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環境因子についてはどのように考えられてきている？

- 1980年代にヘロインの不純物MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) によるパーキンソニズム, 黒質神経細胞脱落の報告があり, 環境因子, ミトコンドリア機能異常がパーキンソン病の病態に関与することが明らかにされました¹⁾。
- 一般の環境因子では, 危険因子として頭部外傷歴, 金属曝露, 農薬などが, そして保護的因子としてカフェイン, 喫煙が報告されています。
- しかしながら, 環境因子については曝露歴, 曝露基準の評価が難しく, 現時点でもパーキンソン病にどこまで関与しているのか, 確定的な知見として明らかにされてはいません。

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遺伝的因子についてはどのように考えられてきている？

- 一方, アイスランドにおける全国民を対象とした遺伝子解析疫学調査により, 50歳以上のパーキンソン病患者の配偶者の発症率に比べ同胞, 子どもなど血縁者の発症危険率は有意に高く, パーキンソン病の発症には遺伝的因子が関わっていることが示唆されました。
- 環境因子と遺伝的因子の関与を考える上で大切な知見として, 一卵性双生児の研究があります。PET (positron emission tomography) での異常所見とも併せると, 一卵性双生児間では疾患一致率は100%ではないものの, 二卵性双生児の疾患一致率に比べ約3倍以上高いと報告されました。遺伝的因子を背景に, 環境因子との相互作用のもとパーキンソン病が発症することが示唆されるとともに, 遺伝子の関与がかなり大きいものと考えられました。

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家族性パーキンソン病の原因遺伝子はどこまで明らかになっているの？

●→ α -synuclein (PARK1)

- 遺伝的因子に関し, 1997年 α -synuclein (PARK1) が常染色体優性遺伝性パーキンソン病 (ADPD) の家系からその原因遺伝子として同定され²⁾, α -シヌクレイン (synuclein) はパーキンソン病の病理学的診断マーカーとしてのレビー (Lewy) 小体の主要構成成分であることが報告されました。

- ついで α -synuclein の遺伝子重複による発現過剰は、レビー小体形成および臨床的重症度と相関することも報告され、パーキンソン病では α -synuclein がレビー小体の形成および臨床像に大きく関わっていることが示唆されました。

● parkin (PARK2)

- α -synuclein の同定の翌1998年、2番目の原因遺伝子として常染色体劣性遺伝性パーキンソン病 (ARPD) の家系から parkin (PARK2) がわが国で単離され³⁾、ユビキチン・プロテアソーム系におけるユビキチンリガーゼとしての機能が報告されました。
- Parkin 蛋白の機能喪失、ユビキチン・プロテアソーム系の機能低下から発症に至る機序も考えられ、それらの発見はとても大きなインパクトとなりました。
- そしてこれら家族性パーキンソン病の原因遺伝子のレビー小体の形成、パーキンソニズムの発症機序への関与について、分子遺伝学的研究が盛んに行われてきています。家族性パーキンソン病の遺伝子産物の分子機構の解明が、パーキンソン病の原因、発症機序の解明につながるものと期待されているわけです。

● その他の原因遺伝子

- その後ここ10年ばかりの間でも、家族性パーキンソン病研究の進歩は目覚ましいものがあり、新しい遺伝子座や遺伝子の報告が続いています。2011年には新規原因遺伝子として VPS35 と EIF4G1 が報告されました。
- これまでに PARK1 ~ 18 までが遺伝子座として報告され、ADPD として α -synuclein, UCH-L1, LRRK2, VPS35, EIF4G1, ARPD として parkin, PINK1, DJ-1 と計8個の原因遺伝子が報告されています。
- そのほか PARK シリーズ、パーキンソン病関連遺伝子として、ATP13A2, GIGYF2, HTRA2/OMI, PLA2G6, FBXO7 など報告され(表1)、これら遺伝子の発見により、遺伝子解析、蛋白の機能解析がさらに広がり、新しい知見がどんどん増えてきています。

5

孤発性パーキンソン病の原因遺伝子異常はどこまで明らかになっているの？

- 家族性パーキンソン病のうち、メンデル遺伝形式に従う単一遺伝子異常によるパーキンソン病とともに、原因遺伝子変異の浸透率の低さのため、見かけ上孤発性に発症するパーキンソン病の一群があることがわかってきています。

表1 ▶ パーキンソン病関連遺伝子の一覧

	染色体上の位置	遺伝子	遺伝形式
PARK1	4q21-q23	SNCA (α -synuclein)	常優/感受性遺伝子
PARK2	6q25.2-q27	PRKN (<i>parkin</i>)	常劣
PARK3	p13	unknown	常優
PARK4	(4p15)	SNCA	常優
PARK5	4p14	UCH-L1	常優
PARK6	1p36-p35	PINK1	常劣
PARK7	1p36	DJ-1	常劣
PARK8	12p11.2-q13.1	LRRK2	常優/感受性遺伝子
PARK9	1p36	ATP13A2	常劣
PARK10	1p	unknown	感受性遺伝子
PARK11	2q37.1	GIGYF2	常優
PARK12	Xq21-q25	unknown	感受性遺伝子
PARK13	2p12	HTRA2 (<i>OMI</i>)	常優
PARK14	22q13.1	PLA2G6	常劣
PARK15	22q12.3	FBXO7	常劣
PARK16	1q32	unknown	感受性遺伝子
PARK17	16q12	VPS35	常優
PARK18	3q27	EIF4G1	常優
	1q21	GBA	感受性遺伝子
	12q24.1	ATXN2 (<i>SCA2</i>)	常優
	17q21.1	MAPT	常優/感受性遺伝子
	4p16	GAK	感受性遺伝子
	6p21.3	HLA-DRB5	感受性遺伝子
	4p15	BST1	感受性遺伝子

常優＝常染色体優性遺伝 (AD)

常劣＝常染色体劣性遺伝 (AR)

● ADPDの場合

- ▶ たとえば、ADPDの原因遺伝子のうち α -synuclein の2重複変異は孤発性パーキンソン病の約1～2%未満に認め、LRRK2 G2019S変異も白人の1.6%を占めるとする報告があります。これらは浸透率が低い(遺伝子変異を持っていても発症しない)ことで説明されます。北アフリカ人では実にパーキンソン病の30～40%にLRRK2 G2019S変異を認めたとの報告もあります。

● ARPDの場合

- ▶ ARPDの原因遺伝子のうち、*parkin*変異は45歳以下発症の孤発性パーキンソン病

の約20%を占め、*PINK1*では孤発性パーキンソン病の約1%というデータがあります。

- *parkin*, *PINK1*, *ATP13A2*, *PLA2G6*などのヘテロ変異が発症の原因になる、または危険因子になるかどうかは、いろいろな報告があり、まだはっきりとした結論が得られていませんが、ヘテロ変異例におけるPETでの異常所見も示されてきており、発症に関与している可能性も示唆されてきています。
- 孤発性パーキンソン病の解析症例の蓄積はまだ十分とはいえず、未知の遺伝子変異が数多く存在するものと思われます。

6

パーキンソン病の感受性遺伝子、危険因子となる遺伝的因子は？

- 病的遺伝子変異のみならず、 α -synucleinや*LRRK2*のいくつかの一塩基多型(SNP)が孤発性パーキンソン病の危険因子となり、感受性遺伝子としても働くことが報告されてきており、家族性パーキンソン病と孤発性パーキンソン病の発症には共通の機構があると考えられます。
- ゲノムワイドの関連解析では、 α -synucleinなどいくつかの遺伝子が感受性遺伝子となることが示されてきています。さらには最近、常染色体劣性遺伝性疾患のGaucher病の原因遺伝子である*GBA*ヘテロ変異が、パーキンソン病の危険因子となることが報告されています。
- 日本人の孤発性パーキンソン病では、現在わかっている*LRRK2* G2385Rおよびいくつかの*GBA*ヘテロ変異だけでも、ともにパーキンソン病患者の約10%に認める頻度の高い危険因子であることがわかっています。
- また、*LRRK2*のほかの変異群が危険因子や保護的因子にもなっていることが、最近報告されました。
- 発症の原因とはならないものの危険因子や保護的因子となる、多くの遺伝的因子の組み合わせによる孤発性パーキンソン病の発症も考えられることから、パーキンソン病は“多因子遺伝性疾患”として考えられるようになってきています。

7

遺伝子からみたパーキンソン病の原因・病態研究の進歩

- 人種差はありますが、これまで蓄積された知見からは、全パーキンソン病のうち家族性パーキンソン病と考えられる症例は5~10%、その他孤発性パーキンソン病の中で単一原因遺伝子異常によるものは約5~20%、危険因子となる遺伝子異常

が明らかにされているものは約10～20%と見積もられます。

- すなわち、全パーキンソン病の少なくとも約20%、5人に1人は何らかの形で発症に関与する遺伝的因子が明らかにされており、もはや特発性ではないことが明らかにされているとも考えられます。
- これら遺伝子解析結果に基づき、関連遺伝子の正常機能および異常機能の解析、動物モデルの研究が進んでいます。必ずしも遺伝子異常がなくとも、神経変性のカスケードにおいてこれらの遺伝子産物が働いていることを示す知見も積み重ねられてきています。
- 神経変性過程におけるARPDの遺伝子産物そのもの(Parkin, PINK1, DJ-1など)のミトコンドリアへの関与も明らかにされつつあります。パーキンソン病の危険因子である加齢に伴い、ミトコンドリアDNAの欠失変異が多くなり黒質神経細胞障害をきたすことも想定されてきており、加齢因子と遺伝的因子が密接に関わっていることも示されてきています。
- このようにパーキンソン病の分子遺伝学的研究は日進月歩であり、その知見が孤発性パーキンソン病の発症メカニズムの解明に大きく貢献することが現実味を帯びてきており、今後もパーキンソン病の遺伝子からの研究が果たす役割は大きいといえます。

