Apoptosis of NG108-15 Cells Induced by Buprenorphine Hydrochloride Occurs via the Caspase-3 Pathway

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Apoptosis of NG108-15 neuroblastoma x glioma hybrid cells (NG108-15 cells) is induced by a morphine alkaloid derivative, buprenorphine hydrochloride (Bph). In a previous report, we used various apoptosis inhibitors to identify the "death pathway," and found that caspase inhibitors Ac-YVAD-CHO (Ac-Tyr-Val-Ala-Asp-CHO) and Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-CHO) did not inhibit this particular apoptosis. Here, we tested Z-VAD-FMK (Z-Val-Ala-Asp[OMe]-CH₂F) and Z-DEVD-FMK (Z-Asp[OMe]-Glu-[OMe]Val-Asp[OMe]-CH₂F) for their ability to inhibit Bph-induced NG108-15 apoptosis. These tri- or tetra-peptide caspase inhibitors have a fluoromethyl ketone in their C-terminus instead of an aldehyde, and thus are more permeable than Ac-YVAD-CHO and Ac-DEVD-CHO. Our observations of DNA ladder formation, cell morphology changes, and caspase-3 activities all indicated that these cell membrane-permeable caspase inhibitors completely inhibited the apoptosis, providing strong evidence that this apoptosis occurs through the caspase cascade "death pathway." Our previous report also showed that pretreatment of NG108-15 cells with TPCK (N-tosyl-l-phenylalanyl chloromethyl ketone) prevented DNA fragmentation and decreased the cell viability in Bph-induced apoptosis. The comparison of caspase-3 activities in Bph-induced samples with or without TPCK pretreatment revealed that caspase-3 was activated in both samples. Taken together, these results indicate that the Bph-induced apoptosis of NG108-15 cells occurs via the conventional caspase-dependent death pathway and that TPCK pretreatment results in a DNA ladder-deficient apoptosis.

Key words NG108-15; buprenorphine hydrochloride; apoptosis; caspase-3; TPCK (N-tosyl-l-phenylalanyl chloromethyl ketone)

Some extracellular stimuli can induce a self-destructive pathway in cells called apoptosis. If the apoptosis is induced in non-proliferative nerve cells, serious or even life-threatening malfunctions of the nervous system can result. We have been interested in the vulnerability of nerve cells, and found previously that a morphine alkaloid derivative, buprenorphine hydrochloride (Bph) induces apoptosis in NG108-15 neuroblastoma x glioma hybrid cells (NG108-15 cells).1

The death cascade of apoptosis has been the subject of intense study, and figures prominently in several prestigious reviews.2-5 Regardless of the inductive agent used (interestingly, Bph is used clinically as an analgesic), the immediate effectors are known to be DNases (such as a CAD6), which cause DNA degradation in nuclei. The caspase family of proteases act just upstream of the DNases in the death cascade. By the end of 1999, 14 caspase-family proteases had been reported. Among them, caspases-1 and -3 have been shown to play a pivotal role in the apoptotic pathway that leads to chromosomal DNA fragmentation.7-9

We have previously used various apoptosis inhibitors to identify the pathway induced by Bph in NG108-15 cells, and found that Ac-YVAD-CHO (Ac-Tyr-Val-Ala-Asp-CHO) and Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-CHO) did not completely inhibit DNA ladder formation, even at final concentrations of 200 μM. However, pretreating NG108-15 cells with TPCK (N-tosyl-l-phenylalanyl chloromethyl ketone), which inhibits a wide range of serine proteases, completely inhibited DNA ladder formation. Despite the disappearance of the DNA ladder, TPCK-pretreated NG108-15 cells still died. Thus, in our previous paper, these observations led us to propose that in this case, NG108-15 cell death occurred via a previously unreported death pathway. This pathway might involve an unidentified serine protease whose activity is inhibited by TPCK, that might act as a switch, shifting the cell from an apoptotic death pathway to some other death pathway (probably necrosis).10

Historically, apoptosis is defined by a set of morphological observations, including cell shrinkage and fragmentation, chromatin condensation, and apoptosis-specific DNA fragmentation.10-12 On the other hand, a "new type" or "deviant type" of cell death has also been reported.13,14 In some cases, cell death exhibits all the typical morphological characteristics of apoptosis, except that the DNA ladder formation is absent. In other cases, cell necrosis, which has been thought to occur by a different mechanism from the "death cascade" of apoptosis, has been reported to use some of the same machinery as the apoptotic death pathway.15,16

In this study, we sought to identify the apoptotic pathway induced by Bph, by testing the effects of different apoptosis inhibitors and by measuring caspase activity directly. The results of these studies have led us to conclude that Bph-induced apoptosis in NG108-15 cells is executed by caspase-3, and that the cell death observed with TPCK pretreatment is an apoptosis that occurs without DNA ladder formation.

