制することなく、神経保護作用を誘導することが示唆されている（Nitta et al., 2004)。

また、タクロリムスの神経保護作用に関する重要な因子は FKBP52（Hsp56）であることが示唆されいる（Gold et al., 1999)。本研究ではHsc70およびHsp70とLeu-Ileが結合することが示されたが、FKBP52とHsc70およびHsp70はHsp90と共にステロイド受容体に結合し、複合体を形成しているという報告があることから（Bagchi et al., 1991; McLaughlin et al., 2002)、タクロリムスとLeu-Ileはこれらの複合体に何らかの作用をおよぼすことによって神経保護作用を誘導している可能性が考えられる。

E．結論

神経保護作用を持つ疎水性ジペプチドのLeu-Ileは、Hsc70と結合して、その作用を発現していると考えられた。

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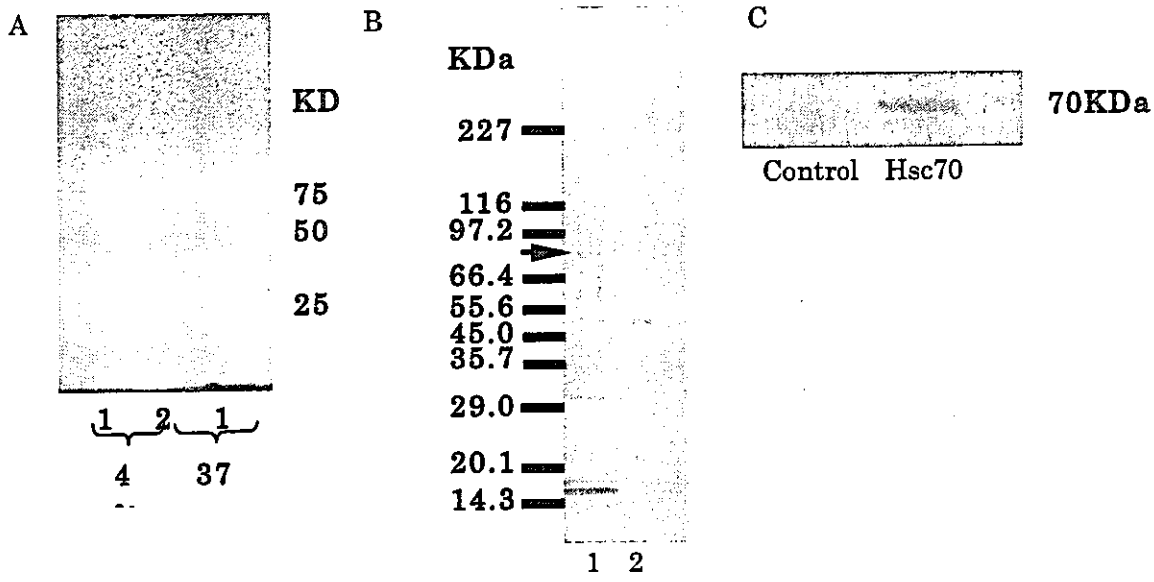

Fig.1 Isolation of Leu-Ile-binding protein
(A): Lane 1: FITC-NH-LI-COOH was reacted with mouse brain homogenate at 4℃ or 37℃. Lane2: NH2-LI-(FITC)-COOH was reacted with mouse brain homogenate at 4℃ or 37℃. The electrophoretogram was visualized by the fluoro-scanner. (B): Mouse brain homogenate was reacted with Affigel-10 coupled (lane1) or uncoupled (lane 2) with Leu-Ile and the Leu-Ile-binding proteins were purified. The Leu-Ile-binding proteins of mass spectrometry were done electrophoresis and stained by silver stain. Lane 1; The Leu-Ile-binding proteins. Lane 2; control. Arrow shows the protein analyzed by mass spectrometry. (C): The same samples as mass spectrometry were stained with antibody to Hsc70.

Table. 1 Mascot search result

1. gil123647 Mass: 71045 Total score: 496 Peptides matched: 10
Heat shock cognate 71 kDa protein
Check to include this hit in error toleranant search or arohive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Rank	Peptide
7	541.29	1080.57	1080.56	0	0	22	1	LLQDFFNGK
9	600.35	1198.68	1198.67	0	0	54	2	DAGTIAGLNVLR
12	627.32	1252.63	1252.61	0	0	64	1	FEELNADLFR
18	744.37	1486.73	1486.69	0	0	52	1	TTPSYVAFTDTER
20	808.93	1516.85	1615.78	0.1	0	52	1	SFYPEEVSSMVLTK
21	825.42	1648.82	1648.79	0	0	30	1	NQVAMNPTNTVFDAK
29	1130.61	2259.2	2259.14	0.1	0	62	1	SINPDEAVAYGAAVQAAILSGDK
30	759.75	2276.22	2276.13	0.1	0	24	1	GPAVGIDLGTTYSCVGVFQHGK
35	971.19	2910.55	2910.53	0	0	67	1	NVLIFDLGGGTFDVSILTIEDGIFEVK
36	999.85	2996.53	2996.45	0.1	0	70	1	TLSSSTQASIEIDSLYEGIDFYTSITR