8

パーキンソン病におけるさらなる遺伝的因子の解明は可能？

- 十分可能であり、実際現在も短期間のうちに新たな知見がどんどん積み重ねられています。ただ、世界的にも網羅的大規模解析のデータにはまだまだ乏しい状況です。
- 人種差もありますが、これまでの多施設間の遺伝子解析結果からは、ADPDの大多数、ARPDの約40%が未知の遺伝子異常によって引き起こされていると考えられており、新規原因遺伝子の同定も含めた新たな研究の発展が望まれるところです。
- また、危険因子となる遺伝子変異や多型も複数わかってきており、ゲノムワイドの関連解析による感受性遺伝子の同定も世界中で行われてきました。さらに、次世代シーケンサーにより網羅的に高速に遺伝子解析がなされるようになってきており、新規原因遺伝子も同定されてきています。今後孤発性パーキンソン病における遺伝的因子の知見もますます重要になってくるものと思われ、積極的に解析されてきています。

9

パーキンソン病に対する遺伝子からのアプローチは
日常診療から始まっている

- パーキンソン病は common disease であり、臨床医が日常の外来で孤発性パーキンソン病はもちろん、家族性パーキンソン病 (ADPD, ARPD) の患者さんに遭遇することも決して稀ではありません。忙しい診療の中でも、血族婚の有無など詳細な家族歴を聴取することは重要で、パーキンソン病の家系内で非発症者と思われる人に軽微なパーキンソニズムが潜在的に存在していることもあり、家系内メンバーの直接の診察所見に基づいた情報収集が有用になってくる場合もあります。
- しかも ADPD や ARPD の中には浸透率が低い場合も多く、見かけ上孤発性と思われる症例でも遺伝子変異が認められる場合が少なくありません。したがって、家族歴がなくても遺伝性パーキンソン病といえることが少なからずあります。少なくともパーキンソン病の5人に1人に具体的な遺伝的因子が示されてきていますが、まだまだ未知の遺伝的因子は多いと考えられます。

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1人の患者さんからパーキンソン病の原因、病態、治療についての
研究へ、そしてまた臨床へ

- したがって、1例1例の臨床情報に基づいた解析結果から clinical base で遺伝的因子の知見を蓄積することは、今後ますます重要になってくるものと思われます。そのことにより、正確な早期診断、早期治療、予防的治療や、遺伝情報に基づき薬剤応答性、副作用の出やすさなどを考え、個人個人に最も合った形で治療法を決定するオーダーメイド医療の一般化が可能となってきます。
- 今後、iPS細胞の研究の発展・応用も大変期待されます。
- このように patient oriented ということを原点とし、家族性および孤発性パーキンソン病のさらなる症例解析データの蓄積、解析研究の成果が多くの患者さんに還元できるよう、臨床に立ち返る形で診断治療に応用されてきています。

11

おわりに

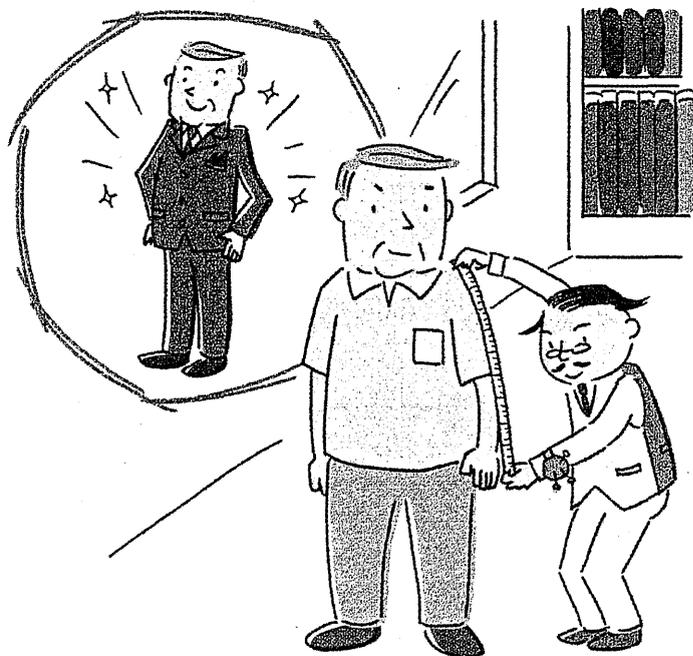
- 以上のように、パーキンソン病において、遺伝子、遺伝的因子は大きな発症の原因または背景となり、神経変性の共通経路で何らかの働きをしていることも明らかにされてきています。その知見から分子遺伝学的研究がさらに大きく発展し、パーキンソン病の根本の原因、発症機序の解明、治療の開発につながるのも、それほど遠

い将来のことではないと期待されます。

◀文献▶

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富山弘幸



Caffeine induces apoptosis by enhancement of autophagy via PI3K/Akt/mTOR/p70S6K inhibition

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Key words: apoptosis, autophagy, PI3K/Akt/mTOR/p70S6K, ERK1/2, caffeine

Abbreviations: PI3K, phosphoinositide-3 kinase; 4E-BP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; 3-MA, 3-methyladenine; MEFs, mouse embryonic fibroblasts; p70S6K, 70-kDa ribosomal protein S6 kinase; PI, propidium iodide; MPP⁺, 1-methyl-4-phenylpyridinium

Caffeine is one of the most frequently ingested neuroactive compounds. All known mechanisms of apoptosis induced by caffeine act through cell cycle modulation or p53 induction. It is currently unknown whether caffeine-induced apoptosis is associated with other cell death mechanisms, such as autophagy. Herein we show that caffeine increases both the levels of microtubule-associated protein 1 light chain 3-II and the number of autophagosomes, through the use of western blotting, electron microscopy and immunocytochemistry techniques. Phosphorylated p70 ribosomal protein S6 kinase (Thr389), S6 ribosomal protein (Ser235/236), 4E-BP1 (Thr37/46) and Akt (Ser473) were significantly decreased by caffeine. In contrast, ERK1/2 (Thr202/204) was increased by caffeine, suggesting an inhibition of the Akt/mTOR/p70S6K pathway and activation of the ERK1/2 pathway. Although insulin treatment phosphorylated Akt (Ser473) and led to autophagy suppression, the effect of insulin treatment was completely abolished by caffeine addition. Caffeine-induced autophagy was not completely blocked by inhibition of ERK1/2 by U0126. Caffeine induced reduction of mitochondrial membrane potentials and apoptosis in a dose-dependent manner, which was further attenuated by the inhibition of autophagy with 3-methyladenine or *Atg7* siRNA knockdown. Furthermore, there was a reduced number of early apoptotic cells (annexin V positive, propidium iodide negative) among autophagy-deficient mouse embryonic fibroblasts treated with caffeine than in their wild-type counterparts. These results support previous studies on the use of caffeine in the treatment of human tumors and indicate a potential new target in the regulation of apoptosis.

Introduction

Caffeine has a diverse range of pharmacological effects.¹ In addition to its various effects on the cell cycle and growth arrest, higher (4–10 mM) concentrations of caffeine can induce apoptosis in several cell lines, such as 10 mM caffeine in human neuroblastoma cells,² 4 mM caffeine in human pancreatic adenocarcinoma cells³ and 5 mM caffeine in human A549 lung adenocarcinoma cells.⁴ Although caffeine has been reported to modulate cell cycle checkpoints and perturb molecular targets of the cell cycle, the exact mechanism of caffeine-induced apoptosis remains unclear.¹

Autophagy is a key mechanism in various physiopathological processes, including tumorigenesis, development, cell death and survival.^{5,6} It has also been shown to have a complex relationship with apoptosis, especially in tumor cell lines.⁷ Several reports

have shown that autophagy not only enhances caspase-dependent cell death, but is also required for it.⁸ In contrast, it has also been shown that autophagy plays an important role in promoting cell survival against apoptosis.⁷ Caffeine has been reported to inhibit some kinase activities, including various forms of phosphoinositol-3 kinase and mammalian target of rapamycin (mTOR).^{9,10} Recently, in food spoilage studies involving yeast, caffeine has been shown to induce a starvation response,¹¹ which is a key regulator of autophagy causing its induction. However, the exact mechanism by which caffeine induces autophagy is still unknown.

Here we report that higher concentrations of caffeine enhance autophagic flux in a dose-dependent manner in various cell lines. Furthermore, we show that caffeine-induced autophagy is mainly dependent on PI3K/Akt/mTOR/p70S6 signaling and eventually results in apoptosis.

