active ingredients in the herb European and American feverfew (*Tanacetum parthenium*), has been shown to inhibit NF-κB (3 – 5). Moreover, parthenolide induces apoptosis through the activation of caspase-3 and necrosis through the disruption of the cell membrane in HL60 cells (20, 21). Parthenolide has been thought to be one of the promising chemotherapeutic agents because parthenolide triggers apoptosis in sarcomatoid hepatocellular carcinoma SH-J1 cells and also other hepatoma cell lines at low doses of 5 – 10 μM (22). In the present study, we have found that arucanolide had a more potent cytotoxic effect compared to parthenolide as reflected in IC_{50}s against HL60 or SW480 cell lines. Furthermore, we found that the cytotoxicity was due to apoptosis mediated by loss of mitochondrial membrane potential and the concurrent AIF release in HL60 cells. Many chemopreventive agents act through induction of apoptosis which would result in inhibition of the carcinogenesis process. During the last several years, it has become increasingly clear that mitochondria play a major rate-limiting role in apoptosis. The decision /effecter phase of the apoptotic process converges on mitochondria, where permeabilization of mitochondrial membranes is triggered; and apoptosis inducing factors such as cytochrome c, AIF, and endonuclease G are released. It has been reported that released AIF was gathered around the nuclei in the cytoplasm and partly translocated into nuclei after the treatment of the apoptogenic dolichyl monoposphate in U937 cells and that both caspase-3 and 8 inhibitors blocked the DNA fragmentation (23). AIF causes chromatin condensation and large scale DNA fragmentation of approximately 50 kb (24). Surely, it is not clear whether AIF directly contributes to DNA ladder formation. In our present study, it was shown that arucanolide-induced apoptosis was mediated by the mitochondrial pathway, and that the released AIF level was time-dependently increasing after exposure to arucanolide. Accordingly, our data raise the possibility that AIF, but not caspases, may play a crucial role in DNA ladder formation by one or more yet undefined mechanisms. It was also to be noted that the mechanism of apoptosis induced by arucanolide was different from that of parthenolide. Parthenolide inhibits the activation of NF-κB (3 – 5), but arucanolide did not affect NF-κB.

Traditionally, sesquiterpene lactones have been used as folk medicines because of their various bioactivities. For example, Tenulin and Helenalin were reported to have an ability to inhibit of DNA synthesis of Ehrlich ascites cells and P-388 cells (25 – 27). Artemisinin, a sesquiterpene lactone with an endoperoxide group, was used as an anti-malarial drug and was effective against both drug-resistant and cerebral malaria-causing stains of *Plasmodium falciparum* (28). Artemisinin inhibits nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells through the inhibition of NF-κB activation (29). Costunolide, a naturally occurring sesquiterpene lactone, reduced the frequency of colonic aberrant crypt foci induced by azoxymethane (30) and was reported to cause a strong growth inhibition against HL60 cells with apoptotic chromatin condensation (31). Furthermore, costunolide suppresses gene expression of hepatitis B virus surface antigen in human hepatoma cells (32).

Parthenolide has been shown to improve endotoxic shock by reducing plasma nitrate/nitrite level and to reduce lung neutrophil infiltration in the rat sepsis model because it attenuated inducible nitric oxide synthase by inhibiting NF-κB activity (33, 34). Patel et al. reported that parthenolide mimicked the effects of IκB-α by inhibiting DNA binding activity of NF-κB and manganese superoxide dismutase (Mn-SOD) expression, which leads to an enhancing paclitaxel-induced apoptosis of breast cancer cells (35). It was also reported that apoptosis by germacranolides such as tatridine A diacetate and ineupatorolide A was accompanied by an early release of cytochrome c from mitochondria, followed by both activation of caspase-3 and fragmentation of poly (ADP-ribose) polymerase-1 (36). Thus, the mechanism of arucanolide-induced apoptosis was found to be clearly distinct from that of other sesquiterpene lactones.

The levels of p-p44/42, p-JNK, and p-p38 were changed within 24 h after the arucanolide-treatment; however, their levels were restored over 24-h after treatment. We did not conclude that these changes were related to the apoptotic signals. We are making ongoing investigations about this.

IC_{50} of arucanolide in mitogen-stimulated normal peripheral blood lymphocytes was observed at 2.3 μM, but that of parthenolide was 1.1 μM (Table 1b), suggesting that arucanolide is more plausible than parthenolide for application as a chemopreventive agent.

As to the structure-activity relationship of germacranolides tested, the cytotoxic activity observed in the present study was as follows: arucanolide > (parthenolide) > calealactone A > 2,3-epoxy-juanislamin > 2,3-epoxy-calealactone A = calealactone B. The presence of a double bond at C-2 and C-3 seems to be preferable to an epoxyl group. Esterification sometimes enhances the bioactivity as observed for aconitine. Arucanolide is esterified at C-8 and C-9 with two organic acids, which influences the cytotoxic activity. Hydrogenation of a side chain moiety often reduces the bioactivity, which is applied to a 2-methylbutylric acid moiety at C-9 in calealactone A and 2,3-epoxy-calealactone A. The carbon chain length of the organic acid is also important.

As seen in arucanlide, the acetyl group at C-8 may be more efficient in terms of the activity than a 2-methylacryl group.

We would conclude that arucanolide is more advantageous as a chemopreventive agent than parthenolide. Further experiments are required to assess the anticancer effect of arucanolide in an animal model and also to define the detailed mechanisms at the molecular level, which are under current progress in our laboratory.

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Special Issue

Monoamine Oxidases: Molecular, Pharmacological and Neurotoxicological Aspects

A Tribute to Prof. Merton Sandler

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Dopamine-Derived Salsolinol Derivatives as Endogenous Monoamine Oxidase Inhibitors: Occurrence, Metabolism and Function in Human Brains

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Abstract

Salsolinol, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, is an endogenous catechol isoquinoline detected in humans by M. Sandler. In human brain, a series of catechol isoquinolines were identified as the condensation products of dopamine or other monoamines with aldehydes or keto-acids. Recently selective occurrence of the (R)enantiomers of salsolinol derivatives was confirmed in human brain, and they are synthesized by enzymes in situ, but not by the non-enzymatic Pictet–Spengler reaction. A (R)salsolinol synthase catalyzes the enantio-specific synthesis of (R)salsolinol from dopamine and acetaldehyde, and (R)salsolinol N-methyltransferase synthesizes N-methyl(R)salsolinol, which is further oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion by non-enzymatic and enzymatic oxidation. The stepwise reactions, N-methylation and oxidation, induce the specified distribution of the N-methylated and oxidized derivatives in the human nigro-striatum, suggesting that these derivatives may be involved in the function of dopamine neurons under physiological and pathological conditions. As shown by in vivo and in vitro experiments, salsolinol derivatives affect the levels of monoamine neurotransmitters though the inhibition of enzymes related in the metabolism of catechol- and indoleamines. In addition, the selective neurotoxicity of N-methyl(R)salsolinol to dopamine neurons was confirmed by preparation of an animal model of Parkinson's disease in rats. The involvement of N-methyl(R)salsolinol in the pathogenesis of Parkinson's disease was further indicated by the increase in the N-methyl(R)salsolinol levels in the cerebrospinal fluid and that in the activity of its synthesizing enzyme, a neural (R)salsolinol N-methyltransferase, in the lymphocytes prepared from parkinsonian patients. N-Methyl(R)salsolinol induces apoptosis in dopamine neurons, which is mediated by death signal transduction in mitochondria. In addition, salsolinol was found to function as a signal transmitter for the prolactin release in the neuro-intermediate lobe of the brain. These results are discussed in relation to role of dopamine-derived endogenous salsolinol derivatives as the regulators of neurotransmission, dopaminergic neurotoxins and neuro-hormonal transmitters in the human brain.

Keywords: Salsolinol; Monoamine oxidase inhibitor; Parkinson's disease; Neurotoxin; Prolactin

INTRODUCTION

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, Sal) was detected in urine of parkinsonian patients administered with L-DOPA (Sandler et al., 1973). This finding stimulated the studies on Sal derivatives in the brain, and gave new aspects of the endogenous alkaloids, which had been considered to