In addition to these results, a time course experiment revealed that apoptosis occurred as early as 30 min after the administration of Bph to NG108-15 cell cultures. We discuss how this "rapid apoptosis" might occur via an additional death signal transduction pathway.

MATERIALS AND METHODS

Cell Culture NG108-15, a neuroblastoma x glioma hybrid cell, was used in this study. The detailed conditions of

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NG108-15 cell culture are described in our previous paper. In brief, NG108-15 cells were cultured in Dulbecco’s Modified Eagle’s Medium (Nissui, Japan) supplemented with 10% fetal bovine serum, at 37°C in 5% CO2.

**Cell Viability and Morphology Observation** Cell viability was assayed by the trypan blue dye exclusion method. An Olympus model IX-70 inverted phase-contrast microscope was used to observe the morphological changes in NG108-15 cells.

**Inhibitory Experiments** TPCK (N-tosyl-l-phenylalanyl chloromethyl ketone, Sigma, U.S.A.), Z-VAD-FMK (Z-Val-Ala-Asp[OMe]-CH2F, Peptide Institute, Japan), and Z-DEVD-FMK (Z-Asp[OMe]-Glu-[OMe]Val-Asp[OMe]-CH2F, CALBIOCHEM, U.S.A.) were used as reagents for the inhibition of Bph-induced apoptosis in NG108-15 cells. Inhibitor was added to NG108-15 cells 1 h before the administration of Bph (100 μM final concentration), then the cells were kept in a CO2 incubator at 37°C until harvesting time. Cells were harvested at the indicated times after the addition of Bph and washed in phosphate-buffered saline lacking CaCl2 and MgSO4 (PBS(−)). The genomic DNA was then extracted from the cell pellet as described previously and used for DNA ladder-formation assays.

**DNA-Ladder Formation, Caspase-3, and Stress-Activated Protein Kinase/C-Jun N-Terminal Kinase (SAPK/JNK) Assays** The assay of DNA ladder formation over time was conducted as described previously. We adjusted the total cell number (about 2×10^6 cells per lane) instead of the total DNA amount.

For caspase-3 activity, NG108-15 cells with or without TPCK pretreatment (10 μM final concentration) were harvested 2 h after the administration of Bph (100 μM). A colorimetric assay for caspase-3 was conducted using an APOALERT® Caspase-3 Assay Kit (CLONTECH, California) following the manufacturer’s protocol. This assay is based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate, DEVD (Asp-Glu-Val-Asp)-pNA.

To assess SAPK/JNK activity, cells were prepared as described above for the caspase-3 assays, then tested using an SAPK/JNK Assay Kit (New England Biolabs, U.S.A.), following the manufacturer's protocol.

**RESULTS**

**Time Course of Bph-Induced Apoptosis in NG108-15 Cells** Figure 1 shows the time course of DNA ladder formation after the administration of Bph to NG108-15 cells. Since we showed in our previous paper that three independent indicators of apoptosis (cell shrinkage, chromatin condensation, and DNA ladder formation) are all present in Bph-induced NG108-15 cell death, the DNA ladder-formation assay was used as representative evidence of apoptosis in this time-course experiment.

Cells were harvested 30 min, and 1, 2, 3, and 4 h after the administration of Bph, and assayed for DNA ladder formation by electrophoresis on a 1.8% agarose gel. We had already shown in our previous study that the DNA ladder formation starts as early as 4 h after the administration of Bph. However, here we conducted a more detailed time course experiment, in which earlier time points were taken (Fig. 1). A faint DNA ladder was apparent as early as 30 min after the administration of Bph, and the ladder was clearly identifiable at 1 h. In the morphological observation, almost all of the NG108-15 cells were still attached to the dish surface at 30 min, but at 1 h about 30% of the cells had detached and were floating in the medium. These floating cells displayed the typical apoptotic features of cell shrinkage and chromatin condensation. As the time went on, the changes in cell morphology progressed, and at 4 h, all cells were detached from the dish surface (data not shown).

This detailed time course experiment indicated that the apoptosis induced in NG108-15 cells by Bph is very rapid.

