the cells. c-Jun is activated by c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), which is in the MAPK superfamily and induces apoptosis in many type of cells. Formation of the activated c-Jun/ATF3 heterodimer in the cells might cause the cells to survive. However, the PAI-1 receptor or receptor-like protein(s) that lead to the activation of c-Jun remains unknown.

In PC-12 cells, the MAPK/ERK pathway, following phosphorylation of Trk A, plays a role in the neuroprotective effects of

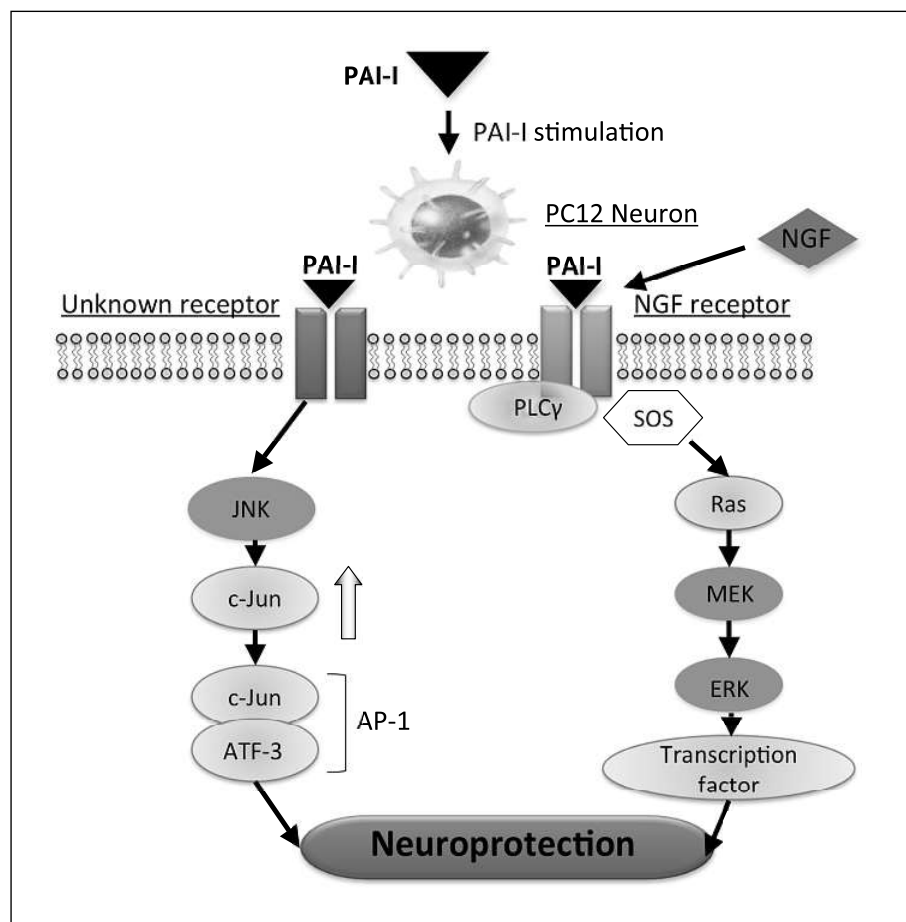


Figure 4: Hypothesis for PAI-1-mediated survival signaling in PC-12 neurons.

PAI-1. PAI-1 increased the phosphorylation levels of both Trk A and ERKs without the aid of NGF (43). It is not certain that PAI-1 alone can promote the differentiation of PC-12 cells. Incubation of undifferentiated PC-12 cells with PAI-1 did not cause the neurite formation (43). However, at four days the cells incubated with PAI-1 exhibited neurite-like structures. Why did PAI-1 need so much time to exhibit NGF-like activity, in spite of its ability to phosphorylate Trk A? One possibility is that in undifferentiated PC-12 cells, PAI-1 can promote the activation of existing Trk A, but, unlike NGF, cannot increase the number of NGF receptors. Failure of PAI-1 to increase the localization of Trk As on PC-12 cell surfaces may retard the cells' differentiation to neurons. Thus, PAI-1's role in the differentiation of neurons in the CNS seems to be smaller than the role of NGF. These findings support the notion that, when neurons are exposed to cellular stresses, astrocytes release PAI-1 to increase the neuron's chances to survive not only by activating the MAPK/ERK pathway via Trk A phosphorylation, but also by activating the c-Jun/ AP-1 pathway (Fig. 4).

Melchor et al. (42) have demonstrated the significance of tissue-type PA-plasmin system in AD using mouse models of the disease. Their results show that the tissue-type PA-plasmin system aids in the clearance of A β peptide and that A β also induces up-regulation of PAI-1, which results in the acceleration of A β -induced neurodegeneration as a serpin. It is important to note

that, although some research groups report the neuroprotective roles of PAI-1 (12, 13), Melchor et al. (42) demonstrated PAI-1's proapoptotic role in AD. This discrepancy reveals that t-PA can play roles in both neuro-protection and neuro-degeneration like a double-edged sword. However, our findings for PAI-1's role in the CNS (43) suggest that the elevated levels of PAI-1 in the AD mouse models, on the other hand, may serve to protect neurons from A β -induced neuron death by activating the c-Jun/AP-1 survival pathway.

Finally, the low-density lipoprotein receptor-related protein (LRP) is the main receptor in the brain for apolipoprotein E, and its gene is located on chromosome 12, the site of a potential AD locus (50). PAI-1 can stimulate cell migration by binding to the LRP, where Jak/Stat signaling system is activated (51). It is unknown whether the neuro-protective effects observed in PC-12 cells is explained by the LRP-evoked signaling. But, LRP seems at present unlikely to act as the neuronal PAI-1 receptor we proposed to activate directly the c-Jun pathway. Further studies about the proposed novel role of PAI-1 in the CNS may provide a basis for the development of new approaches to overcome some neurodegenerative diseases.

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