Expression of Functional Nitric Oxide Synthase for Neuritogenesis in PC12h Cells

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We have previously demonstrated that a natural iridoid compound, genipin, induces neurite outgrowth mediated by nitric oxide (NO) production in PC12h cells. However, genipin could not induce neurite outgrowth by PC12 cells, the parental cells of PC12h cells. The difference in neuritogenic response to genipin may be due to a lack of neuronal NO synthase (NOS) protein, most likely neuronal NOS, in PC12 cells. In this study, we have investigated whether neuronal NOS protein innately expressed in PC12h cells plays any functional role in neuritogenesis. L-Lysine and L-norvaline, inhibitors of arginase which uses the same substrate as NOS, significantly induced neurite outgrowth in PC12h cells but not in PC12 cells. In PC12h cells, L-lysine-induced neurite outgrowth was completely inhibited by a nonselective NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) but was not inhibited by its negative isomer D-NAME, suggesting that up-regulation of NOS activity by arginase inhibitors with increasing intracellular concentrations of substrate induces neuritogenesis in NOS-expressing cells. Thus, it is concluded that innately expressed neuronal NOS has a functional role in neuritogenesis in PC12h cells.

Key words —— nitric oxide synthase, arginase, neurite outgrowth, genipin, PC12h cell

INTRODUCTION

It has been reported that nitric oxide (NO) has a variety of biological functions, such as vasodilation,[1] neurotransmission,[2] inhibition of platelet aggregation[3] and cell survival.[4] In a previous paper, we reported that several NO donors markedly induced neurite outgrowth in rat pheochromocytoma PC12h cells.[5] NO is synthesized from arginine by NO synthase (NOS). Three isoforms of NOS are known; neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). On the other hand, arginase is an enzyme in the urea cycle that produces urea and ornithine from arginine, which is the same substrate used by NOS. It has been proposed that arginase can physiologically down-regulate NO production by decreasing the intracellular arginine concentration.[6] Therefore, it is conceivable that inhibition of arginase activity will induce NOS activation followed by NO production (Fig. 1).

Genipin is a natural iridoid compound. We have previously described the biological activities of the compound, such as its neuritogenic activity in PC12h cells[7] and neuroprotective activities in cultured rat hippocampal neurons exposed to β-amyloid peptide[8] and mouse neuroblastoma Neuro2a cells deprived of serum.[9] With respect to the mechanism of neuritogenesis by genipin, we have described the NO-guanosine 3′,5′-cyclic monophosphate (cGMP)-cGMP-dependent protein kinase (PKG) signaling pathway in PC12h cells.[10] Moreover, we have suggested that genipin directly binds to and activates nNOS *in vitro*.[11] However, in PC12 cells, which are the parental cells of PC12h cells, however, genipin did not exert any neuritogenic activity.[12] The difference in neuritogenic response to genipin between PC12h and PC12 cells may be due to a difference in the expression level of nNOS protein.[12] That is, PC12h cells innately express the protein at a low level, whereas PC12 cells do not express the protein in the resting state.

In the present study, we attempted to confirm whether a low level of nNOS protein in PC12h cells has any functional role in neurite outgrowth using arginase inhibitors.

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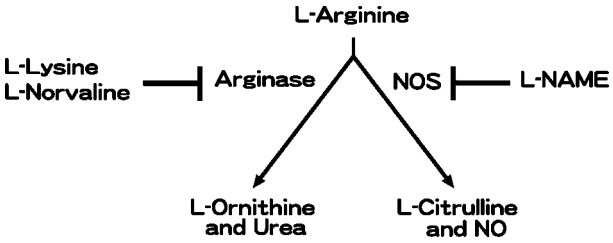

Fig. 1. Enzymatic Reactions of Arginase and NOS

Both arginase and NOS use L-arginine as the common substrate. L-lysine and L-norvaline inhibit arginase, and L-NAME inhibits NOS.

MATERIALS AND METHODS

Materials —— Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Japan) and dissolved in dimethylsulfoxide (DMSO). L-Lysine, L-norvaline, N^G-nitro-L-arginine methyl ester (L-NAME), and N^G-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma Chemical Co. (U.S.A.). L-Lysine was dissolved in distilled water. L-Norvaline, L-NAME and D-NAME were dissolved in phosphate-buffered saline (PBS).