Proteins matching the same set of peptides:
gil123647 Mass: 71077 Total score: 496 Peptides matched: 10
dnak-type molecular chaperone hsc70 - mouse

dnak-type molecular chaperone hsc70 – mouse

MSKGPAVGIDLGTTYSCVGVFQHGK VEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPTNTVF
DAKRLIGRRFDDAVVQSDMKHWPFMVVNDAGRPKVQVEYKGETKSFYPEEVSSMVLTKMKEIAEAYL
GKTVTNAVVTVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKKVGAERNVLIFDLGGGT
FDVSILTIEDGIFEVISTAGDTHLGGEDFDNRMVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTL
SSSTQASIEIDSLYEGIDFYTSITRARFEELNADLFRGTLDPVEKALRDAKLDKSQIHDIVLVGGSTRIPKI
QKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGDK SENVQDLLLLDVTPLSLGIETAGGVMTVLIK
RNTTIPTKQTQTFTTYSDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGI
LNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAEKYKAEDEKQRDKVSSKNSLESYAFNMKATV
EDEKLQGKINDEDKQKILDKCNEIISWLDKNQTAEKEEFEHQQKELEKVCNPIITKLYQSAGGMPGGMP
GGFPGGGAPPSGGASSGPTIEEVD

The spectra of MALDI-TOF/TOF-MS were interpreted with the Mascot software.

Ten peptides matched the fragments (in blue letters) of Hsc70, and the sequence of Hsc70 was formatted.