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Results and Discussion

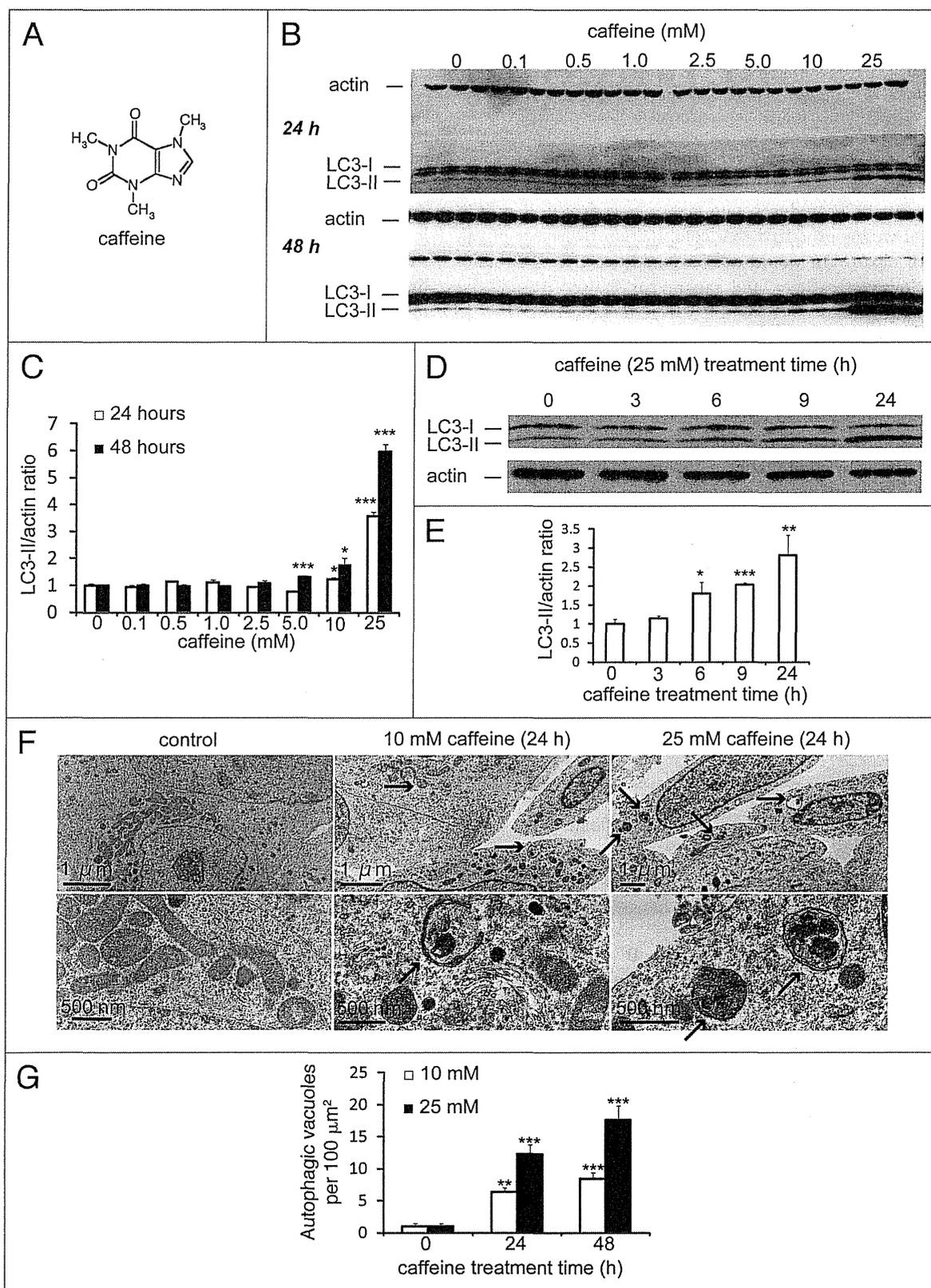
Caffeine (Fig. 1A) is a widely used psychoactive drug that has been used for centuries to increase alertness and energy. It has been reported that caffeine induces autophagy in *Zygosaccharomyces bailii* in association with a starvation response, caused by a unknown mechanism.¹¹ However, it remains unknown whether caffeine affects autophagy in mammalian cells. To determine if caffeine regulates autophagy at a steady state, we first examined levels of the microtubule-associated protein 1 light chain 3 (LC3)-II, which is an LC3-phosphatidyl-ethanolamine conjugate and a promising autophagosomal marker.¹² LC3-II levels (compared to actin loading controls) increased with 5–25 mM caffeine treatment over 48 hours in SH-SY5Y (Fig. 1B and C), PC12D and HeLa cells (Suppl. Fig. S1A and B). The LC3-II/actin ratio also increased in a time-dependent manner in SH-SY5Y (Fig. 1D and E) and HeLa cells (data not shown). Using an electron microscopy technique, the numbers of autophagic vacuoles (AVs) were markedly increased in SH-SY5Y cells treated with 10 or 25 mM caffeine, but not in the control (Fig. 1F and G). Morphometric analysis revealed that the number of AVs per 100 μm^2 of SH-SY5Y cytoplasm in control (Mean \pm standard deviation: 1.3 ± 0.50), whereas that in caffeine-treated cells (10 mM: 8.0 ± 0.82 ; 25 mM: 15 ± 1.9) for 24 hours. Expression levels of p62, a well-known autophagic substrate, were also decreased by caffeine treatment in SH-SY5Y (Fig. 1H and I) and HeLa cells (Suppl. Fig. S1C and D). Furthermore, 10 mM caffeine treatment markedly increased the number of EGFP-LC3-positive vesicles in SH-SY5Y cells transiently transfected with EGFP-LC3 (data not shown) and HeLa cells stably expressing EGFP-LC3 (Figs. 1J and K).^{12,13} This effect was confirmed by the observation that caffeine administration also increased the number of vesicles positive to endogenous LC3 (Suppl. Fig. S1E).

Endogenous LC3 is post-transcriptionally processed into LC3-I, which is found in the cytosol. LC3-I is in turn lipidated to LC3-II, which then associates with autophagosome membranes.¹⁴ LC3-II can accumulate due to increased upstream autophagosome formation or impaired downstream autophagosome-lysosome fusion. To distinguish between these two possibilities, we assayed LC3-II in the presence of E64D plus pepstatin A or bafilomycin A1, which inhibits lysosomal proteases or blocks downstream autophagosome-lysosome fusion and lysosomal proteases, respectively.^{15,16} Caffeine significantly increased LC3-II levels in the presence of E64d plus pepstatin A or bafilomycin compared to E64d plus pepstatin A or bafilomycin alone in (Fig. 2A and B; Suppl. Fig. S1F and G) and HeLa cells (Fig. 2C and D; Suppl. Fig. S1H and I). A saturating dosage of bafilomycin A1 was used in this assay and no further increases in LC3-II levels were observed when cells were treated with higher concentrations. Similar results were observed in PC12D cell lines (data not shown). To confirm the caffeine effect on autophagic flux, we assessed the numbers of autolysosomes and autophagosomes in HeLa cells. The ratio of the numbers of autolysosomes (positive to both LC3 and LAMP2) to autophagosomes (positive to LC3) was increased by 10 mM caffeine treatment for 48 hours (Fig. 2E). Quantification data using ImageJ also showed significant

increase of the ratio (Fig. 2F). These results strongly indicate that high concentration of caffeine treatment enhances autophagic flux.

The class I phosphatidylinositol 3-phosphate kinase (PI3K)/Akt/mTOR/p70ribosomal protein S6 kinase (p70S6K) signaling pathway and the Ras/Raf-1/mitogen-activated protein kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway are two well-known pathways involved in the regulation of autophagy. Both are associated with tumorigenesis and often activated in numerous types of tumors.¹⁷ Therefore, we examined the effect of caffeine on both of these pathways, using western blotting, according to the protocol by Inoki and colleagues.¹⁸ After a 24 hour treatment with caffeine, there was a significant decrease in the levels of phosphorylated p70 S6 kinase, S6 ribosomal protein and 4E-BP1, compared with total normal levels in SH-SY5Y (Fig. 3A), HeLa and PC12D cells (data not shown). Consistent with these results, nonphosphorylated 4E-BP1 proteins were increased by caffeine treatment (Fig. 3A). To further investigate the upstream inhibition of mTOR by caffeine, we examined Ser473 phosphorylation of Akt, which measures both Akt/mTOR and mTORC2 activity. As shown in Figure 3B, treatment with caffeine also decreased the level of phosphorylated Akt in SH-SY5Y cells, which was consistent with a previous report.¹⁹ Similar findings were obtained in HeLa (Suppl. Fig. S2A) and PC12D cells (data not shown). Subsequently, we examined whether caffeine increases the phosphorylation of ERK1/2, a key regulator of autophagy downstream of Akt. As shown in Figure 3C, treatment with caffeine increased phosphorylated ERK1/2. The effects of caffeine on mTOR inhibition were initially detected 3 hours after the addition of caffeine and reached a maximal level after 6 hours in SH-SY5Y (Fig. 3D) and 9 hours in HeLa cells (Suppl. Fig. S2B and C).