**Inhibition of Bph-Induced Apoptosis in NG108-15 Cells by Z-VAD-FMK and Z-DEVD-FMK** Z-VAD-FMK and Z-DEVD-FMK, specific inhibitors for caspases-1 and -3 respectively, were tested for their ability to inhibit Bph-induced apoptosis of NG108-15 cells. The effects of these inhibitors on DNA ladder formation were observed, and in addition, cell viability assays by trypan blue dye exclusion and microscopic morphological observation were conducted. One hour before the administration of Bph, each caspase inhibitor was added to the cell culture to a final concentration of 200 μM. After the addition of Bph, the cells were incubated for another hour, then harvested and tested for DNA ladder formation (Fig. 2A).

The result of the DNA ladder-formation assay using the caspase-1 inhibitor, Z-VAD-FMK is shown in Fig. 2A. Untreated, intact NG108-15 cells showed no ladder formation (lane 2), whereas treatment with Bph resulted in the formation of an obvious ladder (lane 3). Bph-treated NG108-15 cells that had been pretreated with 200 μM of Z-VAD-FMK showed complete inhibition of the DNA ladder formation (lane 4). Neither Z-VAD-FMK alone (lane 5) nor vehicle (dimethyl sulfoxide, DMSO) induced any morphological symptoms of cell death.
Fig. 2. Inhibition of Bph-Induced Apoptosis by Z-VAD-FMK in NG108-15 Cells

A): Inhibition of DNA Ladder Formation by Z-VAD-FMK

Lane 1: 3X174/Hinc II-digested molecular weight markers. Z-VAD-FMK (200 μM final concentration) was added to the NG108-15 culture 1 h before the administration of Bph, then the culture was incubated one more hour, harvested, and subjected to electrophoresis and staining as described for Fig. 1. Lane 2: DNA sample from the untreated NG108-15 cells. Lane 3: DNA sample from Bph-treated NG108-15 cells that were not pretreated with Z-VAD-FMK. Lane 4: DNA sample from Bph-treated NG108-15 cells that were pretreated with Z-VAD-FMK. Lane 5: DNA from cells treated with 200 μM Z-VAD-FMK alone then incubated for 1 h. The vehicle (DMSO) did not induce any apoptotic changes in NG108-15 cells.

B): The Time Course of DNA Ladder Formation by Z-VAD-FMK Pretreatment

Leftmost lane: 3X174/Hinc II-digested molecular weight markers. The sampling times are indicated at the top of each lane as follows: T0, 0 h; T1, 1 h; T2, 2 h; T3, 3 h; and T4, 4 h. Z-VAD-FMK (200 μM final concentration) was added to the cultured NG108-15 cells 1 h before the administration of Bph (T1). Samples of NG108-15 cells were harvested every hour to assay for DNA ladder formation.

C): The Viability of Bph-Treated NG108-15 Cells with Z-VAD-FMK Pretreatment

The viability of NG108-15 cells was evaluated using the trypan blue dye exclusion method. Z-VAD-FMK (200 μM final concentration) was added 1 h before the administration of Bph to NG108-15 cells (T1). Cell viability was then assayed every hour through the duration of the experiment. Cell viability is expressed as the mean ± S.D. of values obtained from three independent experiments.

Figures 2B and 2C indicate the inhibitory effect of Z-VAD-FMK on Bph-induced apoptosis in NG108-15 cells over time. No DNA ladder formation was observed at any time up to 4 h. The viability of NG108-15 cells remained at about 80% during the 4-h experimental term (Fig. 2C), and no increase in floating, dead cells was seen (data not shown).

As with Z-VAD-FMK, Z-DEVD-FMK pretreatment completely inhibited the DNA ladder formation seen with Bph treatment alone (Fig. 3, lanes 3, 4). Also similar to the inhibitory experiment for Z-VAD-FMK, microscopic examination of the cells throughout the duration of the experiment indicated no morphological symptoms of apoptosis and no increase in floating dead cells. The viability of the NG108-15 cells remained at more than 80% throughout the 4-h experiment (data not shown). We therefore concluded that pretreatment of NG108-15 cells with Z-DEVD-FMK before the addition of Bph completely inhibited this particular apoptosis.

The results from Figs. 2 and 3 strongly suggested that the apoptosis induced in NG108-15 cells by Bph occurs via the death pathway that includes caspases-1 and -3.