A

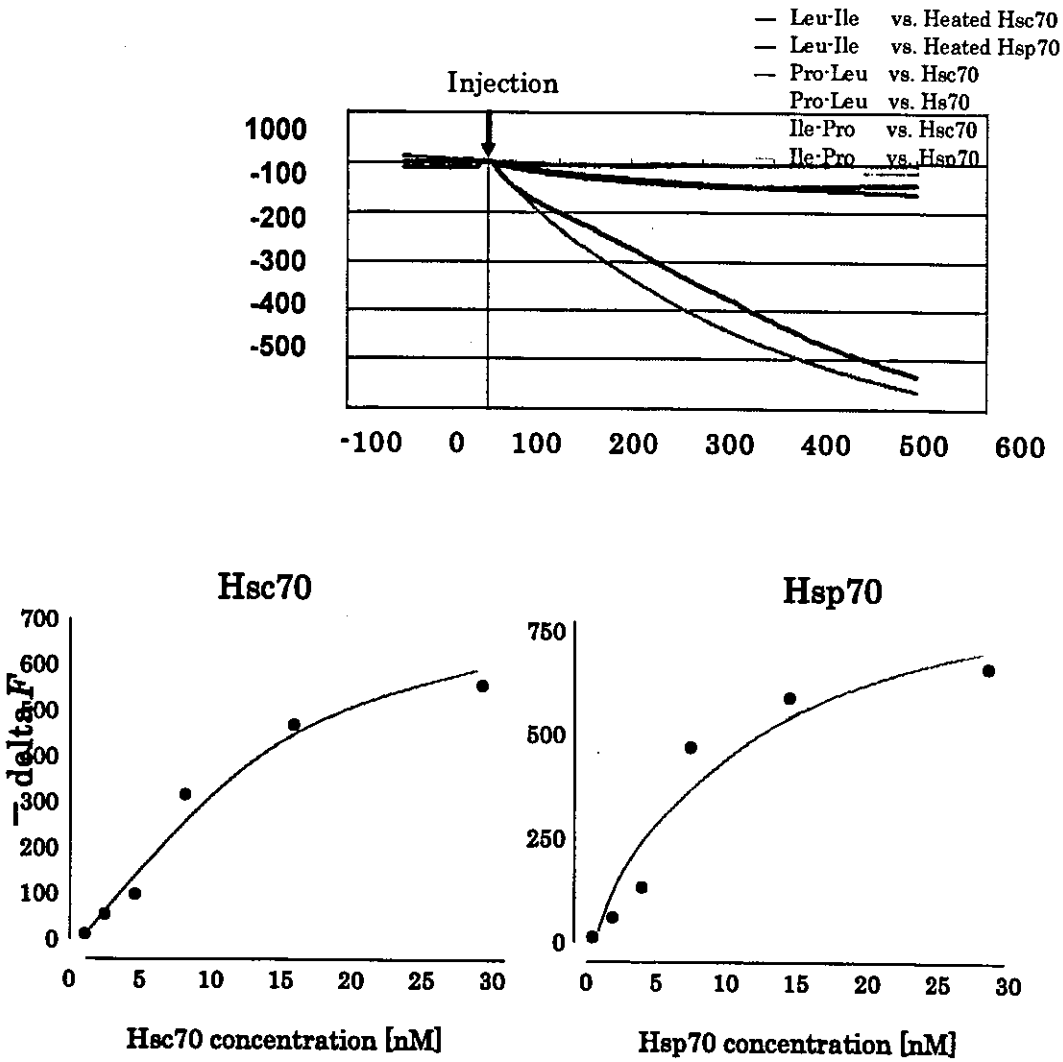

Fig 2 Affinity of Hsc70 or Hsp70 to Leu-Ile by the quartz crystal microbalance.

(A): Leu-Ile, Pro-Leu or Ile-Pro were immobilized on a quartz crystal microbalance (QCM) plate, and the plates were soaked in 6 ml of PBS at 25℃. Hsc70, Hsp70, heat denatured Hsc70 or Hsp70 were applied to the equilibrated solution and the frequency changes of the QCM were recorded. (B): The affinity of Hsc70 or Hsp70 and Leu-Ile was indicated by the frequency changes (-delta F) of QCM. Affinity constants (Kd; Kinetic dissociation) were calculated with the software of AQUA.

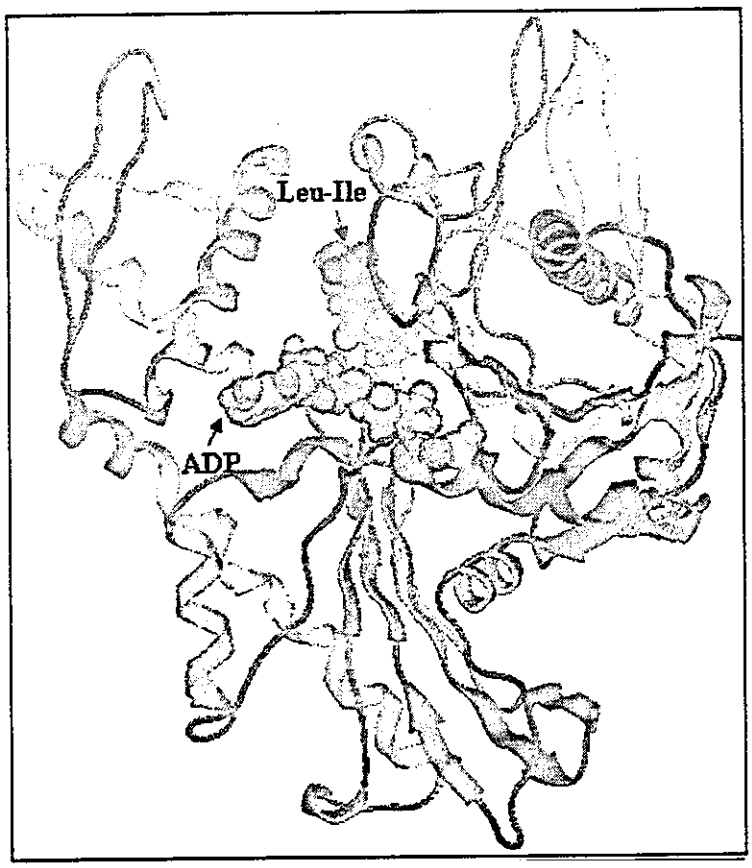

Fig.3 The expected binding site of Leu-Ile in Hsc

Molecular structures of Hsc70 ATPase domein (Flaherty et al.,1990) was indicated. The black and red arrows indicate adenosine diphosphate, and Leu-Ile, respectively. Gold BG, Densmore V, Shou W, Matzuk MM, Gordon HS: Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. J Pharmacol Exp Ther, **289**: 1202-1210 (1999)

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G 知的財産権の出願・登録状況
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Akt 刺激薬 出願番号　特願2005-51829
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1、　実用新案登録
なし
2、　その他
なし

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Oxidative Stress in Mitochondria: The Involvement in Neurodegenerative Diseases