Caffeine has been shown to inhibit PI3K and components of the PI3K/Akt pathway.^{9,20} Next, we performed experiments to confirm whether caffeine-induced autophagy is activated through the PI3K/Akt pathway. Insulin or insulin-like growth factor upregulates PI3K and its downstream targets including Akt and mTOR, resulting in the inactivation of autophagy.^{21–23} As shown in Figure 4A and B, insulin treatment for 30 minutes significantly phosphorylated Akt at Ser473, whereas the phosphorylation was completely abolished by additional treatment with caffeine. No significant differences of the LC3-II/actin ratio between caffeine treatment and caffeine treatment with insulin were observed. Also, caffeine and Akt1/2 inhibitors did not have additive effects on the levels of LC3-II/actin ratio compared to the single treatment of caffeine or Akt inhibitors (Fig. 4C and D). To further confirm the caffeine effects on this pathway, cells were transiently transfected with myristoylated Akt (myr-Akt), a constitutively active form of Akt.²⁴ Caffeine treatment of both cells transfected with control vector and myr-Akt markedly decreased the levels of the phosphorylated Akt (Fig. 3E), indicating that caffeine directly inhibits the Akt phosphorylation. If caffeine facilitates autophagy through PI3K/Akt and ERK1/2 signalings, the autophagy should be partially blocked by ERK1/2 inhibition using the mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor, U0126. U0126 significantly but mildly



reversed the levels of LC3-II/actin ratio (Fig. 4F and G). The failure of U1026 to reverse completely the caffeine effect can be explained by the autophagy induction through Akt/mTOR signaling. In addition, only Akt knockdown with inducible short

hairpin RNAs (shRNAs) to specifically and stably knock down all three Akt isoforms sufficiently increases autophagic flux.²⁵ Therefore, we concluded that the caffeine-induced autophagy is mainly dependent on the PI3K/Akt/mTOR pathway.

Figure 1A–G (See opposite page). Caffeine increases autophagic flux in various cell lines. (A) Structural formula of caffeine. (B and C) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting (B) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (C) was performed using three independent experiments. (D and E) SH-SY5Y cells treated with 25 mM caffeine for 3–24 hours were analyzed by immunoblotting (D) with antibodies against LC3 and actin. Densitometry analysis of LC3-II levels relative to actin (E) was performed using three independent experiments. (F) Electron microscopic examination of SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours. Autophagic vacuoles accumulating in the cytoplasm are shown by arrows. (G) Morphometric analysis of autophagic vacuoles was performed with 30 different areas of the cytoplasm of control and caffeine-treated cells.

Because caffeine induces autophagy dependently of mTOR inhibition, we hypothesized that combination treatment of caffeine with rapamycin would not have additive effects on autophagy. However, caffeine and rapamycin showed an additive effect on the enhancement of LC3-II/actin ratio compared to the single treatment of caffeine or rapamycin (Fig. 5A and B). Several lines of evidences support the hypothesis that resistance to rapamycin results from a positive feedback loop from mTOR/S6K1 to Akt, resulting in enhancement of Akt phosphorylation at Ser 473.^{26–28} Recently, mutual suppression of the PI3K/Akt/mTOR pathway by combination of rapamycin with perifosine, an Akt inhibitor, induces synergistic effects on autophagy-induced apoptosis as well as enhancement of autophagy, suggesting that dual inhibition of the PI3K/Akt/mTOR by rapamycin with caffeine would be also a rational treatment for cancer.²⁹

Several anti-cancer agents are known to inhibit the PI3K/Akt/mTOR/p70S6K pathway and simultaneously activate ERK1/2, resulting in induction of autophagy in tumor cell lines.^{30,31} The upregulation of this process has beneficial effects in neurodegenerative diseases, such as Parkinson and Huntington diseases, whereas an excess of autophagy can lead to cell death.^{32,33}

Therefore, we decided to investigate whether caffeine-induced autophagy rescues or induces cell death. Using PC12D cells treated with 1-methyl-4-phenylpyridinium (MPP⁺), a well-established Parkinson disease model,³⁴ we determined that 1 mM caffeine treatment was not sufficient for the induction of autophagy (Suppl. Fig. S4 and B) and promoted increased cell viability, whereas >2.5 mM caffeine decreased cell viability (Fig. 6A). In addition, a significant decrease in cell viability was noted in cells treated with >2.5 mM caffeine without MPP⁺. Also, mitochondrial membrane potentials assessed by JC-1 were significantly preserved by 1 mM caffeine treatment compared to the control with MPP⁺, while those were lost by >5 mM caffeine treatment (Fig. 6B and Suppl. Fig. S5A). These data suggest that caffeine-induced autophagy is not protective in these cell lines and leads to cell death.

Autophagy and apoptosis may act independently in parallel pathways or may influence one another.⁷ To confirm the relationship between these pathways in cells treated with caffeine, we examined caffeine effects on the cell cycle with a propidium iodide (PI) staining assay. Treatment with 2.5–10 mM caffeine increased the percentage of cells in the sub-G₁ peak, which is indicative of

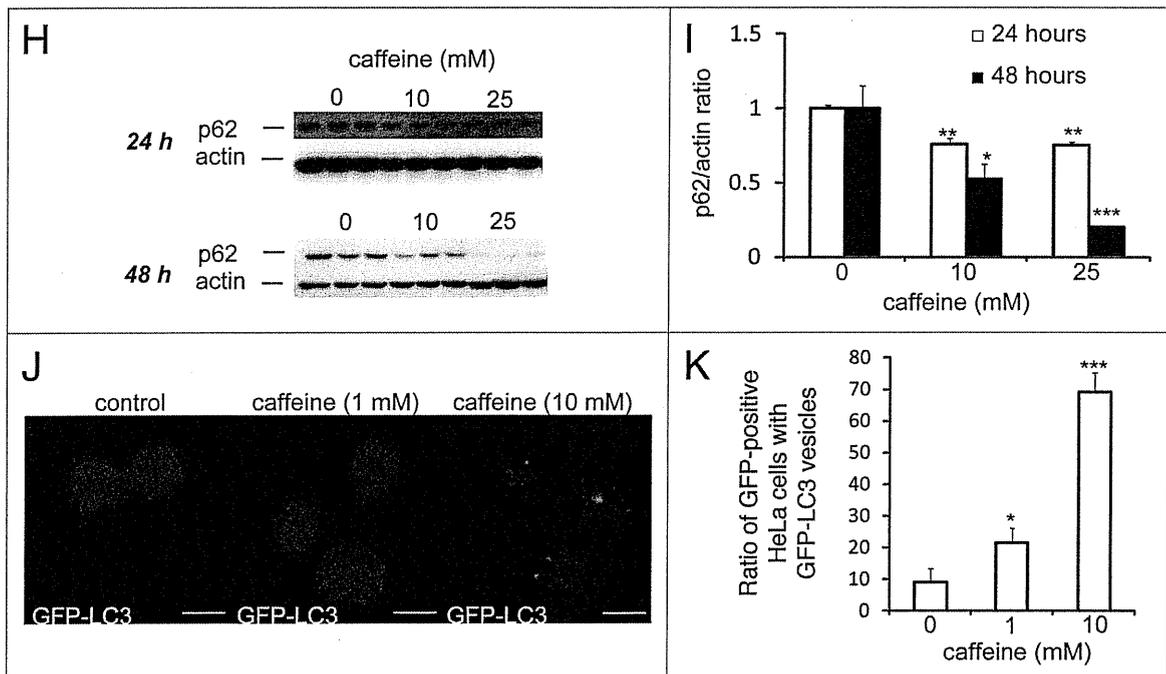


Figure 1H–K. Caffeine increases autophagic flux in various cell lines. (H and I) SH-SY5Y cells treated with various concentrations of caffeine for 24 or 48 hours were analyzed by immunoblotting with antibodies against p62 and actin. Densitometry analysis of p62 levels relative to actin (I) was performed using three independent experiments. (J and K) HeLa cells stably expressing EGFP-LC3 were treated with various concentrations of caffeine for 24 hours and analyzed using confocal microscopy. The percentage of EGFP-positive HeLa cells with >5 EGFP-LC3 vesicles was assessed (K) described previously in reference 43. Error bars, S.D.; **p* < 0.05; ***p* < 0.01.