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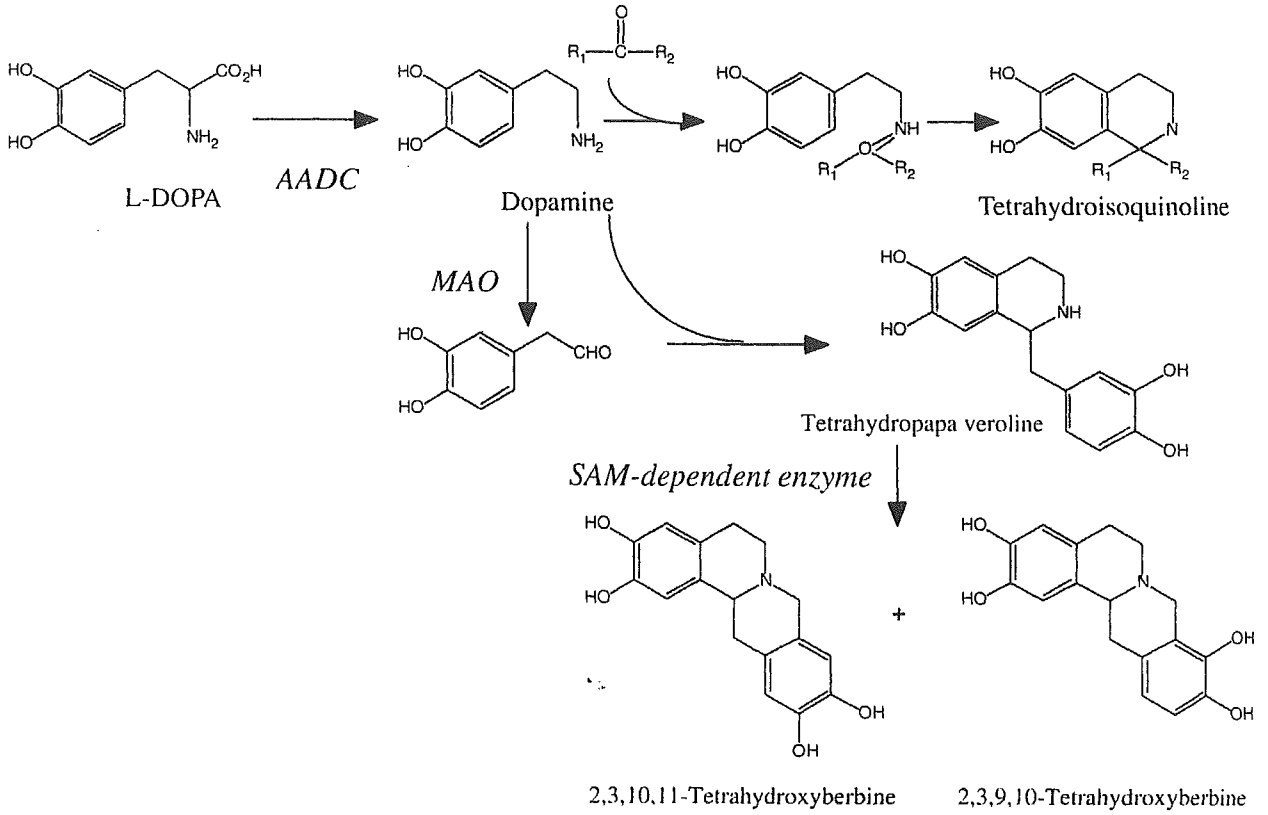

Fig. 1. Chemical synthesis of catechol isoquinolines from dopamine. L-DOPA is decarboxylated into dopamine by aromatic L-amino acid decarboxylase (AADC), which condensed with aldehydes into tetrahydroisoquinolines. Dopamine is oxidized by monoamine oxidase (MAO) into 3,4-dihydroxyphenylacetaldehyde, which generates tetrahydropapaveroline (THP) by condensation with dopamine. 2,3,10,11-Tetrahydroberbine and 2,3,9,10-tetrahydroberbine are produced from THP by *S*-adenosyl-L-methonine (SAM)-dependent enzymes.

occur only in plants. As shown in Fig. 1, the non-enzymatic Pictet–Spengler reaction, which involves the condensation of β-arylethanolamines with carbonyl compounds, produces Sal derivatives from dopamine with aldehydes or keto-acids. In human tissues three classes of monoamine-derived alkaloids have been reported; dopamine-derived catechol 1,2,3,4-tetrahydroisoquinolines (TIQs), and β-phenethylamine-derived TIQs without catechol structure and indoleamine-derived β-carbolines.

In normal non-alcoholic subjects and alcoholics, Sal and *O*-methylated Sal were found in urine, cerebrospinal fluid and brains (Collins, 1980; Sjöquist et al., 1981). A series of Sal-related isoquinolines were identified in human tissues as summarized in Table 1. In addition, in adrenal tissue 1-methyl-4,6,7-trihydroxy-TIQ and 1,2-dimethyl-4,6,7-trihydroxy-TIQ were detected as the condensation product of norepinephrine or epinephrine with acetaldehyde (Cohen and Collins, 1970). In the urine of phenylketonuric children, a TIQ carboxylic acid, 3′,4′-deoxynorlaudanosaline-carboxylic acid (DNLCA) was detected (Lasala and Coscia, 1979), which is derived from dopamine and phenylpyruvic acid. In addition, 3,4-dihydroxyphenylacetaldehyde, an oxidation product of dopamine by monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4, MAO), reacts with dopamine to produce tetrahydropapaveroline (1-(3′,4′-dihydroxybenzyl)-1,2,3,4-tetrahydro-6,7-dihydroxyisoquinoline, norlaudanosoline, THP) (Holtz et al., 1964). 3′-*O*-Methyl norlaudanosolinecarboxylic acid (Coscia et al., 1977) and tetrahydroberberines (THBs), produced from THP by *S*-adenosyl-L-methionine (SAM)-dependent enzymes, 2,3,10,11-THB and 2,3,9,10-THB were also identified in urine of parkinsonian patients receiving L-DOPA therapy (Cashaw et al., 1974).

Until recently isoquinolines had been considered to occur as racemic forms in humans, which are generated by non-enzymatic condensation of monoamines. However, more accurate and simpler chromatographic methods for analysis of Sal enantiomers proved the predominant occurrence of the (*R*)enantiomers in mammalian tissues, suggesting that Sal might be synthesized enzymatically.

This reviews summarizes the recent advances in our understanding on the occurrence, synthesis and

Table 1
Concentrations of salsolinol derivatives in human brain, CSF and IVF

Salsolinol derivatives	Tissue	Content (pmol/g wet weight)	Reference
Salsolinol	CSF: parkinsonian	<5[a]	Moser and Kämpf (1992)
	Striatum: control	19 ± 10	Ung-Chhun et al. (1985)
	Alcoholic	58 ± 21	
	Hypothalamus: control	43 ± 13	Ung-Chhun et al. (1985)
	Alcoholic	87 ± 49	
Salsolinol-1-carboxylic acid	Striatum	161 ± 69	Ung-Chhun et al. (1985)
	Hypothalamus	83 ± 42	
6 or 7-Methyl-salsolinol	CSF: control	1.3 ± 0.3[a]	Dordain et al. (1984)
	Parkinsonian	0.6 ± 0.2	
	Alcoholic	1.1 ± 0.3	
	Striatum: control	9 ± 6	Ung-Chhun et al. (1985)
	Alcoholic	11 ± 6	
	Hypothalamus: control	8 ± 3	Ung-Chhun et al. (1985)
	Alcoholic	2 ± 1	
2(*N*)-Methyl-norsalsolinol	CSF: parkinsonian	15, 26, 60[a]	Moser and Kämpf (1992)
1,2-Dimethyl-6,7-dihydroxyisoquinolinium ion	Substantia nigra	254 ± 59.0	Maruyama et al. (1997a)
	IVF	16.0 ± 16.5 nM	Maruyama et al. (1996b)

The data mentioned here were not determined for the enantiomeric characters.
[a] pmol/ml.

function of monoamine-derived isoquinolines in the human brains.

BIOSYNTHESIS OF (*R*)SALSOLINOL DERIVATIVES IN HUMAN BRAIN

Sal has an asymmetric center at first position and exists as (*R*)- and (*S*)enantiomer. Dostert and his colleagues found that in urine from healthy volunteers the (*R*)enantiomer of Sal is predominant, and proposed the biosynthesis pathway, as shown in Fig. 2 (Dostert et al., 1990). The condensation of dopamine with pyruvic acid yields 1-carboxyl-Sal, which is detected in human urine, CSF and caudate nucleus, but the enantomeric structure was not determined. The decarboxylation of 1-carboxyl-Sal produces 1,2-dehydrosalsolinol, which was also identified in human urine (Dostert et al., 1990). However, the enzymatic reduction of 1,2-dehydrosalsolinol or the decarboxylation of 1-carboxyl-Sal into optically active Sal has never been shown in animals or plants. This hypothesis of (*R*)Sal synthesis had not been fully confirmed.

More recently a quite sensitive method of Sal enantiomers has been developed by use of high-performance liquid chromatography (HPLC) with chiral columns and electrochemical detection (Maruyama et al., 1997a; Naoi et al., 1998b). Table 2 summarizes the results in analyses of Sal derivatives in human materials. Only the (*R*)enantiomers of Sal and *N*-methylated Sal occur in the human brain, cerebrospinal fluid (CSF) and intraventricular fluid (IVF), and the (*S*)enantiomers were not detected (Maruyama et al., 1996a,b, 1997c). Then, we isolated two enzymes involved in the synthesis of optically active Sal derivatives in the human brain. As shown in Fig. 2, a (*R*)salsolinol synthase catalyzes the enantio-selective synthesis of (*R*)Sal and 1-carboxyl(*R*)-Sal from dopamine with acetaldehyde or pyruvic acid (Naoi et al., 1996b). The decarboxylation of 1-carboxyl(*R*)Sal into 1,2-dehydrosalolsinol was confirmed to occur by a non-enzymatic reaction. However, the enantio-selective conversion of the latter into (*R*)Sal was not confirmed by use of human brain samples. The *N*-methylation of (*R*)salsolinol into *N*-methylsalsolinol (*N*MSal) is catalyzed by two *N*-methyltransferases with different optimum pH, at pH 7.0 and 8.4. *N*M(*R*)Sal is enzymatically oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$) by an oxidase sensitive to semicarbaside (Naoi et al., 1995) and also non-enzymatically by autoxidation (Maruyama et al., 1995). Table 3 summarizes the enzymatic characteristics of the enzymes participating the synthesis and metabolism of (*R*)Sal derivatives.

