Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3

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Received 27 January 2000; received in revised form 13 March 2000

Edited by Masayuki Miyasaka

Abstract Anandamide (arachidonoylethanolamide), an endogenous cannabinoid receptor ligand has been suggested to have physiological role in mammalian nervous system. However, little is known about the role of anandamide on neuronal cells. Here, we demonstrate that anandamide causes death of PC-12 cells, showing marked DNA condensation and fragmentation, appearance of cells at sub-G_0/G_1 and redistribution of phosphatidyl serine, the hallmark features of apoptosis. Anandamide raised intracellular superoxide level and CPP32-like protease activity in PC-12 cells markedly. Furthermore, antioxidant *N*-acetyl cysteine prevented anandamide-induced superoxide anion formation and cell death, implying that intracellular superoxide is a novel mediator of anandamide-induced apoptosis of PC-12 cells.

Key words: PC-12 cell; Anandamide; Superoxide; CPP32-like protease; Apoptosis

1. Introduction

Programmed cell death, or apoptosis, is a normal physiological process that occurs during embryonic development, as well as in the maintenance of tissue homeostasis. Neuronal apoptosis is required for the development of the nervous system but also occurs during pathophysiological states [1]. Extensive neuronal cell death is observed after acute brain injury, including stroke [2] and trauma [3], is thought to contribute to neurodegeneration diseases, such as Parkinson's disease and Alzheimer's disease [4]. Additionally, neuronal apoptosis is induced in response to agent that causes increase in intracellular superoxide anion (O^{-2}) and resulting hydrogen peroxide (H_2O_2). Antioxidant *N*-acetyl cysteine prevents neuronal apoptosis induced by oxidative stress [5].

Anandamide, an arachidonic acid derivative has been isolated and subsequently identified as endogenous cannabinoid receptor ligand (CB), which is expressed abundant in brain [6]. Anandamide acts as a neuromodulator and may control intercellular communication in astrocytes by regulating gap-junction permeability [7,8]. Anandamide functions as a carrier of arachidonic acid in the modulation of hepatic fatty acid metabolism [9]. Anandamide hyperpolarizes the membrane potential of the smooth muscle cells of endothelium-intact arteries [10]. On the other hand, anandamide can be produced rapidly from circulating blood cells, such as macrophage cell line by lipopolysaccharide (LPS) during septic shock, and may circulate in the blood [11]. Apart from this, anandamide release is also induced by platelet activating factor following hemorrhagic shock [12]. Recently, we have shown that anandamide is one of the possible causative factors of hypotension induced by endotoxin shock [13]. Administration of anandamide into mice develops hypothermia, hypomotility and catalepsy [14]. Anandamide exerts an immunoregulatory role in the central nervous system [15]. Thus, our understanding about the role of anandamide in pathophysiological state is almost limited to the immune system. Hence, the role of anandamide on neuronal cells remains elusive. As anandamide can be released and synthesized from macrophage, platelets and endothelium, and can quickly reach to central nervous system as well, we hypothesized synthesis and subsequent release of anandamide can be enhanced in some pathophysiological states in brain, such as stroke, cerebral hemorrhage or disruption of blood-brain barrier. In this study, we tested the effects of anandamide on rat pheochromocytoma (PC-12) cell line, which has been suggested to be a useful model for neurobiological and neurochemical studies [16]. Our data show for the first time that anandamide leads to the generation of intracellular superoxide anion, which, in turn, triggers downstream signals culminated to the caspase-3 activation, resulting in apoptosis of PC-12 cells.

2. Materials and methods

2.1. Materials

Anandamide, propidium iodide (PI), superoxide dismutase (SOD), *N*-acetyl cysteine (NAC), phorbol myristate acetate (PMA), cytochrome *c* and RNAse were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). CPP32-like protease activity assay kit was purchased from MBL (Nagoya, Japan), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was bought from Dojindo (Kumamoto, Japan). Win-102 (functionally active) and Win-109 (functionally inactive) were purchased from Research Biochemical Inc. (USA). Stock solution of anandamide (10 mM) was prepared in ethanol, and stored in aliquots at −80°C.

2.2. Cell culture

PC-12 cells were cultured in RPMI 1640 (Gibco BRL, USA) medium supplemented with heat-inactivated at 56°C 10% horse serum and 5% fetal bovine serum (FBS) plus appropriate antibiotics at 37°C in the presence of 95% air and 5% CO_2 in collagen coated dishes. Human breast cancer cell line MCF-7 was maintained in Dulbecco's minimum essential medium (DMEM) (Gibco BRL, USA) containing 10% FBS.