What Is the Significance of the Prevention of DNA Ladder Formation in TPCK-Pretreated NG108-15 Cells? We previously found that, among various apoptosis inhibitors tested, only TPCK (10 μM) inhibited DNA ladder formation in Bph-induced NG108-15 cells.3 Interestingly, even though DNA ladder formation was prevented by TPCK pretreatment, the cells still died, similar to the cells that did not receive TPCK pretreatment. To investigate the nature of the inhibitory effect of TPCK in this system, a time-course experiment with sampling points every 30 min or 1 h was conducted (Fig. 4). The DNA ladder formation was completely inhibited by TPCK pretreatment as early as 30 min after the addition of Bph (Fig. 4A). TPCK alone did not cause any cellular damage, as indicated by the DNA ladder formation (Fig. 4A) and cell viability (Fig. 4B) assays.

However, unlike the DNA ladder formation, Bph-induced NG108-15 cell death was not prevented by TPCK-pretreatment (Fig. 4B). In addition, a comparison of the DNA ladders formed in the absence and presence of TPCK (“Bph only” and “TPCK+Bph” in Fig. 4A) indicates smeared DNA
signals in the high-molecular-weight region in the “TPCK + Bph” samples. In other words, this could mean that only “low-molecular-weight” DNA ladder formation was prevented by TPCK in Bph-induced apoptosis in NG108-15 cells. Some studies have reported that the typical DNA ladder is the result of two types of DNA fragmentation, one of relatively high-molecular-weight DNA and the other of low-molecular-weight DNA. Thus, we should consider the possibility that at least two DNases could take part in the Bph-induced apoptosis in NG108-15 cells. The DNase responsible for the low-molecular-weight DNA ladder formation may not be crucial for this apoptosis, because NG108-15 cells did not survive, even when low-molecular-weight DNA ladder formation was prevented.

An increase in caspase activity during cell death is thought to be part of the direct evidence that death is occurring through apoptosis. We have measured caspase-3 activity directly in the Bph-induced apoptosis of NG108-15 cells with or without TPCK pretreatment (Fig. 5). The assay is based on the colorimetric detection of p-nitroanilide (pNA) that is released from DEVD-pNA by the enzymatic action of caspase-3. Caspase-3 activity is expressed as nanomol of pNA released per minute per milligram protein of cell lysate (Fig. 5). Caspase-3 was clearly activated in Bph-induced apoptosis, both with and without TPCK pretreatment. The amount of pNA released with or without TPCK pretreatment was 2.0–2.5 nM, which was 4 to 5 times higher than that released using lysates from untreated NG108-15 cells or cells that had been treated with Bph and a caspase-3 inhibitor. The significantly higher caspase-3 activity in Bph-treated samples both with and without TPCK pretreatment was reproduced in three independent assays. Figure 5 shows the results of one representative experiment. We therefore concluded that TPCK pretreatment results in another type of apoptosis, in which caspase-3 is still activated, but no DNA ladder is formed.

**Assay of SAPK/JNK Activity**

It is well known that stress activated protein kinase or c-Jun N-terminal kinase (SAPK/JNK) activity is up-regulated by some apoptotic events. Especially in Fas/FasL-stimulated apoptosis, the “death signal” runs through the caspase-8 and SAPK/
JNK route to ultimately cause DNA fragmentation.\textsuperscript{25,26} SAPK/JNK is also activated in some cases by various extracellular stress factors. There are two similarities between our apoptosis system and that of Fas/Fasl apoptosis; in both cases apoptosis progresses very rapidly, and both are characterized as "stress-induced apoptosis."\textsuperscript{27} Thus, to clarify the pathway of signal transduction in our Bph-induced apoptosis system, we tested for SAPK/JNK activation using an SAPK/JNK Assay Kit.\textsuperscript{8} We measured SAPK/JNK activity in Bph-induced NG108-15 cells with and without TPCK pretreatment. The principal of the assay is based on Western blotting, using antibodies that specifically recognize phosphorylated c-Jun in the c-Jun-SAPK complex.

Figure 6 shows the result of one SAPK/JNK assay. No differences in phosphorylated c-Jun signals were observed among untreated NG108-15 cells, Bph-treated NG108-15 cells, and TPCK-pretreated Bph-treated NG108-15 cells. This result was reproducible, and indicated that at least in our system, the stress-activated protein kinase did not appear to directly contribute to Bph-induced apoptosis in NG108-15 cells.