More recently, we reported that only the (*S*)enantiomer of THP was detected in human brains (Sango et al., 2000). The concentrations varied from 0.12 to 0.28 pmol/g wet weight of brain tissues, and were much lower than those of dopamine (450–5440 nmol/g wet

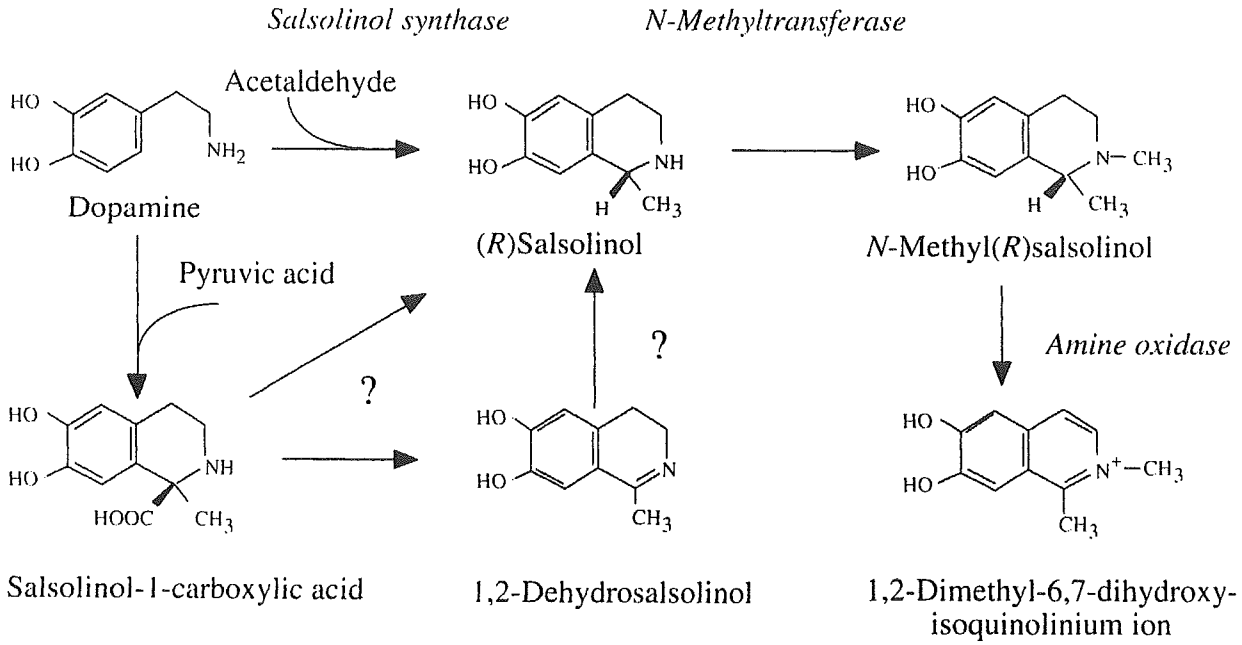

Fig. 2. Biosynthesis pathway of (*R*)salsolinol derivatives in human brain. The enzymatic condensation of dopamine with acetaldehyde or pyruvic acid is catalyzed by (*R*)salsolinol synthase to yield (*R*)Sal and (*R*)Sal-1-carboxylic acid. However, the enantio-selective synthesis of (*R*)Sal from (*R*)Sal-1-carboxylic acid and 1,2-dehydrosalsolinol was not confirmed. *N*-Methyltransferase catalyzes the *N*-methylation of (*R*)Sal into *N*M(*R*)Sal, which is further oxidized into 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$).

Table 2
Concentrations of (*R*)- and (*S*)enantiomer of salsolinol derivatives in human tissues, detected by HPLC

Salsolinol derivatives	Tissue	Concentration	Reference
(*R*)Salsolinol	Urine	1.95–11 ng/ml	Dostert et al. (1989)
		10.2–51.75 μg per day	Strolin Benedetti et al. (1989a)
		16.2–103.3 pmol/ml	Dostert et al. (1990)
	Alcoholic	47.5–231.1 nmol per day	Dostert et al. (1991)
	Plasma	0.397 ng/ml	Baum and Rommelspacher (1994)
		0.4–4.2 pmol/ml	Haber et al. (1996)
	Frontal cortex	134 ± 125 pmol/g	Maruyama et al. (1997a)
	Caudate	73.3 ± 79.9	
	Putamen	37.8 ± 23.0	
	Substantia nigra	94.5 ± 78.7	
	IVF	0.39 ± 0.21 nM	Maruyama et al. (1996b)
(*S*)Salsolinol	Urine	0–23.3 pmol/ml	Dostert et al. (1990)
	Alcoholic	56.1–139.5 nmol per day	Dostert et al. (1991)
	Plasma	0.4–3.1 pmol/ml	Haber et al. (1996)
	Brain regions, CSF, IVF	Not detected	Maruyama et al. (1996b)
N-Methyl(*R*)salsolinol	Caudate	65.7 ± 88.3 pmol/g	Maruyama et al. (1997a)
	Putamen	110 ± 126	
	Substantia nigra	76.6 ± 23.0	
	CSF		
	Parkinsonian	8.32 ± 2.89 nM	Maruyama et al. (1996a)
	Control	4.53 ± 2.08 nM	
	IVF	9.15 ± 9.08 nM	Maruyama et al. (1996b)

Measured by GC-MS.

Table 3
Characteristics of enzymes related to the metabolism of *N*-methyl-(*R*)salsolinol

Enzymes	Characteristics	Substrate
(*R*)Salsolinol synthase	Molecular weight: 34.3 kDa Optimum pH: 7.4	Dopamine Acetaldehyde Pyruvic acid
Neutral (*R*)salsolinol *N*-methyltransferase	Methyl donor: SAM[a] Molecular weight: 35.6 kDa Optimum pH: 7.0	(*R*)Sal, norsalsolinol
N-Methyl(*R*)salsolinol oxidase	Sensitive to semicarbazide, not to clorgyline, (−)deprenyl	*N*M(*R*)Sal, *N*M(*S*)Sal, *N*-methylnorsalsolinol

These enzymes were isolated from the cytosol soluble fraction of human brain gray matter.
[a] *S*-Adenosyl-L-methionine.

weight) and (*R*)Sal (19–117 pmol/g wet weight). It is not yet clarified whether the synthesizing enzyme is identical with the enzyme catalyzing the synthesis of (*R*)Sal.

THE DISTRIBUTION OF SALSOLINOL DERIVATIVES IN BRAIN REGIONS AND FACTORS AFFECTING THE CONCENTRATIONS

The distribution of Sal, *N*MSal and $DMDHIQ^+$ was examined in four major regions of control brains, the frontal cortex, caudate, putamen and substantia nigra (Maruyama et al., 1997c). *N*M(*R*)Sal and its precursor, dopamine, were found to occur selectively in the nigro-striatum, whereas (*R*)Sal distributes uniformly among the brain regions, as shown in Table 2 and Fig. 3. On the other hand, $DMDHIQ^+$ with chemical structure similar to, a dopaminergic neurotoxin, 1-methyl-4-phenyl-pyridinium ion (MPP^+) was detected only in the substantia nigra (Table 1). These results suggest that their concentrations do not depend on those of dopamine, but on the activity of the synthesizing enzymes. The *N*-methylation of (*R*)Sal by an *N*-methyltransferase, whose activity is higher in the nigro-striatum than in other brain regions (Maruyama et al., 1992), brings about the selective distribution in dopamine neurons. Binding of $DMDHIQ^+$ to melanin may account for the selective accumulation of the isoquinolinium ion in the substantia nigra (Naoi et al., 1994a).

The factors determining the levels of Sal derivatives, the age, alcohol contents in blood, dopamine turnover, and the enzyme activities related to the metabolism, were examined in human brain samples. The levels of (*R*)Sal decreased in the caudate, putamen and substantia nigra according to the age (Maruyama et al., 1997c). A negative correlation was confirmed between the level of *N*M(*R*)Sal in the striatum and the age. The effects of alcohol level in blood on the concentrations of Sal derivatives were examined, and as shown in Fig. 3, the presence of alcohol did not affect the levels of (*R*)enantiomer of Sal and *N*MSal in any brain regions, whereas the (*S*)enantiomers were not detected even in alcoholic brains. These results suggest that the levels of acetaldehyde, a product of ethanol, do not determine those of Sal derivatives in the brain. The level of a dopamine product, homovanillic aid (HVA), or the ratio of HVA/dopamine, an indicator of dopamine turnover, did not correlate with the levels of catechol isoquinolines in the human brain.