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ABSTRACT

In mitochondria, oxidative phosphorylation and enzymatic oxidation of biogenic amines by monoamine oxidase produces reactive oxygen and nitrogen species, which may account for neuronal cell death in neurodegenerative disorders, including Parkinson's and Alzheimer's disease. In these disorders, inclusion body composed of oxidation-modified proteins and lipids is detected specifically for distinct diseases, such as the Lewy body for Parkinson's disease. The relationship between mitochondrial dysfunction, increased oxidative stress, accumulation of oxidation-modified protein, and final cell death of definite neurons in the brain remains to be clarified. In this paper, we review our recent results on interaction among these factors in neurons, using a cellular model of apoptosis induced by peroxynitrite-generating *N*-morpholino sydnonimine (SIN-1) and an inhibitor of complex I, rotenone in human dopaminergic SH-SY5Y cells. In control cells, 3-nitrotyrosine-containing protein produced by peroxynitrite was detected, suggesting that neurons exist in a state of constant oxidative stress. *N*-morpholino sydnonimine induced apoptosis and reduction in ATP level, which is an inhibitor of proteasome, while carbobenzoxy-L-isoleucyl-*γ*-*t*-butyl-glutamyl-L-alanyl-L-leucinal (PSI) increased further. The subunits of mitochondrial complex I were found to contain 3-nitrotyrosine, suggesting that peroxynitrite prefers these enzymes. In addition, rotenone induced mitochondrial dysfunction, and accumulation and aggregation of protein modified with acrolein, an aldehyde product of lipid peroxidation. Rotenone treatment reduced the enzymatic activity of the proteasome system, a major organelle in the degradation of oxidation-modified protein, and it was due to the oxidative modification of 20S *β* subunit of the proteasome. These results are discussed in relation to the interaction between mitochondrial dysfunction, oxidative stress, and proteasome inactivation, resulting in neuronal cell death in neurodegenerative disorders, such as Parkinson's and Alzheimer's disease.

I. OXIDATIVE STRESS AND MODIFIED PROTEIN AS THE MARKER

Oxidative stress has been proposed to induce neuronal death in aging and age-associated disorders (1,2), and mitochondria are a major source of reactive oxygen and nitrogen species (ROS–RNS). The superoxide anion radical generated by oxidative phosphorylation in the mitochondria is one of the most potent ROS and reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), whereas oxidation of biogenic amines by monoamine oxidase in mitochondrial outer membrane produces hydrogen peroxide. Mitochondria are now considered to play a pivotal role in apoptosis (3), which emerges as a common death type of neurons in neurodegenerative disorders, including Parkinson's (PD) and Alzheimer's diseases (AD) (4,5). The role of mitochondria in the process of apoptotic commitment is recognized. In mitochondria, impairment of energy charge and redox, permeability transition (PT), disruption of membrane potential, $\Delta\Psi m$, and release of cytochrome *c* are observed prior to the fragmentation of nuclear DNA, a hallmark of apoptotic morphological features.

Neurodegenerative disorders are characterized by a decline of specified neurons associated with protein deposits typical for each disease. In PD, dopamine neurons in the substantia nigra degenerate progressively with the formation of the Lewy bodies (LB). The pathogenesis of PD remains unknown, and the gene responsible for the sporadic cases has not been identified. PD is considered to represent the final outcome of various genetic and environmental interactions. The vulnerability of dopamine neurons is a consequence of the increased generation of ROS and RNS, reduced antioxidant capacity, high content of iron and dopamine, and possible defect in mitochondrial function. Reactive oxygen species and RNS generated in mitochondria modify bioactive molecules, such as lipids, proteins, DNA, and carbohydrates, either directly or indirectly with peroxidation products of lipids or carbohydrates. Several kinds of modified bioactive molecules have been proposed as markers of oxidative modification by ROS and RNS, as summarized in Table 1. Hydroxyl

Table 1 Oxidative Modification of Protein

Direct modification	Secondary modification
Polymerization (cross reaction)	Modification by lipid peroxidation
Aggregation	Aldehydes
Fragmentation	4-Hydroxynonenal
	Acrolein
Inactivation or activation of enzymes	Malondialdehyde
	Hydroperoxide
Modification of amino acids	Carbonyl production
3-Nitrotyrosine	
Dityrosine	Modification by glycosylation
	Aldehydes
Carbonyl production	Carbonyls

radicals modify tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine residues as preferred targets. Under anaerobic conditions, the hydroxyl radicals promote protein–protein cross-linking through –S–S– and –tyrosyl-tyrosyl– (dityrosine) bonding, and under aerobic conditions, peroxyradicals induce fragmentation of the polypeptide chain. In addition, proline, arginine, and lysine are particularly sensitive to metal-catalyzed oxidation and are converted to carbonyl derivatives.

Oxidative modification produces aggregated and cross-linked proteins, which are resistant to proteolytic degeneration and are difficult to be removed from the cells. Accumulation of the modified proteins may impact on a variety of cellular pathways by changing the enzymatic, regulatory, and transporting potencies of cellular specific protein, in addition to taking up space in limited cellular volume. The level of the oxidized protein may reflect the balance between the generation of ROS–RNS and degradation of modified protein, in which the ubiquitin–proteasome system plays a key role (6).

One of the most active RNS is peroxynitrite ($ONOO^-$) (7), which is unstable, but its protonated peroxynitrous acid (ONOOH) is extremely reactive (8), which generates hydroxyl radical by homolytic cleavage (9). The main targets of nitration are sulfhydryl and hydroxyl residues in cysteine,