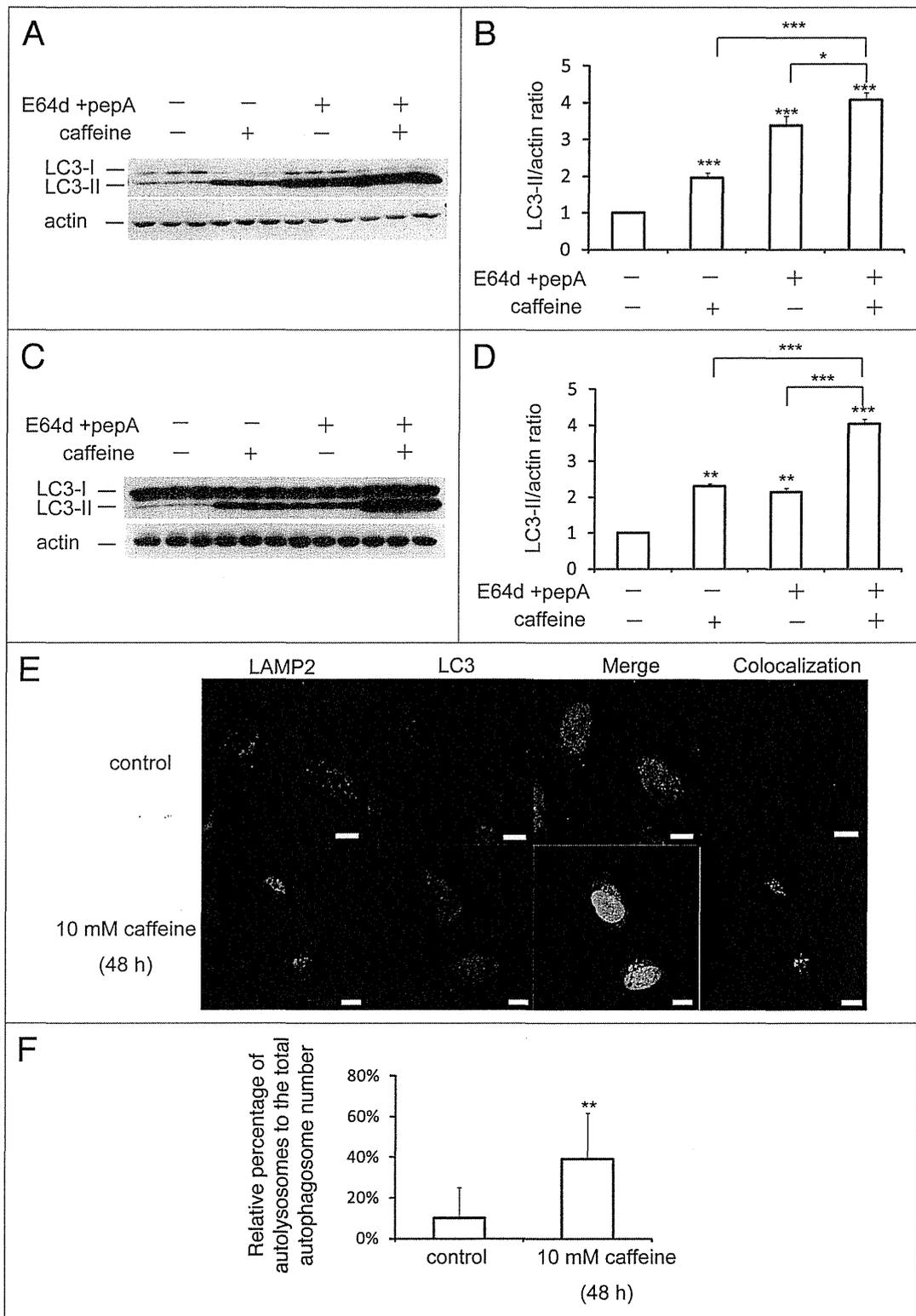


Figure 2. Caffeine does not block autophagosome-lysosome fusion. (A–D) SH-SY5Y (A) or HeLa (C) cells treated with 10 mM caffeine with or without E64d (10 μ g/ml) and pepstatin A (10 μ g/ml) were analyzed by immunoblotting with antibodies against LC3 and actin. Densitometry analysis of LC3 levels relative to actin in SH-SY5Y (B) and HeLa (D) cells was performed using three independent experiments. (E and F) HeLa cells treated with various concentrations of caffeine for 48 hours were analyzed using confocal microscopy (E). Number of the autolysosomes and autophagosomes were automatically counted using ImageJ "Colocalization" Plugin and the ratios were calculated (F).

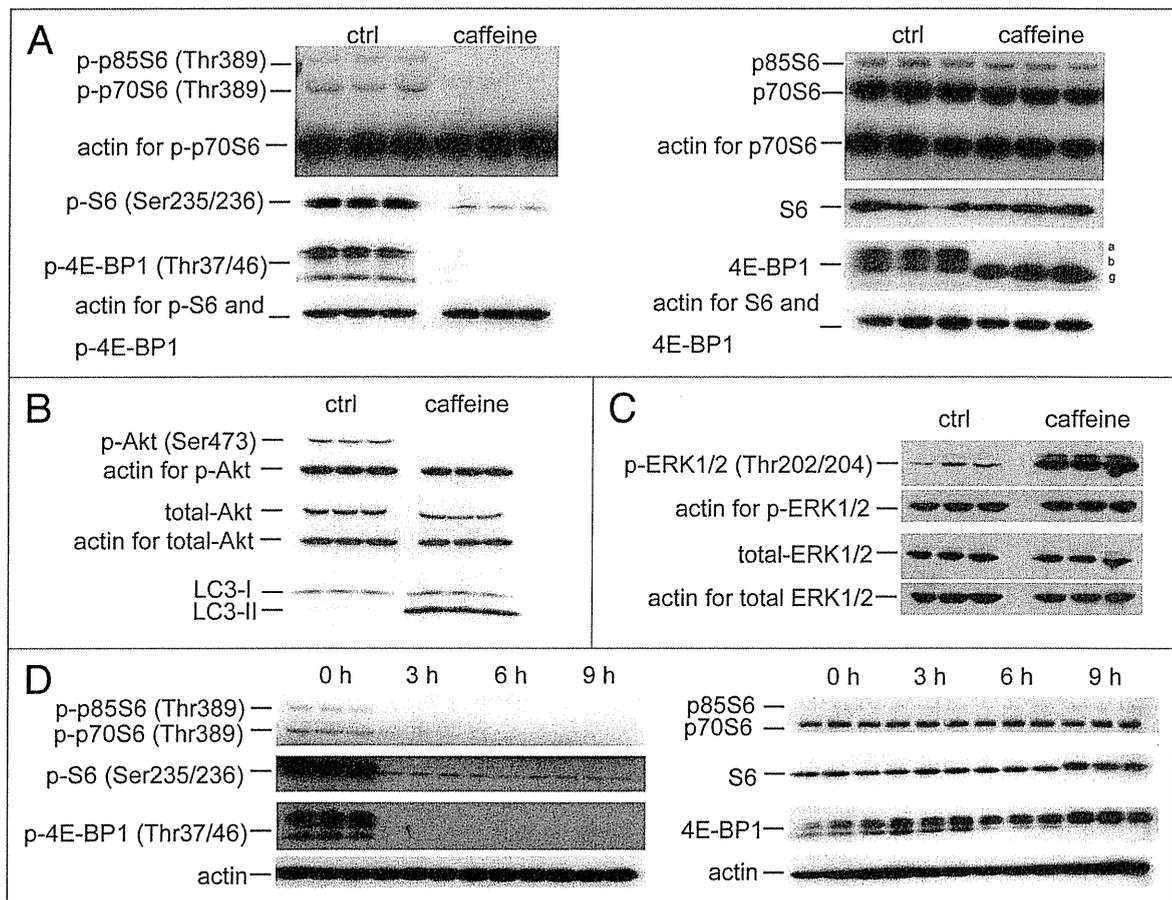


Figure 3. Caffeine inhibits the Akt/mTOR/p70S6 signaling pathway and activates ERK1/2 signaling. (A and B) SH-SY5Y cells treated with or without 10 mM caffeine for 24 hours were analyzed for mTOR activity by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 (A), Akt (B) and actin. (C) SH-SY5Y cells treated with or without 10 mM caffeine for 0, 3, 6 or 9 hours were analyzed by immunoblotting for levels of phosphor- and total ERK1/2 and actin. (D) SH-SY5Y cells treated with 10 mM caffeine for various time periods were analyzed by immunoblotting for levels of phosphor- and total p70 ribosomal S6 protein, S6, 4E-BP1 and actin.

apoptosis (Fig. 6C). To confirm whether caffeine-induced cell death is apoptotic, we examined the activity of caspase-3, a well-known inducer of apoptosis. Treatment with 10 mM caffeine markedly increased levels of cleaved caspase-3 and decreased full-length caspase-3 in PC12D cells (Fig. 6D), consistent with previous reports on the induction of apoptosis by caffeine.³⁵⁻³⁷

To test whether caffeine-induced apoptosis is dependent on autophagy, we determined whether the inhibition of autophagy by 3-methyladenine (3-MA) or Atg7 siRNA knockdown affects caffeine-induced cytotoxicity in PC12D cells. Treatment with 1 or 5 mM 3MA or Atg7 knockdown significantly decreased the percentage of cell death or cells with reduced mitochondrial membrane potentials caused by caffeine treatment (5 or 10 mM) (Fig. 6E and F and Suppl. Fig. S6B). As can be seen from the increased caffeine-induced apoptosis shown in Figure 6A and C, our data suggests that caffeine-induced autophagy is necessary for apoptotic cell death. To further confirm this, we compared autophagy-deficient mouse embryonic fibroblasts (MEFs), lacking the *Atg7* gene (*Atg7*^{-/-}), without LC3-II expression (Suppl. Fig. S4E), and matched wild-type (*Atg7*^{+/+}) MEFs, in which autophagy is induced by caffeine in a dose-dependent

manner (Suppl. Fig. S4C and D). As expected, the level of caffeine-induced cell death (positive to trypan blue staining) in *Atg7*^{-/-} MEFs was less than that in *Atg7*^{+/+} MEFs (Fig. 7A). The numbers of early apoptotic cells (annexin V positive, PI negative) were significantly increased in both a time-dependent and dose-dependent manner by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Fig. 7B–D). Also, apoptotic or necrotic cells (annexin V positive) were significantly increased by caffeine treatment of *Atg7*^{+/+} MEFs compared to *Atg7*^{-/-} MEFs (Suppl. Fig. S6). Together, these results indicate that caffeine-induced autophagy partly occurs upstream of apoptosis and is not a protective response to caffeine.