We examined the effects of the activities of the enzymes related to the metabolism on the levels of Sal derivatives in brain regions, the frontal cortex, caudate, putamen, substantia nigra and thalamus (Naoi et al., 1997). The activities of (*R*)Sal synthase, (*R*)Sal *N*-methyltransferase with the optimal pH at 7.0 (neutral) and at 8.4 (alkaline), and of *N*M(*R*)Sal oxidase were compared with the levels of (*R*)Sal, *N*M(*R*)Sal and $DMDHIQ^+$. A good correlation was confirmed between the activity of neutral (*R*)Sal *N*-methyltransferase in the striatum (putamen and caudate) and the concentration of $DMDHIQ^+$ in the substantia nigra, as shown in Fig. 4. There was no significant correlation between the activities of other enzymes and the levels of isoquinolines in the these brain regions. These results clearly show that the enzymatic activity of *N*-methyltransferase regulates the level of *N*M(*R*)Sal, a neurotoxin, in the nigro-striatum of human brains. In addition, it is suggested that *N*M(*R*)Sal is synthesized in the striatum, transported to the substantia nigra and oxidized into $DMDHIQ^+$, which accumulates as conjugated with melanin. This is comparable with the results observed in a rat model of Parkinson's disease, where $DMDHIQ^+$ was detected in the substantia nigra

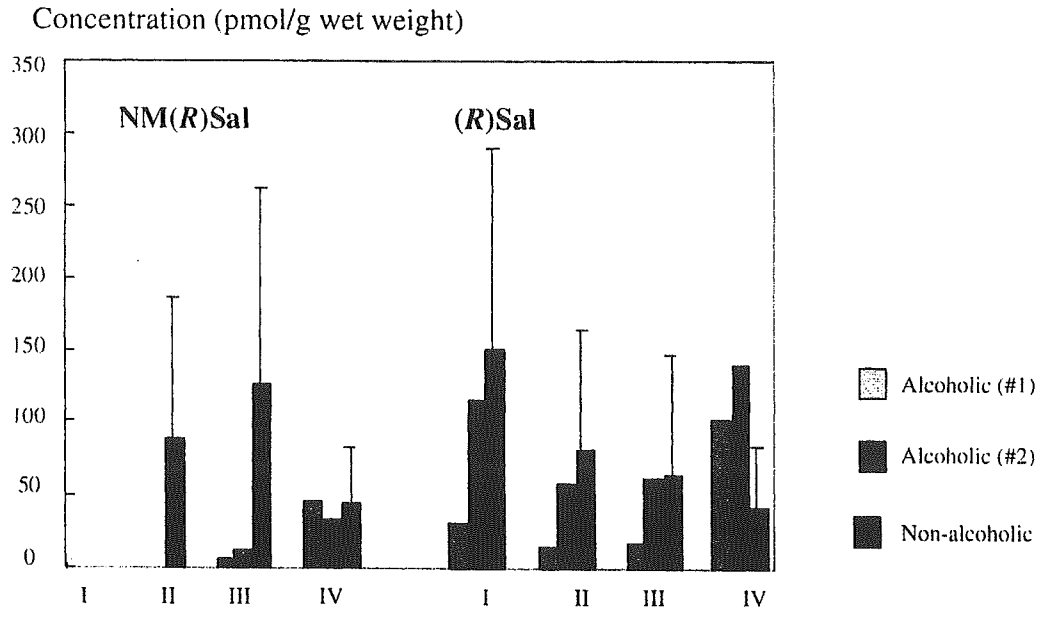

Fig. 3. Concentration of (*R*)salsolinol ((*R*)Sal) and *N*-methyl(*R*)salsolinol (*N*M(*R*)Sal) in the four regions of control human brains, and the effects of ethanol in blood. I: frontal cortex; II: caudate; III: putamen; IV: substantia nigra. In peripheral blood of alcoholic case 1 and 2, 3.3 and 0.1 mg/ml of ethanol were detected. The results of non-alcoholic were the mean ± S.D. of seven cases without ethanol in blood. In frontal cortex, *N*M(*R*)Sal was not detected in any sample.

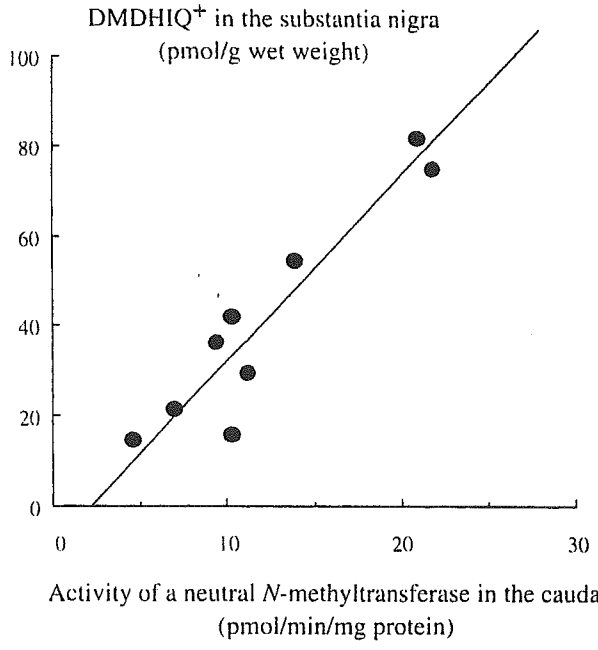

Fig. 4. The effect of the activity of a neutral (*R*)salsolinol *N*-methyltransferase in the caudate on the levels of 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion ($DMDHIQ^+$) in the substantia nigra. The frontal cortex, caudate, putamen and substantia nigra of nine control human brains were analyzed for the levels of (*R*)Sal, *N*M(*R*)Sal and $DMDHIQ^+$, and the activities of (*R*)Sal synthase, neutral and alkaline (*R*)Sal *N*-methyltransferases. Significant correlation was confirmed between the $DMDHIQ^+$ level in the substantia nigra and the activities of neutral *N*-methyltransferase in the caudate ($r = 0.905$, $P < 0.001$) and putamen ($r = 0.616$, $P < 0.05$).

after injection of *N*M(*R*)Sal in the striatum (Naoi et al., 1996a).

SALSOLINOL DERIVATIVES AS INHIBITORS OF TYPE A MONOAMINE OXIDASE

The inhibition of MAO by Sal was first reported by Yamada (1971). Sal inhibited MAO activity in rat brain stem and liver, and the inhibition was competitive to serotonin, a substrate specific for type A. Sal and THP inhibited rat brain MAO in vitro and the inhibition was competitive to serotonin and non-competitive to benzylamine, a substrate of type B MAO (Collins et al., 1973; Meyerson et al., 1976). A series of (*R*)- and (*S*)Sal were examined for the effects on the activities of type A and B MAO, as summarized in Table 4. The (*R*)enantiomers of Sal and *N*MSal inhibited MAO-A more potent than the (*S*)enantiomers, and the inhibition to type A MAO was competitive to the substrate, and to type B was noncompetitive (Dostert et al., 1989; Minami et al., 1993). The oxidized $DMDHIQ^+$ is the most potent inhibitor of MAO-A, followed by *N*M(*R*)Sal, (*R*)Sal and *N*-methylnorsalsolinol (Naoi et al., 1994b). The presence of hydroxyl groups at sixth and seventh position and substitution of a hydrogen group at first position with a methyl or dihydroxybenzyl group are required for the inhibition, whereas

Table 4
The K_i value of salsolinol derivatives to type A and B monoamine oxidase in human brain synaptosomes

Salsolinol derivatives	K_i values (μM) for monoamine oxidase	
	Type A	Type B
(*R*)Salsolinol	37.9	68.3
(*S*)Salsolinol	149.5	149.5
2(*N*)-Methyl(*R*)salsolinol	36.1	433.3
2(*N*)-Methyl(*S*)salsolinol	81.3	No inhibition
Norsalsolinol	No inhibition	No inhibition
2(*N*)-Methynorsalsolinol	61.4	289
1,2(*N*)-Dehydrosalsolinol	322.3	No inhibition
1-Carboxyl(*R*)salsolinol	421.3	No inhibition
1,2(*N*)-DM-6,7-DHIQ^{+a}	9.21	No inhibition
2(*N*)-M-6,7-DHIQ^{+a}	44.4	No inhibition
2(*N*)-M-1-carboxyl(*R*)salsolinol[b]	No inhibition	No inhibition

[a] 1,2(*N*)-Dimethyl- and 2(*N*)-methyl-6,7-dihydroxyisoquinolinium ion.
[b] 2(*N*)-Methyl-1-carboxyl(*R*)salsolinol.

the absence of a methyl group or a presence of a carboxy group at first position in addition to a methyl group depletes the inhibitory activity. The structure–activity relationship of isoquinoline derivatives was reported in details by Bembenek et al. (1990) and Thull et al. (1995).