DISCUSSION

Whether it occurs in vivo or in vitro, once an unprecedented type of cell death is found to be apoptotic, a central goal of the study should be to identify the "death initiators" and "death executioners." Recent apoptosis studies revealed that the executioners are responsible for the proteolytic cleavage of a number of cellular proteins, ultimately leading to the characteristic morphological changes and DNA fragmentation that are often associated with apoptosis.\textsuperscript{28}

We showed previously that NG108-15 cells will die of apoptosis induced by a morphine alkaloid derivative, buprenorphine hydrochloride (Bph).\textsuperscript{13} Using this model system to examine the vulnerability of nerve cells, we studied the pathway by which this death signal occurs. One basic way to dissect the pathway used in a focused apoptosis system is to observe the effects of several apoptosis inhibitors on the action of the death executioners. In our previous study, caspase-1 and -3 inhibitors Ac-VDAD-CHO and Ac-DEVD-CHO inhibited Bph-induced apoptosis, but not completely.\textsuperscript{11} In this study, we investigated the apoptosis pathway in more detail. Because a type of cell death that is not prevented by caspase inhibitors has been reported,\textsuperscript{29} we thought it possible that a deeper analysis of the Bph-induced apoptosis in NG108-15 cells could reveal a new route among several death cascades.

Here, we have extended the inhibitor studies of our previous report using the cell membrane-permeable caspase inhibitors Z-VAD-FMK\textsuperscript{30} and Z-DEVD-FMK instead of Ac-VDAD-CHO and Ac-DEVD-CHO,\textsuperscript{7} respectively. Our results clearly indicated that the apoptosis was completely blocked by these inhibitors. Furthermore, we also observed the complete inhibition of caspase-3 activation by Ac-DEVD-CHO in the experiment shown in Fig. 5, in which Ac-DEVD-CHO was directly added to the cell lysate from apoptosis-induced NG108-15 cells. From these results, we have concluded that Bph-induced apoptosis in NG108-15 cells occurs through the conventional caspase-1 and -3 pathway.

We also observed that TPCK did not affect caspase-3 activity but it did inhibit a certain DNase. TPCK pretreatment before Bph-induced apoptosis caused the disappearance of the DNA ladder in the low-molecular-weight range, although the NG108-15 cells still died. Interestingly, smeared DNA signals in the high-molecular-weight range were not inhibited by TPCK. In another system, Chow et al. reported the involvement of multiple proteases in the Fas-mediated apoptotic cascade in T lymphocytes.\textsuperscript{31} Thus, a hypothesis could be presented that at least one serine protease and two DNases are involved in the Bph-induced apoptotic cascade in NG108-15 cells. Furthermore, a TPCK-sensitive serine protease, which could directly regulate a DNase responsible for low-molecular-weight DNA ladder formation, might be located on a "branch" route of the caspase-3 death cascade. On the other hand, a different DNase, responsible for high-molecular-weight DNA ladder formation might be involved in the "backbone" route of the caspase-3 death cascade, which directly promotes the cell death in Bph-induced NG108-15 apoptosis.

Generally, Fas/Fasl is known to induce a rapid apoptosis in cells,\textsuperscript{32} and may play a role in "acute apoptosis," a sometimes life-threatening condition associated with the rejection of transplanted organs.\textsuperscript{33} As we have found that Bph-induced apoptosis is also very rapid (Fig. 1) and that Bph is a stress factor for NG108-15 cells, we investigated the relationship between this Bph-induced apoptosis and typical Fas/Fasl apoptosis. A stress-activated protein kinase (SAPK) or c-Jun N-terminal kinase (JNK) is a known component of the Fas/Fasl-caspase-8 pathway. Therefore, we tried to detect the SAPK/JNK activity by Western blotting using anti-phosphorylated-c-Jun antibodies. We found no increase in SAPK/JNK activity in Bph-induced apoptosis with or without TPCK pretreatment, suggesting that the Fas/Fasl route does not take part in this system. However, more detailed investigations, such as a direct assay of Fas induction on the NG108-15 cell membrane and/or a direct assay of caspase-8 activity should be performed before we can resolve this issue.

We are also interested in identifying the "initiator" of the Bph-induced apoptosis in NG108-15 cells. Although Bph is a morphine alkaloid derivative, our previous attempts to inhibit this apoptosis by naloxone, an opioid receptor antagonist and naltrindole, a δ-opioid receptor antagonist, failed.\textsuperscript{11} Because of its high hydrophobicity,\textsuperscript{34} it is likely that Bph
passes easily through the cell membrane into the cytoplasm. Therefore, an investigation of Bph's effect on the mitochondrial membrane would be a first priority in our future study of Bph-induced apoptosis in NG108-15 cells.

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