Cell Culture —— PC12h cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (v/v) horse serum and 5% (v/v) precolostrum calf serum. The cells were plated at a density of 2×10^3 cells/cm^2 on 35 mm-diameter dishes coated with collagen type I. After 24 hr of culture, the medium was replaced by serum-free DMEM/Ham's F12 medium supplemented with 30 nM sodium selenate, 5 μg/ml transferrin, 5 μg/ml insulin, and 20 nM progesterone containing vehicle for control or the appropriate test compound as specified in the results.

Neurite Outgrowth Assay —— Neurite outgrowth in PC12h cells was evaluated by measuring the length of the longest neurite in individual cells, as previously reported.[7] The neurite length of 100 cells was averaged to evaluate the neurite outgrowth for each treatment.

Statistical Analysis —— Statistically significant differences between groups were estimated by analysis of variance (ANOVA) followed by Scheffe's test. A $p < 0.01$ was considered to indicate a significant difference.

RESULTS

Neurite Outgrowth by Arginase Inhibition

In a previous paper, we demonstrated that nNOS protein was detectable in Western blot analysis in PC12h cells but was not detectable in PC12 cells in the resting state.[12] As shown in Fig. 1, it is possible that the increase in L-arginine, which is the substrate of nNOS, by arginase inhibitors results in nNOS activation. To confirm whether nNOS actually acts as a neuritogenic effector, we examined the neurite-promoting effect of arginase inhibitors using L-lysine[13] and L-norvaline, which is more specific than L-lysine.[14] As shown in Fig. 2, L-lysine significantly induced neurite outgrowth in a concentration-dependent manner in PC12h cells but not in PC12 cells after 24-hr treatment. Similar results were observed in L-norvaline-treated PC12h and PC12 cells (Fig. 3). These results suggest that these arginase inhibitors induce the up-regulation of (n)NOS activity via the suppression of arginase activity in PC12h cells.

Inhibition of Arginase Inhibitor-Induced Neurite Outgrowth by a NOS Inhibitor

To confirm whether the arginase inhibitor-in-

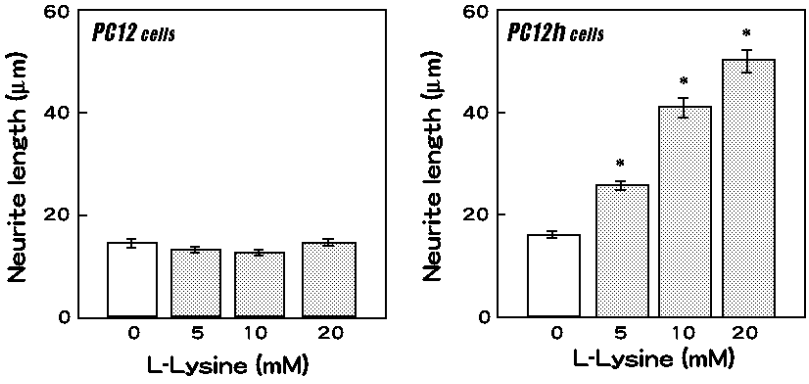

Fig. 2. Neuritogenesis Induced by L-Lysine in PC12 and PC12h Cells

Each cell line was treated with L-lysine (5–20 mM) or its vehicle (0 mM) for 48 hr. Neurite length was then measured as described in MATERIALS AND METHODS. Each value is expressed as the mean ± S.E.M. ($n = 100$). *$p < 0.01$ *vs.* 0 mM.