To examine the effects of Sal derivatives on the neurotransmitter levels in the brains, in vivo microdialysis studies were carried out. The analyses of monoamine metabolites in the microdialysate in the rat striatum indicated that Sal derivatives inhibited MAO and COMT activities in situ and the levels of 3,4-diphenylacetic acid (DOPAC) and homovanillic acid (HVA) reduced markedly, and 5-hydroxyindolacetic acid (5-HIAA) most significantly, as shown in Fig. 5 (Maruyama et al., 1993). The (*R*)enantiomers were more potent than the (*S*)enantiomers, and the presence of a catechol structure and a methyl group at 1 or 2(*N*) position increased the effects, as in the case of the in vitro experiments. These results demonstrate

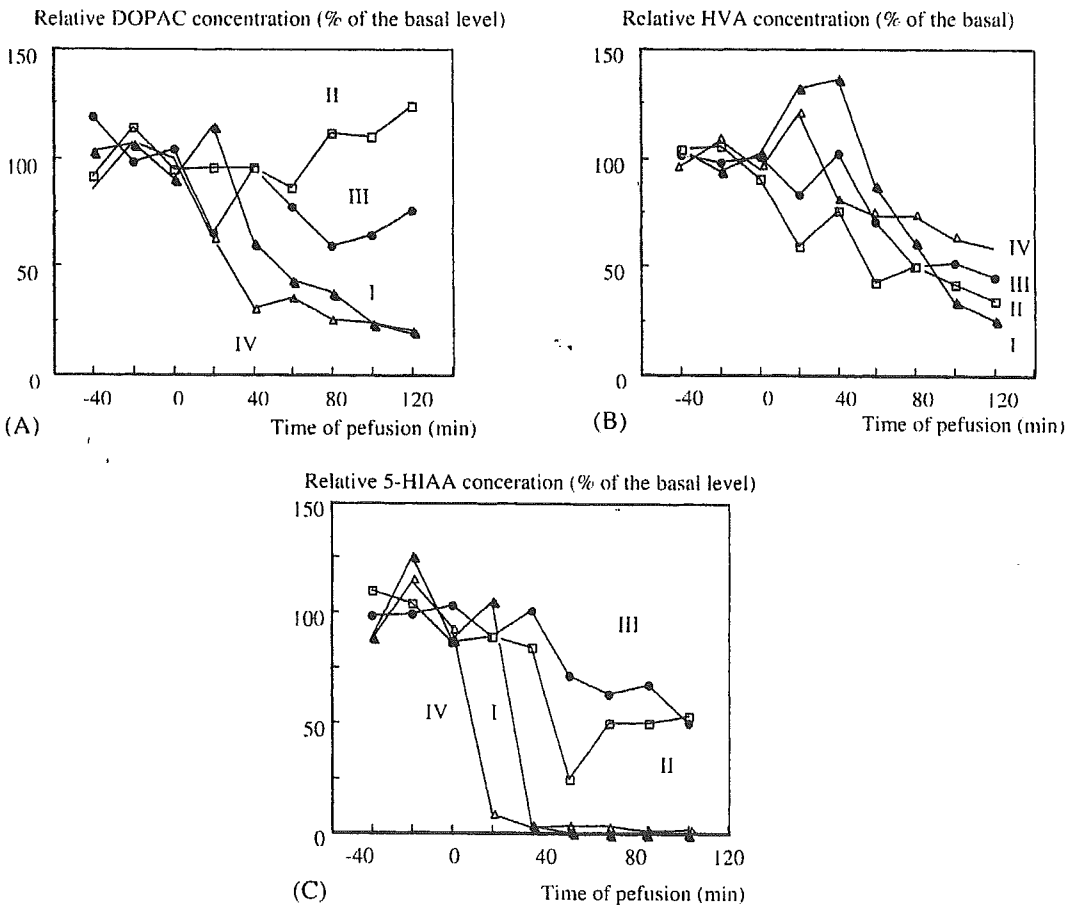

Fig. 5. In vivo effects of (*R*)salsolinol derivatives on the metabolism of catechol- and indoleamines in the rat striatum; in vivo microdialysis studies. Rat striatum was injected with isoquinoline solution in saline and collected samples were analyzed by HPLC with multi-electrochemical detectors: (A) the levels of 3,4-dihydroxyphenylacetic acid (DOPAC); (B) homovanillic acid (HVA); and (C) 5-hydroxyindolacetic acid (5-HIAA), expressed as percent of the basal level. I: (*R*)Sal; II: *N*M(*R*)Sal; III: norsalsolinol (6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline); IV: 2(*N*)-methylnorsalsolinol.

that Sal derivatives inhibit type A MAO more markedly than type B in the brain, also by in vivo experiments.

THE EFFECTS OF SALSOLINOL DERIVATIVES ON THE METABOLISM OF MONOAMINES

Sal and related TIQs inhibit the enzymes participating in the metabolism of monoamines. Sal inhibits a rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase (tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2, TH), prepared from rat brain. The inhibitor constant, K_i, value for Sal was 14 μM and the inhibition was competitive to a cofactor, 6,7-dimethyl-5,6,7,8-tetrahydropterin (Weiner and Collins, 1978). The naturally occurring cofactor of TH, L-*erythro*-5,6,7,8-tetrahydrobiopterin, induces allostery to the enzyme polymers and to change the affinity to the biopterin itself. The (*R*)enantiomer of Sal deletes the allostery of TH to the biopterin and reduces the activity more markedly than the (*S*)Sal (Minami et al., 1992). Norlaudanosolinecarboxylic acids and other TIQ carboxylic acids are potent inhibitors of TH, and the inhibition was non-competitive with respect to its substrate, tyrosine, and its cofactor, 6-methyltetrahydropterin (Coscia et al., 1980). These TIQs inhibited also dopamine-β-hydroxylase (3,4-dihydroxy-phenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1). One or more free hydroxyl groups of salsolinol and THP were methylated by catechol-*O*-methyltransferase (*S*-adenosyl-L-methionine: catechol *O*-methyltransferase, EC 2.1.1.6, COMT) (Collins et al., 1973). They are competitive inhibitors of dopamine *O*-methylation in vitro. COMT was inhibited by other TIQs, 1-benzyl and 1-methyl derivatives of 6,7-dihydroxy-3,4-dihydroisoquinolines, and the inhibition was non-competitive to substrate (Cheng et al., 1987).

Sal derivatives inhibit a rate-limiting enzyme in indoleamine synthesis, trytophan hydroxylase (L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14,16,2, TPH) (Matsubara et al., 1994). Sal derivatives inhibited markedly the activity of TPH, and $DMDHIQ^+$ was an extremely potent inhibitor. The inhibition was non-competitive in terms of either the biopterine cofactor or the substrate, L-tryptophan. On the other hand, the condensation products of indoleamines with acetaldehyde, β-carbolines, did not inhibit the TPH activity.

N-METHYL(*R*)SALSOLINOL AS AN ENDOGENOUS NEUROTOXIN TO DOPAMINE NEURONS: POSSIBLE INVOLVEMENT TO THE PATHOGENESIS OF PARKINSON'S DISEASE

Studies with 1-methyl-4-phenyl-1,2,3,6-tetrahydrpyridine (MPTP) (Langston et al., 1983) suggest that endogenous and xenobiotic neurotoxins may cause parkinsonism in humans. To find dopaminergic neurotoxins, the catechol isoquinolines were injected into the striatum of male Wistar rats, and the behavioral, biochemical and histo-pathological effects were examined. Among catechol isoquinolines, *N*M(*R*)Sal was the most cytotoxic to dopamine neurons, causing behavioral, histopathological and biochemical changes in the nigro-striatum, which were quite similar to those observed in parkinsonian patients (Naoi et al., 1996a, 1998a). After 1 week continuous injection of *N*M(*R*)Sal in the striatum, the number of dopamine neurons stained with anti-TH antibody reduced markedly in the substantia nigra, but necrotic reaction was not observed, suggesting that *N*M(*R*)Sal caused apoptotic cell death selectively in dopamine neurons of the rat model.

The involvement of *N*M(*R*)Sal in the pathogenesis of Parkinson's disease was proved by analyses of CSF from parkinsonian patients (Maruyama et al., 1996a). The *N*M(*R*)Sal levels in CSF from parkinsonian patients were significantly higher than in control, as summarized in Table 2. In parkinsonian CSF, the presence of Sal (Moser and Kämpf, 1992) and *N*-methylnorsalsolinol (2(*N*)-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) (Moser et al., 1995) was reported, but it was not confirmed whether they increased in Parkinson's disease (Muller et al., 1999).

These results suggest that the metabolism of *N*M(*R*)Sal in the brain may be altered in Parkinson's disease. To confirm this point, the activities of enzymes related to the metabolism of *N*M(*R*)Sal were examined in lymphocytes prepared from parkinsonian patients (Naoi et al., 1998c). Among enzymes, only the activity of a neutral (*R*)Sal *N*-methyltransferase was significantly higher in parkinsonian lymphocytes. Recently the correlation was confirmed between the neutral (*R*)Sal *N*-methyltransferase activity in lymphocytes and the *N*M(*R*)Sal concentration in CSF from untreated parkinsonian patients. The results support our view that the activity of a neutral (*R*)Sal *N*-methyltransferase in the brain may determine the neurotoxin level in the substantia nigra of parkinsonian brains.