In various tumor cell lines, higher concentrations of caffeine alone induce p53-dependent G₁ phase arrest and under certain conditions apoptosis can also occur in a p53-independent manner.¹ Furthermore, disruption at the G₂/M checkpoint by caffeine allows cells time to repair DNA damage by driving them through mitosis, eventually resulting in apoptosis.^{36,38,39} Consistent with these reports, the results of our study indicate that increased concentrations of caffeine treatment cause a dose-dependent increase in apoptosis. More recently, autophagy, a process long known to

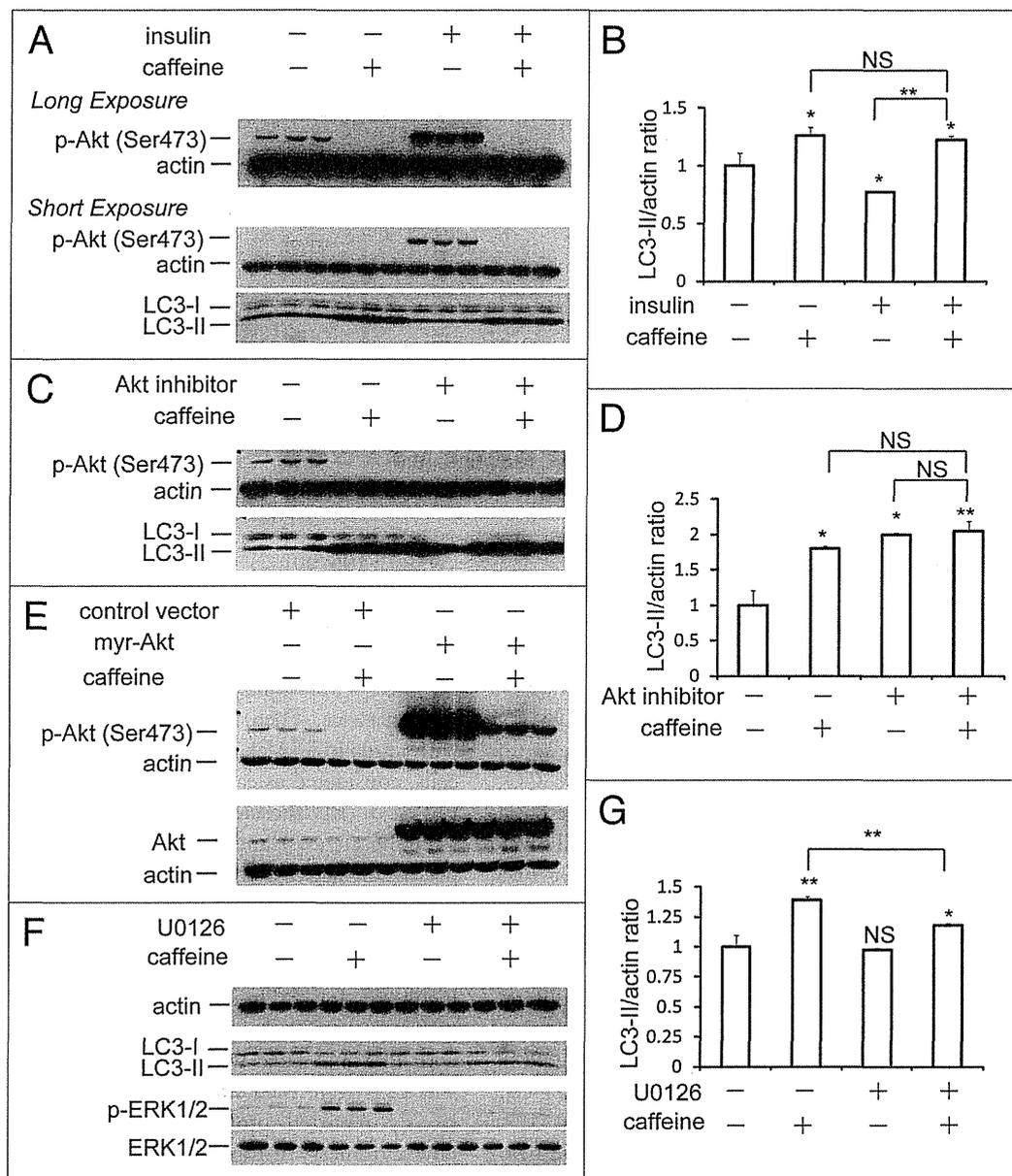
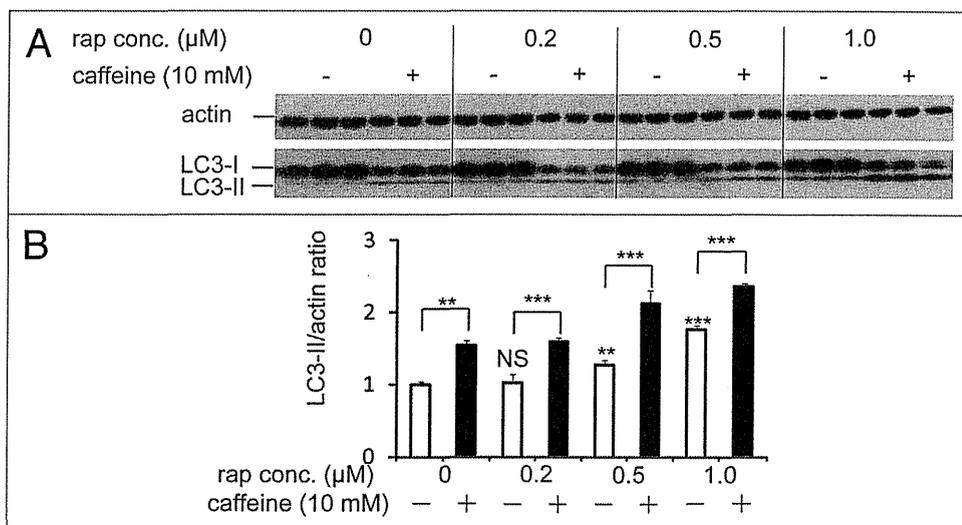


Figure 4. Caffeine-induced autophagy is dependent on PI3K/Akt/mTOR pathway. (A) SH-SY5Y cells treated with 25 mM caffeine for 3 hours followed by treatment with or without 200 nM insulin for 30 minutes were analyzed by immunoblotting. (B) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (C) SH-SY5Y cells treated with 25 mM caffeine, 50 μ M Akt1/2 inhibitors or 25 mM caffeine with 50 μ M Akt1/2 inhibitors for 6 hours were analyzed by immunoblotting. (D) Densitometry analysis of LC3-II levels relative to actin was performed using three independent experiments. (E) SH-SY5Y cells were transfected for 24 hours with either a control plasmid DNA (pcDNA3.1) or a plasmid encoding constitutively active Akt (myr-Akt), and then treated with H₂O or 10 mM caffeine for 6 hours. Immunoblotting was performed using antibodies against Akt, p-Akt (Ser 473) and actin. (F) SH-SY5Y cells treated with 25 mM caffeine with or without 20 μ M U0126 for 6 hours were analyzed by immunoblotting using antibodies against actin, LC3, p-ERK and ERK. (G) Densitometry analysis was performed using three independent experiments. Error bars, SD; * $p < 0.05$; ** $p < 0.01$; N.S., not significant.

provide a survival advantage to cells undergoing nutrient deprivation and other stresses, has also been linked to the cell death process.⁷ The cross-talk between apoptosis and autophagy is complex and sometimes contradictory; however, it is critical to the overall fate of the cell. In this study, we have shown that autophagy is induced by higher concentrations of caffeine without starvation, mainly via the inhibition of PI3K/Akt/mTOR/p70S6K signaling. Likewise, when caffeine-induced autophagy is blocked by 3-MA

treatment or *Atg7* knockout, apoptosis is partially attenuated, suggesting that caffeine-induced autophagy occurs upstream of caffeine-induced apoptosis. It also indicates the involvement of other pathways in caffeine-induced apoptosis. These results provide new insight into the effects of caffeine on cell death and survival and its use as a possible intervention strategy for the upregulation of apoptosis by a harnessing of its autophagic activity in tumor treatment.

Figure 5. Rapamycin treatment with caffeine has an additive effect on enhancement of autophagy. (A) SH-SY5Y cells treated with various concentrations of rapamycin with or without 10 mM caffeine for 48 hours were analyzed by immunoblotting. (B) Densitometry analysis was performed using three independent experiments. Error bars, SD; * $p < 0.05$; ** $p < 0.01$; N.S., not significant.



Materials and Methods

Cell line. HeLa cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 100 U/ml penicillin/streptomycin (Sigma) at 37°C and 5% CO₂. PC12D and SH-SY5Y cells were maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 5% horse serum and 100 U/ml penicillin/streptomycin at 37°C and 5% CO₂. All experiments with PC12D were performed after differentiation with NGF treatment for 48 hours. *Atg7*^{+/-} and ^{-/-} MEFs were maintained in DMEM (Sigma) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 1% sodium pyruvate (Gibco, 11360), 1% non-essential amino acid (NEAA) and 4.2 μl 2% beta-mercaptoethanol at 37°C.

To establish a HeLa GFP-LC3 stable cell line, proliferating HeLa cells were transfected with a GFP-LC3 plasmid.¹⁴ Forty-eight hours post-transfection with Lipofectamine 2000 (Invitrogen), positive stable clones were selected by growing cells with G418 (400 μg/ml) for 2 weeks and maintained in DMEM (Sigma) supplemented with 10% FBS (Sigma), 100 U/ml penicillin/streptomycin and 200 μg/ml G418 at 37°C and 5% CO₂. All cellular experiments were performed with cells cultured in complete medium with FBS as explained above.