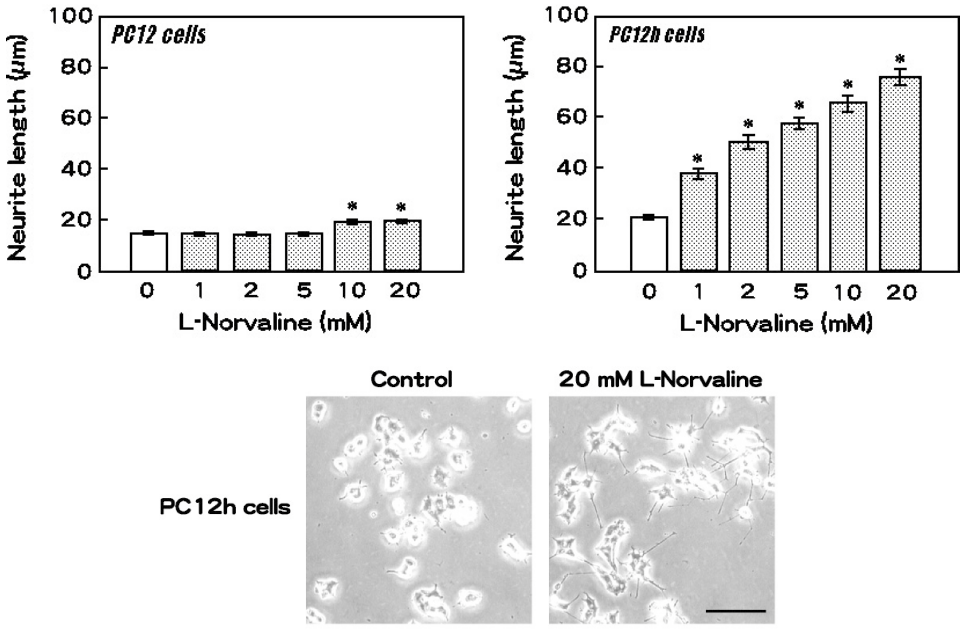

Fig. 3. Neuritogenesis Induced by L-Norvaline in PC12 and PC12h Cells

Each cell line was treated with L-norvaline (1–20 mM) or its vehicle (0 mM) for 48 hr. Neurite length was then measured as described in MATERIALS AND METHODS and photographs were taken (scale bar = 100 μm). Each value is expressed as the mean ± S.E.M. (n = 100). *$p < 0.01$ *vs.* 0 mM.

duced neurite outgrowth was definitely mediated by NOS activation, we examined the effects of a NOS inhibitor on neurite outgrowth. The L-lysine-induced neurite outgrowth was almost totally blocked by L-NAME, a nonselective NOS inhibitor,[15] in PC12h cells (Fig. 4). In contrast, D-NAME, the inactive isomer of L-NAME,[16] did not have any inhibitory effect in PC12h cells (Fig. 4). Neither L-NAME nor D-NAME had any effect in PC12 cells, which was to be expected from the results in Fig. 2 and 3 (Fig. 4). These results confirm that arginase inhibitor-induced neurite outgrowth depends on NOS activation in PC12h cells.

DISCUSSION

In this study, inhibition of arginase was found to induce neurite outgrowth in NOS-expressing PC12h cells and to be mediated by up-regulation of NOS activity.

We have previously reported that genipin induces neurite outgrowth through the NO-cGMP-PKG signaling pathway in PC12h cells.[7,10] Moreover, it seems that this pathway is triggered by direct binding of genipin to nNOS, suggesting that nNOS is very important for genipin-induced neuritogenesis.[11] In PC12 cells, however, the responsiveness to genipin in neuritogenesis was markedly less, the cause of which was believed to be undetectable levels of nNOS protein in the cells.[12] We attempted to determine whether nNOS protein in PC12h cells had any functional role in neurite outgrowth.

L-Arginine is a common substrate for both NOS and arginase. Therefore, our hypothesis was that arginase inhibitors would up-regulate NOS activity by blocking L-arginine depletion and hence inducing neurite outgrowth in PC12h cells. As was expected, arginase inhibitors induced neurite outgrowth in PC12h cells expressing nNOS but not in PC12 cells expressing only a slight amount. In PC12h cells, furthermore, the observed neurite outgrowth was extremely inhibited by a NOS inhibitor, indicating that an arginase inhibitor most certainly up-regulates NOS activity. Thus, we believe that a low level of expression of nNOS in PC12h cells most likely acts on and is sufficient for neurite outgrowth. In support of this, it has been reported that nerve growth factor (NGF)-primed PC12 cells, which express nNOS protein, extended significant neurites in response to genipin.[12]

It has been reported that NO production is reciprocally regulated by arginase activity. For example, arginase activity potentiates airway constriction by inhibition of NO production.[17] Arginase prevents NO-dependent apoptosis of RAW 264.7 cells.[18] Moreover, L-citrulline which is a by-product of the NOS reaction, can be recycled to L-argin-