Table 5
Cytotoxicity of salsolinol derivatives to SH-SY5Y cells

Salsolinol derivatives	Apoptotic cells (percent of the total cells)	Necrotic cells
Control	4.86 ± 4.34	1.21 ± 1.68
(*R*)Salsolinol	9.55 ± 2.26	1.70 ± 1.41
(*S*)Salsolinol	10.71 ± 4.54	1.33 ± 0.68
N-Methyl(*R*)salsolinol	100[a]	0
N-Methyl(*R*)salsolinol[b]	26.73 ± 4.57[a]	0.85 ± 0.68
N-Methyl(*S*)salsolinol	10.9 ± 3.04	0.28 ± 0.44
DMDHIQ$^+$	28.02 ± 9.09[a]	1.58 ± 1.8
(*R*)-1-Carboxy-Sal	3.73 ± 1.75	1.08 ± 1.10
(*S*)-1-Carboxy-Sal	5.72 ± 2.41	1.01 ± 0.78
Norsalsolinol	34.06 ± 5.05[a]	1.63 ± 1.68
N-Methylnorsalsolinol	8.87 ± 0.73	0.13 ± 0.32
(*R*)-THP	0.52 ± 0.62	40.0 ± 6.71[a]
(*S*)-THP	0.07 ± 0.28	41.1 ± 8.10[a]
N-Methyl-THP	13.72 ± 4.48	0.94 ± 1.35
Tetrahydropapaverine	10.13 ± 4.57	1.79 ± 0.50
Papaveroline	32.30 ± 12.37[a]	67.88 ± 12.37[a]
N-Methylpapaveroline	100[a]	0
N-Methylpapaveroline[b]	30.82 ± 3.06[a]	3.93 ± 1.24

The apoptotic and necrotic cells were assessed by morphological observation of four microscopic fields containing about 200 cells, after staining with Hoechst 33258.

[a] The concentrations used were 500 μM, except 250 μM. The number represents mean ± S.D. of three independent experiments.

[b] Difference from control is statistically significant, $P < 0.01$.

Recently, apoptosis of dopamine neurons was confirmed in the substantia nigra of parkinsonian brains: activation of caspase 3, increase in Bax, and translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into nuclei (Hartmann et al., 2000; Tatton, 2000). *N*M(*R*)Sal induced apoptosis in dopamine neurons, as shown in the rat model, and the mechanism of cell death was studied in human dopaminergic neuroblastoma SH-SY5Y cells (Akao et al., 1999, 2002; Maruyama et al., 1997b, 2001a,b; Naoi et al., 2002). Among catechol isoquinolines, *N*M(*R*)Sal was the most potent to induce DNA damage, whereas *N*M(*S*)Sal, Sal and 1-carboxy-Sal were less cytotoxic, as summarized in Table 5. Apoptosis was initiated by mitochondrial permeability transition (PT) as shown by the collapse in membrane potential, $\Delta\Psi_m$, followed by release of cytochrome *C*, activation of caspase 3, nuclear translocation of GAPDH and final fragmentation of nucleosomal DNA. Only the (*R*)enantiomer of *N*MSal induced $\Delta\Psi_m$ collapse in SH-SY5Y cells, and the (*S*)enantiomer did not (Maruyama et al., 2001a,b). The enantio-specificity to induce apoptosis was confirmed also in isolated mitochondria (Akao et al., 2002). These results suggest the presence of a binding site of *N*M(*R*)Sal in mitochondria outer membrane, which recognizes the enantiomeric structure and initiates apoptotic signal transduction. In the case of THP, the oxidized papaverolines induced apoptosis at the lower concentrations, whereas the reduced tetrahydropapaverolines induced necrosis by the potent inhibition of ATP synthesis in mitochondria (Maruyama et al., 2000).

Racemic Sal (Storch et al., 2000) and 1,2,3,4-tetrahydro-2-methyl-4,6,7-isoquinolinetriol (Willets et al., 1996) were also cytotoxic to dopamine neurons, but a quite different mechanism seems to function in the induction of cell death. These isoquinolines produce reactive oxygen species (ROS) by autoxidation, resulting in metabolic compromise, and necrotic, rather than apoptotic, cell death was induced.

FUNCTION OF SALSOLINOL IN THE BRAIN

The synthesis of Sal from dopamine and acetaldehyde, a product of ethanol, suggested its involvement in alcohol addiction (Cohen and Collins, 1970). The hypothesis that opiates may be biosynthesized from THP-related complex isoquinolines initiated the studies to find the involvement of THP in alcohol addicts possibly mediated by opiates. However, this hypothesis has not been proved after so many contradicting publications concerning the role of Sal and THP in alcoholism.

Recently we found a quite novel function of Sal in the brain. Dopamine as a neuro-hormone is delivered to the anterior lobe through the vascular connection between the hypothalamus and pituitary gland. It inhibits prolactin and maintains mammotropes in their tonically suppressed secretary state (Freeman et al., 2000). Suckling the nipples of lactating mothers elevated plasma prolactin, which may be caused by increased secretion of a prolactin-releasing hormone (PRH) from mammotropes of the anterior lobe of the pituitary galnd. Toth et al. (2001) detected Sal in the neuro-intermediate lobe and median eminence in rats and the levels increased by suckling in lactating rats, as shown in Table 6. The increase was in parallel with plasma prolactin levels. More recently, selective binding of Sal was confirmed in the rat striatum, anterior lobe and neuro-intermediate lobe of the pituitary gland, in addition to the cortex, median eminence and hypothalamus (Homicsko et al., 2002). Their results suggest that vesicular monoamine transporter in dopaminergic terminals may be a target of Sal. The values of the binding constant, K_D, were in the nanomolar ranges. These results suggest that Sal may

Table 6
Concentrations of (*R*)salsolinol in the neuro-intermediate lobe of the pituitary gland and the median eminence

Rats	(*R*)Salsolinol concentration (pmol/mg wet weight)	
	Neuro-intermediate lobe	Median eminence
Control male	1.67 ± 1.27*	3.86 ± 2.19**
Female (ovariectomized)	1.38 ± 0.39	11.96 ± 4.69
Female (ovariectomized + E_2)	6.38 ± 2.20*	6.25 ± 2.63*
Female (sham operated)	30.90 ± 2.6	ND[a]
Female (neuro-intermediate lobe denervated)	2.60 ± 0.6**	ND[a]

Adult male, ovariectomized, overirectomized rats administered with17β-oestradiol (E_2) or primiparous lactating female rats were used (Toth et al., 2001). Sal was measured by HPLC with electrochemical detection (Maruyama et al., 1997a).

[a] Not determined.

* $P < 0.05$ vs. female (ovariectomized).

** $P < 0.05$ vs. female (sham operated).

regulate the function of dopamine neurons as a neurotransmitter and may be a mediator in neuro-endocrine system, though its specified binding sites and intervention in dopamine system.

CONCLUSION

After the discovery of Sal by M. Sandler in 1973, the biosynthesis, metabolism and function of catechol isoquinoline derivatives have been clarified in human brains, as reviewed here. The stereochemical characters of the (*R*)enentiomers induce their selective occurrence and physiological function in human brain. The metabolism in situ, *N*-methylation and oxidation, gives arise novel biological activities, which may be involved in neurodegenerative disorders, such as Parkinson's disease. The recent results on the binding site of Sal suggest further new advances in finding the role of Sal as a mediator of dopamine neurons in the brain. The studies on salsolinol for these near 30 years show that the finding of a new compound in humans can trigger so-wide development of a series of new theories and findings, which should be attributed to M. Sandler.

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N-Propargyl-1 (*R*)-aminoindan, rasagiline, increases glial cell line-derived neurotrophic factor (GDNF) in neuroblastoma SH-SY5Y cells through activation of NF-κB transcription factor

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Abstract

N-Propargyl-l(*R*)-aminoindan, rasagiline, an anti-Parkinson drug, was found to increase the protein and mRNA levels of glial cell line-derived neurotrophic factor (GDNF) in human neuroblastoma SH-SY5Y cells, whereas an analogue without a propargyl residue, aminoindan, did not. GDNF is known to protect dopaminergic neurons in animal and cellular models of Parkinson's disease, and the supplement has been tried for the treatment of degenerating dopamine neurons in Parkinsonian patients. In this paper, intracellular mechanism underlying the induction of GDNF was studied. Rasagiline induced phosphorylation of inhibitory subunit (IκB) of nuclear factor-κB (NF-κB), and translocation of active p65 subunit from cytoplasm into nuclei. Activation of NF-κB was also quantitatively determined by NF-κB p65 transcription assay. Sulfasalazine, an inhibitor of IκB kinase, suppressed the activation of NF-κB and the increase of GDNF by rasagiline simultaneously, further indicating the involvement of the IκB kinase-NF-κB pathway. The results on the activation of the transcription factor by rasagiline are discussed in relation to its possible application as a neuroprotective drug to halt declining of neurons in neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases.

Keywords: Rasagiline; GDNF; NF-κB; Neuroprotection; Anti-Parkinson drugs; Neurotrophic factors

1. Introduction

In neurodegenerative disorders including Parkinson's disease (PD), hitherto treatments are intended to replace deficient neurotransmitters, such as dopamine. Recently, a new therapeutic strategy has been proposed to protect neurons from cell death by attenuating apoptotic signal transduction (Thompson, 1995). Apoptosis is proposed to be a major type of neuronal death in PD, as shown by the activated apoptotic cascade in the nigro-striatum of Parkinsonian brains (Tatton, 2000).