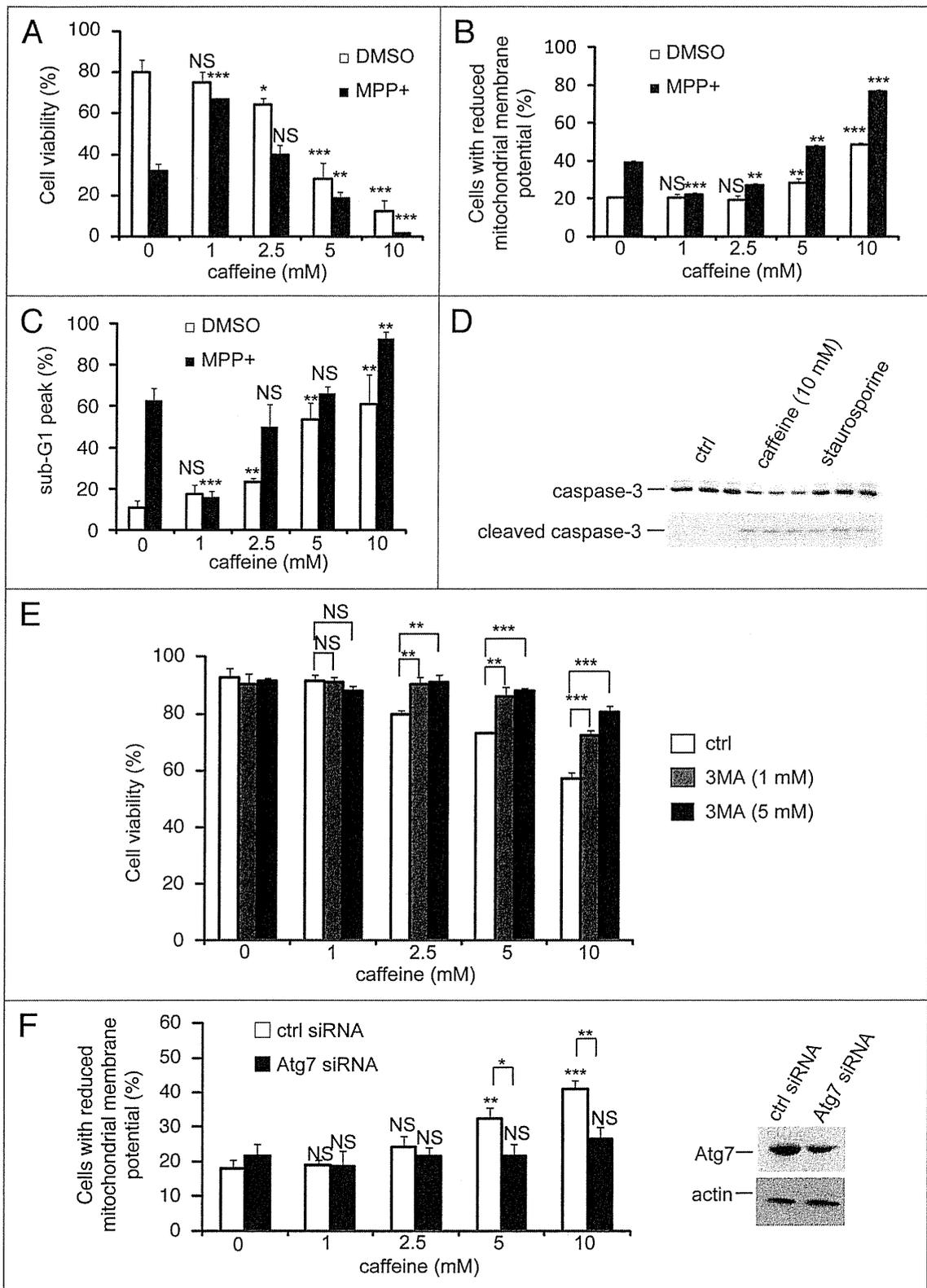
Cell viability assays. A trypan blue dye (Invitrogen, 15250-061) exclusion assay was used to examine cell viability and performed according to previously reported protocols.^{40,41} Changes of mitochondrial membrane potential were assessed also with the lipophilic cationic membrane potential-sensitive dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide)

(Wako, 106-00131) according to the manufacturer's protocol. Detection of early apoptotic cells was determined using an annexin V/propidium iodide (PI) detection kit (Invitrogen), according to the manufacturer's protocol. Briefly, 0.5 × 10⁶ *Atg7*^{+/-} or ^{-/-} MEFs were exposed to caffeine (0–25 mM) for 24 hours and washed twice. Then, they were incubated at room temperature with annexin V/Alexa488 and PI for 15 minutes. Annexin V-PI cells, considered as early apoptotic cells, were enumerated using FACScan (BD Biosciences). Data were analyzed with CellQuest (BD Biosciences) and FlowJo softwares (Tree Star Inc.). Cells positive or negative for annexin V were regarded as apoptotic or non-apoptotic cells, respectively.

Cell cycle analysis. To examine apoptosis, 1.0 × 10⁴ cells/well PC12D cells were seeded onto 96-well culture plate and incubated for 48 h in DMEM with NGF and treated with caffeine for 72 h. The cells were harvested and washed with PBS and fixed with ice-cold 70% ethanol at 4°C for 2 h. The cells were then stained with PI solution according to previously reported protocol.⁴¹ DNA content was analyzed by flow cytometry using FACScan and CellQuest software (BD Biosciences).

Compounds. Compounds used included caffeine (Wako, 031-06792), E64d (Sigma, E8640), pepstatin A (Sigma, P5318), rapamycin (LC Laboratories, R5000), CCI-779 (Selleck Chemicals, S1044), MPP⁺ (Sigma, M0896), bafilomycin A1 (Sigma, B1793), 3-methyladenine (Sigma, M9281), insulin (Sigma, I0516), U0126 (Sigma, U120), Akt1/2 inhibitors (Sigma, A6730), staurosporine (Cell Signaling Technology, 9953) and DMSO (Sigma, D2650).

Figure 6 (See next page). Caffeine induces apoptosis by enhancement of autophagy. (A) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 72 hours, cell viability was measured using trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B) After cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 48 hours, mitochondrial membrane potential was analyzed by JC-1 using a flow cytometry. Data are the means of triplicate experiments. (C) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with DMSO or MPP⁺ for 72 hours, caffeine-induced sub G₁ area was analyzed by propidium iodide staining assay using a flow cytometry. Data are the means of triplicate experiments. (D) PC12D cells were treated with H₂O or caffeine for 24 hours or staurosporine (positive control) for 3 hours and analyzed with immunoblotting for levels of caspase-3 and cleaved caspase-3. (E) After PC12D cells were treated with 0, 1, 2.5, 5 or 10 mM caffeine with or without 1, 3 or 5 mM 3MA for 24 hours, cell viability was measured by trypan blue dye exclusion assay. (F) PC12D cells were transfected with control siRNA or siRNAs targeting *Atg7*. Forty eight hours later, they were treated with 0, 1, 2.5 or 10 mM caffeine for 24 hours and mitochondrial membrane potential was analyzed using JC-1. The knockdown effects on *Atg7* were confirmed by immunoblotting using antibodies against *Atg7* and actin. Data are the means of triplicate experiments. Error bars, S.D. NS, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.



Plasmid DNAs. Myristoylated Akt (21–151), a constitutively active form of Akt, was purchased from Millipore.

siRNA knockdown experiments. PC12D cells were transfected with rat Atg7 siRNAs (Invitrogen, 10620318-9) using Lipofectamine RNAiMAX (Invitrogen, 13778-075) according to the manufacturer's protocol.

Western blotting. Cell pellets were lysed on ice in RIPA buffer for 20 minutes in the presence of protease inhibitor (Roche). Western blotting was performed according to a previously published report.⁴² The antibodies used were as follows: anti-p70 ribosomal protein (Cell Signaling Technology, 2708), anti-ribosomal protein (Cell Signaling Technology, 2217), anti-4E-BP1