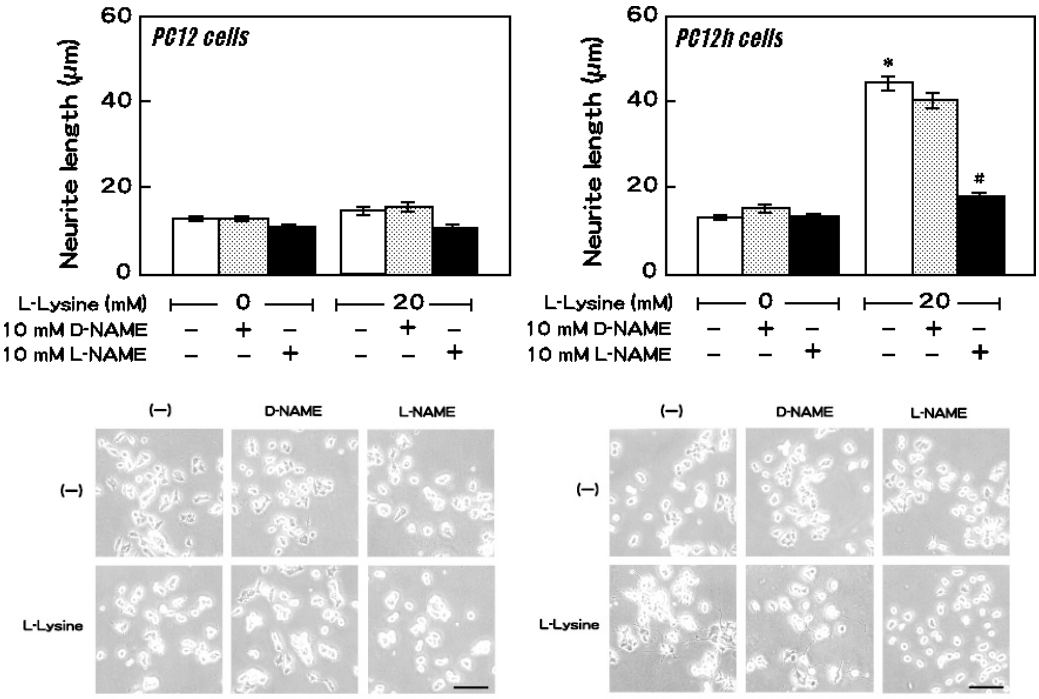

Fig. 4. Involvement of NOS in L-Lysine-Induced Neurite Outgrowth in PC12 and PC12h Cells

10 mM NAME (D- or L-, +) or its vehicle (–) was added to the medium 30 min before treatment with L-lysine (20 mM) or its vehicle (0 mM) for 48 hr. Neurite length was then measured as described in MATERIALS AND METHODS and photographs were taken (scale bar = 100 μm). Each value is expressed as the mean ± S.E.M. (n = 100). *$p < 0.01$ *vs*. treatment with vehicle alone, #$p < 0.01$ *vs*. treatment with L-lysine alone.

ine by the citrulline-NO cycle.[19] Our preliminary examination indicated that PC12h cells showed obvious arginase activity, whereas PC12 cells showed less activity in the resting state. We believe that the lack of responsiveness to arginase inhibitors in neurite outgrowth is due to the lack of not only nNOS but also arginase proteins in PC12 cells. In support of this, it has been reported that PC12 cells do not express even arginase mRNA in the resting state.[20] In contrast to PC12 cells, in PC12h cells both nNOS protein and arginase activity could be observed in the resting state. This finding suggests that NO-dependent neurite outgrowth could be regulated by arginase in PC12h cells, implying arginase may be involved in a novel mechanism of neuritogenesis. It has been reported that arginase II mRNA is present ubiquitously in nNOS-positive neurons.[21] Therefore, we presume that arginase II regulates nNOS-dependent NO production followed by neurite outgrowth in PC12h cells.

In summary, the findings of the present study suggests that a slight amount of nNOS protein detected in PC12h cells is actually involved in neuritogenesis and is an amount sufficient to induce neuritogenesis. Furthermore, the activities of arginase and NOS may be reciprocally regulated in neuritogenesis in PC12h cells.

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