Recently, (-)deprenyl (selegiline) and related propargylamines were reported to prevent the cell death in neurons caused by various stimuli in animal and cellular models of neurodegenerative disorders. We reported that a series of propargylamines with β-phenethylamine, aminoindan and aliphatic structure protected human dopaminergic neuroblastoma SH-SY5Y cells from apoptosis induced by *N*-methyl(*R*)salsolinol [*N*M(*R*)Sal], an endogenous neurotoxin selective to dopamine neurons, 6-hydroxydopamine,

Abbreviations: BDNF, brain-derived neurotrophic factor; ΔΨm, mitochondrial membrane potential; DTT, dithiothreitol; ECF, enhanced chemifluorescence; EIA, enzyme immunoassay; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; HRP, horse radish peroxidase; IκB, inhibitor subunit of NF-κB; MAP, mitogen-activated protein; MEM, minimum essential medium; NF-κB, nuclear transcription factor kappa B; NGF, neurotrophic growth factor; *N*M(*R*)Sal, *N*-methyl(*R*)salsolinol; PBS, phosphate-buffered saline; PD, Parkinson's disease; SOD, superoxide dismutase; TNF-α, tumor necrosis factor α

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and peroxynitrite (Maruyama et al., 2000, 2001a,b, 2002). Among propargylamines, *N*-propargyl-1(*R*)-aminoindan (rasagiline) was found to be the most potent to suppress apoptosis (Maruyama and Naoi, 1999). Rasagiline is a propargylamine with a cyclic benzylamine, and not metabolized into amphetamine-like compounds as in the case with selegiline (Finberg et al., 1996), and it is now under the phase III trials to treat PD (Parkinson Study Group, 2002).

The mechanism of neuroprotection by propargylamines was studied using SH-SY5Y cells, and rasagiline was found to increase mRNA and protein levels of anti-apoptotic bcl-2 and bcl-xL in SH-SY5Y cells (Akao et al., 2002b). In addition, rasagiline treatment increased the activities of superoxide dismutase (SOD) and catalase in the rat brain regions containing dopamine neurons (Carrillo et al., 2000). These results suggest that rasagiline and related propargylamines may induce anti-apoptotic, pro-survival genes by activation of common intracellular signals, such as transcription factors. Nuclear factor-κB (NF-κB) is one of the most important transcriptional factors and its activation was reported to be essential for neuronal survival mediated by tumor necrosis factor-α (TNF-α) and delayed preconditioning (Blondeau et al., 2001; Kaltschmidt et al., 1999; Mattson et al., 2000). The activation of NF-κB was reported to induce pro-survival Bcl-2 and Bcl-xL, SOD, brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF), a selective neurotrophic factor for dopamine neurons (Tomac et al., 1995; Tamatani et al., 1999). NF-κB is a redox-sensitive transcription factor and activated by various pathogenic stimuli, including oxidative stress, ischemic insult and β-amyloid deposition. Commonly, NF-κB consists of two subunits of 65 kDa (p65: RelA) and 50 kDa (p50) or 52 kDa (p52), and is sequestered in the cytoplasm as an inactive complex with NF-κB inhibitory subunit (IκB). Upon stimulation, IκB is phosphorylated, dissociated from the complex and degraded by the ubiquitin-proteasome system. This reaction allows translocation of free, active NF-κB complex into nuclei, where it binds to specific DNA motifs in the promoter/enhancer regions of target genes and activates transcription. However, it has never been reported whether activated NF-κB mediates the anti-apoptotic function of neuroprotective agents, such as propargylamines.

This paper reports the increase of GDNF level by rasagiline in SH-SY5Y cells, in addition to previously reported Bcl-2. The mechanism underlying the gene induction of GDNF was studied in concern to the activation of NF-κB. The possibility to develop a new neuroprotective therapy for PD and other neurodegenerative diseases is discussed.

2. Materials and methods

2.1. Materials

(*R*)(+)-*N*-Propargyl-1-aminoindan (rasagiline) prepared as reported previously (Youdim et al., 1995) was kindly donated by Teva Pharmaceutical Co. (Netanya, Israel). Lactacystin and sulfasalazine were purchased from Sigma (Missouri, St. Louis, USA); NF-κB p65 transcription assay kit (Trans-AM NF-κB) from Active Motif (Carlsbad, CA, USA); enhanced chemifluorescence (ECF) Western blotting kit from Amersham Life Science (Buckinghamshire, UK). The lysis buffer for immunoblotting, RIPA buffer kit (50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitors), was obtained from Boehringer Mannheim (Mannheim, Germany). Anti-NF-κB p65 antibody (#100-4165) was purchased from Rockland (Gilbertsville, PA, USA); anti-IκB and anti-phosphorylated IκB (p-IκB) (Ser32/36) antibodies were from Cell Signaling (Beverly, MA, USA). Dulbecco's modified Eagle minimum essential medium (MEM) and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Cell culture

SH-SY5Y cells were cultured in 75 cm^2 flasks at 37 °C in 95% atmosphere and 5% CO_2 and the culture medium used was Cosmedium-001 (CosmoBio, Tokyo, Japan) supplemented with 5% newborn calf serum.

2.3. Quantitative measurement of the protein and mRNA levels of GDNF

SH-SY5Y cells were cultured in 96-well poly-L-lysine coated plates (2×10^4 cells per well) in Cosmedium-001 with 5% calf serum for 24 h, then the medium was changed to MEM with or without rasagiline or aminoindan (the final concentration, 1 μM–10 nM) for 3 h, or with 100 nM of rasagiline for 3, 6, 18 and 24 h. The effect of sulfasalazine (100 μM), an inhibitor of IκB kinase (Wahl et al., 1998), was examined by adding the inhibitor 30 min before the treatment with rasagiline. After the treatment, the cells and the medium were frozen immediately and stored at −20 °C until analysis. The protein amount of GDNF was quantified as reported previously using the enzyme immunoassay (EIA) (Nitta et al., 2002). GDNF used as a standard was donated by Amgen (Munich, Germany). Anti-GDNF antibody was prepared and purified from rabbit serum (Nitta et al., 1999). The 96-well plates (Falcon 3910, NJ, USA) were incubated with 5 μl of anti-GDNF antibody in 500 μl of 0.1 M Tris–HCl buffer, pH 9.0, per well overnight, washed with the washing buffer (0.1 M Tris–HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% Na_3N, 0.1% bovine serum albumin and 1 mM $MgCl_2$), and then blocked with the washing buffer containing 1% skim milk. Samples or standard in the washing buffer were added to GDNF antibody-coated wells, and the plate was incubated for 12–18 h at 4 °C. The biotinylated secondary antibody was reacted in avidin-conjugated β-galactoside (Boebringer Mannheim) for 1 h. After washing with the washing buffer, the enzyme activity retained in each well was measured by incubation with a fluorescent

substrate, 4-methylumbelliferyl-β-D-galactoside (100 μM) in the washing buffer. The fluorescence intensity of a product, 4-methylumbelliferone, was measured at 360 nm with excitation at 448 nm. Standard curve of human recombinant GDNF from 5.08 pg/ml to 3.3 ng/ml was used.

For the measurement of mRNA of GDNF by reverse transcription-polymerase chain reaction (RT-PCR), the cells were treated with 1 μM–10 nM of rasagiline for 3 h and the total RNA was isolated using a TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). To avoid DNA contamination, the samples were subjected to DNase I (Invitrogen). Single strand cDNA was synthesized from 1 μg total RNA in a volume of 20 μl containing 0.5 μg oligo(dT)12–18 primer (Invitrogen), 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 units of RNasin ribonuclease inhibitor (Promega, Madison, WI, USA), 0.5 mM each of dATP, dCTP, dGTP, and dTTP, 10 mM DTT and 200 units of MMLV reverse transcriptase (SuperScript™ II, Invitrogen). The reaction mixture was incubated for 50 min at 42 °C, terminated by heating for 15 min at 70 °C with 37 cycles. Under this condition, the linearity of mRNA increment to the number of PCR cycles was confirmed. To remove RNA complementary to the cDNA, 2 units of RNase H were added and incubated for 20 min at 37 °C. PCR was performed at an annealing temperature of 57 °C using the following primers: GDNF, 5′ CGGGACTCTAAGATGAAGTTATGGGATGTCGTG-3′ and 5′-GGGTCAGATACATCCACACCGTTTAGCGGAATGC-3′, giving a 651-bp product; GAPDH, 5′-GGAGATTGTTGCCATCAACGAC-3′ and 5′-ATGAGCCCTTCCACAATGCCAAAG-3′, giving a 441-bp product.