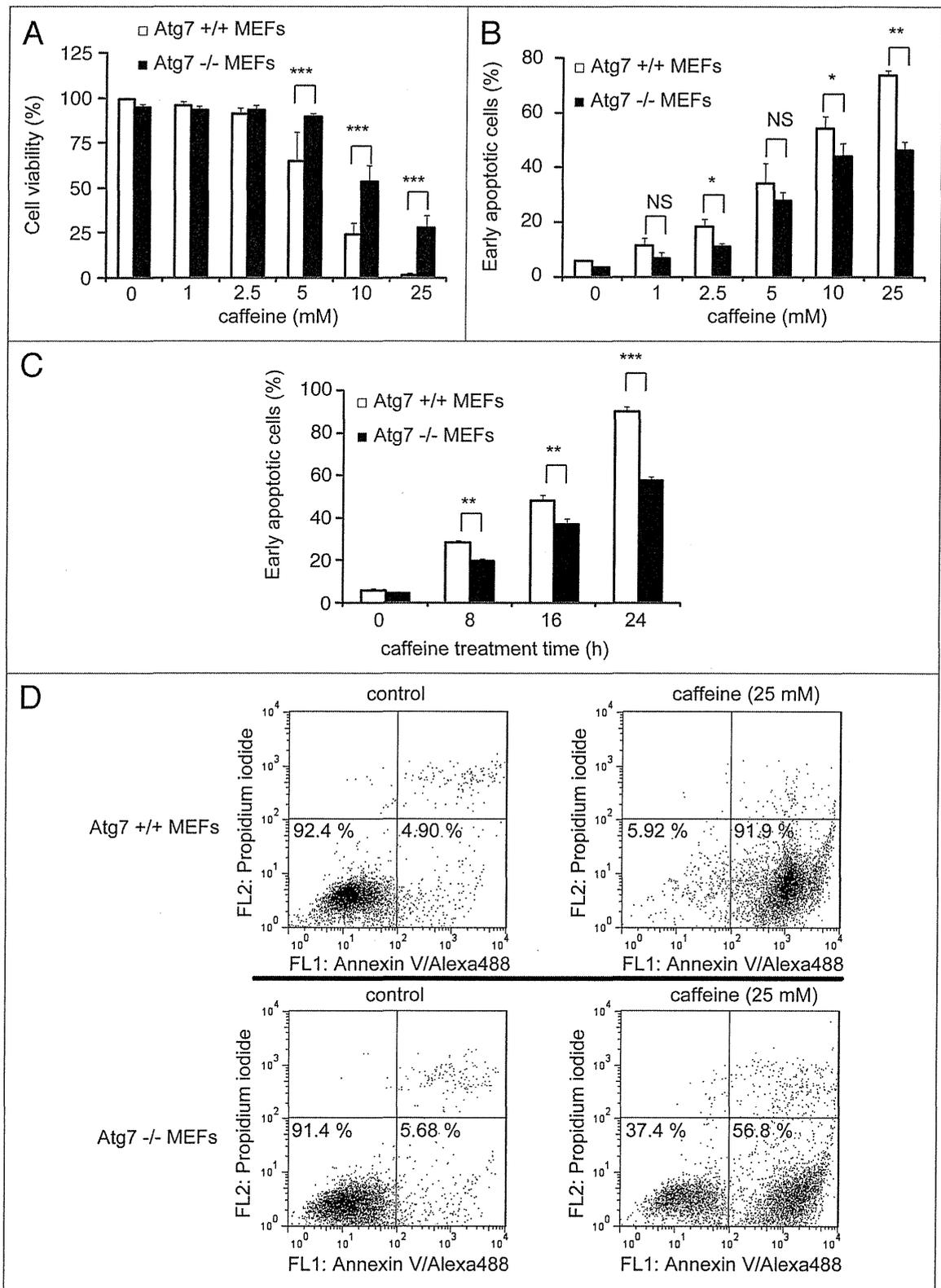


Figure 7. Cells without *Atg7* expression are more resistant to caffeine-induced apoptosis. (A) After *Atg7*^{+/+} or ^{-/-} mouse embryonic fibroblasts (MEFs) were treated with 0, 1, 2.5, 5, 10, 25 mM caffeine for 24 hours, the cell viability was measured by trypan blue dye exclusion assay. Data are the means of triplicate experiments. (B–D) Fluorescence-activated cell-sorting analysis for annexin V/propidium iodide (PI). *Atg7*^{+/+} or ^{-/-} MEFs were cultured with various concentrations of caffeine for 24 hours (B) or with 25 mM caffeine for various times (0, 8, 16 or 24 hours) (C and D). Annexin V/PI staining was subsequently performed to assess early or late apoptosis and necrosis. 5 × 10³ cells were analyzed by flow cytometry and the percentage of early apoptotic cells (annexin V-positive and PI-negative cells, the lower right region in (D)) was determined. Data are the means of triplicate experiments. Error bars, SD. NS, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

(Cell Signaling Technology, 9452), anti-Akt (Cell Signaling Technology, 9272), anti-p44/42 MAP kinase (Cell Signaling Technology, 9102), anti-phospho-p70 ribosomal protein (Thr389) (Cell Signaling Technology, 9205), anti-phospho-S6 ribosomal protein (Ser235/236) (Cell Signaling Technology, 2211), anti-phospho-4E-BP1 (Thr37/46) (Cell Signaling Technology, 9459), anti-phospho-p44/p42 MAPK (Thy202/Tyr204) (Cell Signaling Technology, 9101), anti-Atg7 (Cell Signaling Technology, 2631), anti-phospho-Akt (Cell Signaling Technology, 4060), anti-actin (Millipore, clone C4), anti-LC3 (MBL, clone 4E12), anti-p62 (Progen Biotechnik, GP62-C) antibodies. Antibody signals were enhanced with chemifluorescent methods from GE HealthCare.

Immunofluorescent microscopy. Cells were embedded with 4% paraformaldehyde for 20 minutes. Following this, they were permeabilized with 0.1% Triton-X in 1x PBS. After incubation with 10% FBS and 1% bovine serum albumin in 1x PBS for 30 minutes, cells were immunostained with anti-LC3B (x500) (Sigma, L7543), anti-LAMP2 (x50) (Development Studies Hybridoma Bank, clone H4B4) overnight and incubated with anti-rabbit IgG tagged with AlexaFluor 488 or anti-mouse IgG tagged with AlexaFluor 546 for 1 hour. The cover slips were embedded with VectraShield, stained with DAPI and images were acquired on a Zeiss LSM510 META confocal microscope (63 x 1.4 NA) or a Leica TCS SP5 confocal microscope at room temperature using Zeiss LSM510 v.3.2 software or Leica LAS AF software. Adobe Photoshop 7.0 (Adobe Systems Inc.) was used for subsequent image processing. For colocalization assay in HeLa cells, an appropriate confocal image was taken with Leica LAS AF software. Then, these images were analyzed automatically with the ImageJ "Colocalization" Plugin (Settings: Each threshold: 25, Ratio: 75%) followed by "Analyze particles" (Settings: threshold 25; Pixel: 1) between endogenous LC3 positive and

LAMP2 vesicles. Experiments were done in triplicate at least twice.

Quantification of cells with GFP-LC3 vesicles. HeLa cells stable expressing GFP-LC3 were treated with various concentrations of caffeine for 24 or 48 hours and then fixed as described above. Analyses in triplicate were done for counting the proportion of GFP-positive cells with GFP-LC3 vesicles as previously described in reference 43.

Electron microscopy. SH-SY5Y cells treated with various concentrations of caffeine were prefixed in 2% glutaraldehyde in PBS at 4°C, treated with 1% OsO₄ for 3 hours at 4°C, dehydrated in a graded series of ethanol and flat embedded in epon. Ultra-thin sections were doubly stained with uranyl acetate and observed using a JEOL JEM-2000EX electron microscopy at 80 kV.

Statistical analysis. Densitometry analysis was performed using ImageJ 1.43 on immunoblots from three independent experiments. A t-test was performed with SYSTAT software (Hulinks).

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Note

Supplementary materials can be found at: www.landesbioscience.com/journals/autophagy/article/14074

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Zonisamide reduces cell death in SH-SY5Y cells via an anti-apoptotic effect and by upregulating MnSOD

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ABSTRACT

Zonisamide, originally known as an antiepileptic drug, has been approved in Japan as adjunctive therapy with levodopa for the treatment of Parkinson's disease (PD). Although zonisamide reduces neurotoxicity, the precise mechanism of this action is not known. Here, we show that zonisamide increases cell viability in SH-SY5Y cells via an anti-apoptotic effect and by upregulating levels of manganese superoxide dismutase (MnSOD). These results would give us novel evidences of PD treatment.

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Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by pronounced loss of dopaminergic neurons in the substantia nigra pars compacta. At present, there is no known treatment to suppress the progression of this cell death and the goal of current therapies is only to alleviate symptoms. New therapies are, therefore, required to delay disease progression and to improve the long-term prognosis of PD. Mitochondrial dysfunction due to oxidative stress has been proposed as a major factor in the pathogenesis of PD [3,22] and reduced activity in mitochondrial complex I is associated with PD [13,22]. After complex I blockade with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice, there is a time-dependent and region-specific activation of mitochondrial cytochrome c, which activates caspase-9 and -3, resulting in apoptosis [20].

Zonisamide, originally known as an anticonvulsant agent, has been approved in Japan as adjunctive therapy with levodopa for patients with PD [15,16]. The mechanism of zonisamide to improve Parkinsonism has been reported to modify the turnover and synthesis of dopamine (DA) [18,28]. Moreover, it has been recently reported that zonisamide attenuates MPTP-induced and 6-hydroxydopamine (6-OHDA)-induced neurotoxicity and dopaminergic neuron-specific oxidative stress in mice [1,2,29,30]. Although zonisamide is likely to delay the progress of PD, the exact mechanism of its action remains unclear.

In this study, we show that zonisamide increases the viability of differentiated SH-SY5Y cells by inhibiting activation of proapoptotic molecules and upregulating levels of manganese superoxide dismutase (MnSOD), also known as superoxide dismutase-2 (SOD2).

The following antibodies were used in this study: anti-caspase-3 antibody (rabbit), anti-caspase-9 antibody (rabbit), and anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (rabbit) were obtained from Cell Signaling Technology. Anti-actin antibody (mouse) was from Millipore. Anti-MnSOD antibody (rabbit) was from Upstate.

SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin-streptomycin (Invitrogen-GIBCO) at 37 °C and 5% CO₂. To induce cell differentiation, SH-SY5Y cells were incubated in complete medium plus 10 μM *all-trans* retinoic acid (Sigma) for 72 h. For pharmacological studies, zonisamide (Dainippon Sumitomo Pharma Co.), staurosporine (Cell Signaling Technology), 1-methyl-4-phenyl-pyridium ion (MPP⁺) (Sigma), and dopamine hydrochloride (Sigma) were added at indicated times and concentrations.

Cell viability was assessed by the WST assay; the ability of mitochondrial activity to reduce 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) to formazan using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions.

TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining was performed using the *In situ* Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer's instructions. Cells were then mounted with Vectashield containing DAPI (Vector Lab-

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