2.4. Western blot analysis of NF-κB, IκB and phosphorylated IκB

NF-κB p65 translocated into nuclei was identified by Western blotting of the subcellular fractions using ECF Western blotting kit. SH-SY5Y cells were cultured in 75 cm^2 flasks with Cosmedium-001 and 5% calf serum overnight. The medium was changed to MEM with rasagiline (the final concentrations, 1 and 0.1 μM) and incubated at 37 °C. The cells were harvested and washed twice by PBS, suspended in 10 mM sodium phosphate buffer, pH 7.4, then allowed to stand at 4 °C for 30 min. To the cell suspension, the equal volume of 0.64 M sucrose in 10 mM sodium phosphate buffer, pH 7.4, was added. The sample was homogenized and centrifuged at 800 × *g* for 15 min. The pellet and the supernatant were used as nuclear and cytoplasmic fraction, respectively. The nuclear sample was prepared from pellet dissolved in the RIPA buffer by sonication and centrifugation. The purity of the nuclear fraction was confirmed by detection of the nuclear and cytoplasmic markers, using anti-C23 and anti-14-3-3β antibodies (Santa Crutz Biotechnology, Santa Cruz, CA, USA), respectively. β-Actin and glyceraldehyde-3-phosphate dehydrogenase were used as controls to confirm protein amounts loaded to SDS-polyacrylamide gel electrophoresis.

Since immediate degradation of phosphorylated IκB by proteasome hinders its immunoblot detection, SH-SY5Y cells were pre-treated with 10 μM of lactacystin, a proteasome inhibitor, for 4 h, and then treated with 100 nM of rasagiline for 0, 5, 15, and 30 min according to Casciati et al. (2002). The cells were washed twice with PBS and lysed in the RIPA buffer. β-Actin was used as a loading control. The protein concentration was quantified according to Bradford (1976). The lysate (40 μg protein per well) was subjected to SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel, and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were treated with Block Ace (Yukijirushi, Sapporo, Japan) for 1 h, and incubated with the first antibody overnight at 4 °C. The membranes were incubated with the second antibody conjugated with fluorescein, then with the anti-fluorescein antibody conjugated with alkaline phosphatase, and finally with an ECF substrate. The fluorescence at 540 nm was measured with excitation at 488 nm using a Fluoroimage analyzer (Amersham Pharmacia, Pyscataway, NJ, USA).

2.5. Quantitation of activated NF-κB

Activation of NF-κB was determined by NF-κB binding to κB sites using NF-κB p65 transcription assay kit according to Kretz-Remy et al. (2001), who reported the validity of this method to measure activated NF-κB. The principle of this assay is to measure the binding of activated NF-κB p65 to an oligonucleotide containing the NF-κB consensus-binding site. SH-SY5Y cells were cultured in six-well flasks with Cosmedium-001 containing 5% calf serum for 24 h. After the medium was changed to MEM, the cells were treated with 100 nM of rasagiline for 30–120 min or with 10 μM–10 nM of rasagiline for 60 min. The effect of sulfasalazine, an inhibitor of IκB kinase, was examined by incubation of the cells with the inhibitor (100 μM) for 30 min before rasagiline treatment. The cells were mechanically harvested, washed with PBS twice, lysed in 100 μl of the Lysis buffer [20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Igapal CA-630 (Sigma), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 50 mM DTT and a protease inhibitor cocktail]. Twenty micrograms of protein in the Binding buffer (4 mM HEPES buffer, pH 7.5, containing 120 mM KCl, 8% glycerol, 1% bovine serum albumin, 10 μg/ml Herring sperm DNA, 2 mM DTT) was applied to each well coated with oligonucleotide (5′-GGGACTTTCC-3′) corresponding to the NF-κB binding site, and incubated for 60 min at the room temperature. The wells were washed three times with 10 mM phosphate buffer, pH 7.5, containing 50 mM NaCl and 0.1% Tween 20, then incubated with p65 NF-κB antibody for 60 min, washed again three times, and incubated with horse radish peroxidase (HRP)-conjugated antibody for 60 min. Using a substrate, 3,3′,5,5′-tetramethylbenzidine, the activity of HRP was measured spectrophotometrically at 450 nm with reference at 655 min. Five micrograms of

the extract of Hela cells stimulated with TNF-α for 30 min was used as a positive control. The activation of NF-κB was expressed as percentage of the positive control. To confirm the specificity of the NF-κB binding to the oligonucleotide, 20 pmol of wild or mutated NF-κB oligonucleotide was added in the reaction mixture and the binding activity of NF-κB was compared with that without addition of the oligonucleotide.

2.6. Statistics

Experiments were repeated four times and the data were shown as the mean ± S.D. Difference was statistically evaluated by analysis of variance (ANOVA) followed by Sheffe's *F*-test. A *P*-value <0.05 was considered to be statistically significant.

3. Results

3.1. Rasagiline increased protein and mRNA level of GDNF

The effect of rasagiline on GDNF protein level was examined by the EIA. The assay system of GDNF was specific for GDNF, and no cross-reactivity was observed with other TGF-β super family proteins (Nitta et al., 2002). Rasagiline markedly increased GDNF protein level in SH-SY5Y cells, whereas the level in the culture medium was lower than the detection limit, 0.5 pg/ml. The increase was observed most markedly with 100 nM of rasagiline, but aminoindan did not affect GDNF level at any concentrations used in the experiments (Fig. 1A). Rasagiline at 100 nM increased GDNF protein to six folds of the basal level after 3 h incubation and the level was sustained for 24 h (Fig. 1B). Rasagiline induced GDNF mRNA significantly at 100 nM after 3 h incubation, as shown in Fig. 1C. The concentration increasing the mRNA level corresponded to that of the most optimal to increase GDNF protein level. Rasagiline at higher than 1 μM or lower than 1 nM did not affect the protein and mRNA levels of GDNF, showing an inverted U-shaped curve of concentration-activity relationship. On the other hand, in control and the cells treated with rasagiline at other concentrations, mRNA level of GDNF was lower than the detection limit.

3.2. Activation of NF-κB by rasagiline

To examine the involvement of transcription factors in the induction of prosurvival genes, the effects of rasagiline

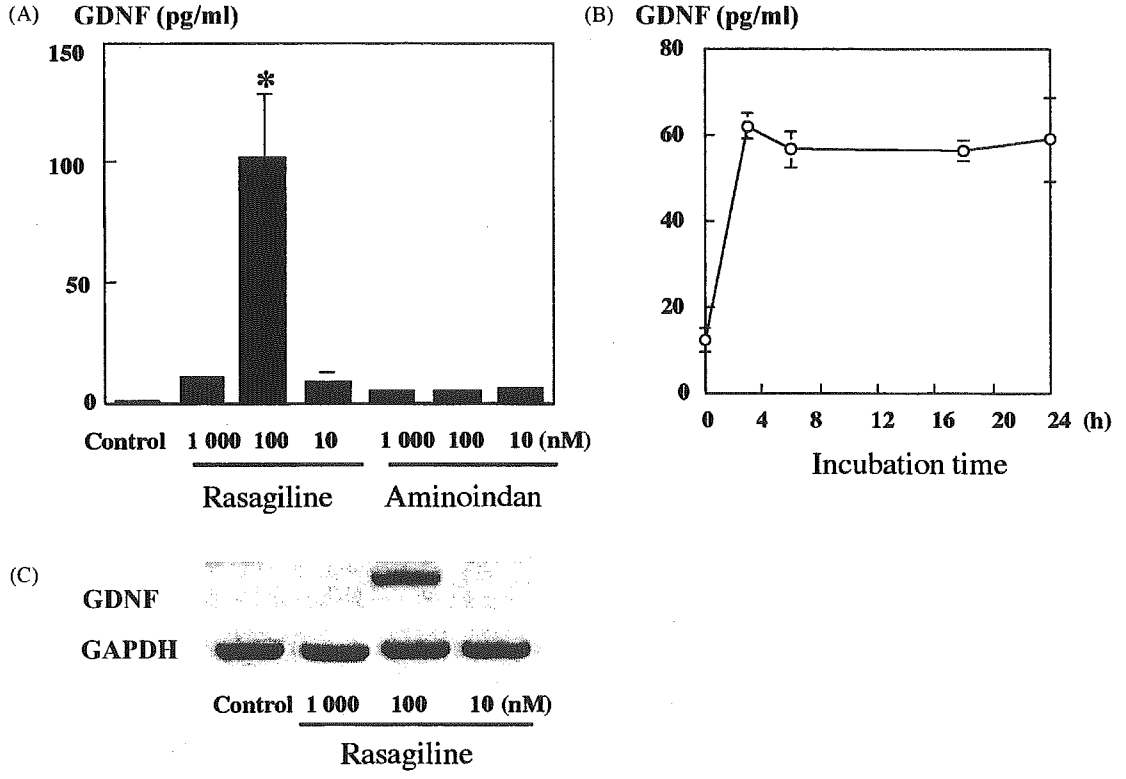

Fig. 1. Effects of rasagiline and aminoindan on the protein and mRNA level of GDNF in SH-SY5Y cells. (A) SH-SY5Y cells were treated with 1 μM–10 nM of rasagiline or aminoindan for 3 h, and GDNF protein level was analyzed by the EIA, as described in Section 2. Each column and bar represent the mean and S.D. of four experiments. (*) The difference from the control is statistically significant ($P < 0.01$) by ANOVA. (B) SH-SY5Y cells were treated with 100 nM of rasagiline for 3–24 h and the amount of GDNF protein was analyzed by the EIA. Each circle and bar represent the mean and S.D. of four experiments. (C) Effect of rasagiline on mRNA level of GDNF. SH-SY5Y cells were treated with 1 μM–10 nM of rasagiline for 3 h. Total RNA was extracted and subjected to RT-PCR for GDNF as described in Section 2. GAPDH was used as control.