2.3. Cell viability test

MTT assays were applied to test the cell viability. Briefly, PC-12 cells were seeded at a density of $2.0–2.5\times10^4$ cells per well. PC-12 cells were treated with anandamide for the indicated concentrations and periods. As anandamide was dissolved in ethanol, control cells were incubated with the indicated percentage of ethanol. Following the treatments, MTT (0.5 mg/ml final concentration) was added to

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PII: S0014-5793(00)01425-3

each well, and incubation was then continued for 3 h. As mitochondrial enzyme converts MTT to insoluble formazon crystal, 100 μl of a solution containing 10% SDS (pH 4.8) plus 0.01 N HCl was added to dissolve the crystal. Absorption values at 570–630 nm were determined with an automatic microtiter plate reader (Immuno Mini NJ-2300, Japan).

$$\text{Cell viability (\%)} = \frac{\text{OD of the treated cells} \times 10}{\text{OD of the non} - \text{treated cells}}$$

2.4. Propidium iodide staining

PC-12 cells were plated onto L-polylysine coated 2-well Lab-Tek chamber (5×10^4/well), and stimulated with anandamide for the indicated periods. After washing with phosphate-buffered saline (PBS), cells were permeabilized with acetone:methanol (1:1) for 10 min at −20°C, and incubated with PI (10 μg/ml) for 30 min at room temperature in the dark. Apoptotic cells were analyzed with fluorescence microscopy (Zeiss, Germany).

2.5. Analysis of cellular DNA content

For analysis of cellular DNA content, PI staining method was used. Briefly, PC-12 cells were plated at a density of 2.5×10^5 per 35 mm dish. After treatment for the indicated periods, cells were extensively washed with PBS, and centrifuged ($200\times g$ for 5 min). The pellet was resuspended in 70% ethanol (2 ml), and the suspension incubated at −20°C for 20 min. Then cells were incubated for 15 min with PI (5 μg/ml) in PBS in the presence of RNase (5 μg/ml). After that DNA content was determined (2×10^4 cells each time) by FACS-can analyzer (Epics, Coulter, USA).

2.6. Determination of phosphatidyl serine (PS) externalization by FACS

To determine the externalization of PS, cells were washed in DMEM twice and plated onto 60 mm dishes at a density of 1×10^6/well in DMEM alone. Cells were treated with anandamide (10 μM) for the indicated periods, harvested and incubated with annexin V-FITC (Immunotech, France) according to the manufacturer's instruction. Annexin V-positive cells were counted by the FACS-can analyzer.

2.7. Assay of caspase activity

Cells plated in 10 cm dishes (2×10^6/dish) were treated with anandamide, and incubated for the times indicated in the figure legends. Following treatment, cells were washed, and lysed with chilled lysis buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 10 mM EGTA, and 10 μM digitonin) and incubated for 10 min on ice, followed by centrifugation (15 000 rpm for 1 min). Then DEVDase activity was assayed at 405 nm using DEVD-pNA as a substrate by a microplate reader (ImmunoMini NJ-2300, Japan) in 96-well dish (Iwaki, Japan) according to the manufacturer's instructions. Fold increase in CPP32-like protease activity was determined by comprising these results with the level of uninduced control after subtracting the background readings from lysates and buffers from the readings of both induced and uninduced samples.

2.8. Superoxide anion assay

Cytochrome *c* reduction method was used to measure the intracellular superoxide by a slight modification of Pick et al. [17]. Briefly, cells (1×10^6/ml) were suspended in Hank's balanced salt solution containing cytochrome *c* (100 μM). From this cell suspension, 100 μl was incubated with anandamide (10 μM final concentration) in the presence or absence of antioxidant agent SOD (300 U/ml) or NAC (0.125–1 mM) at 37°C for the indicated periods. Reaction mixture without cells was used as a blank and PMA (10 μM) was used as a positive control.

3. Results and discussion

3.1. Anandamide causes loss of cell viability of PC-12 cells

The rapidly accumulating data on anandamide demonstrate that anandamide represents a new neurotransmitter system, and plays a significant role in brain [18]. However, few studies have been done on neuronal cells. In the present study, we attempted to investigate the effects of anandamide on PC-12 cells. Fig. 1B shows that in response to anandamide (10 μM) cells rounded, phase became bright, and finally detached from the dish, indicating loss of viable cells in culture. Since cell viability is positively correlated to the degree of MTT reduction, we then evaluated the cell viability of anandamide-treated cells by using MTT reduction assays. Anandamide dose-dependently caused the loss of cell viability of PC-12 cells. Fig. 1C shows that loss of cell viability was not observed

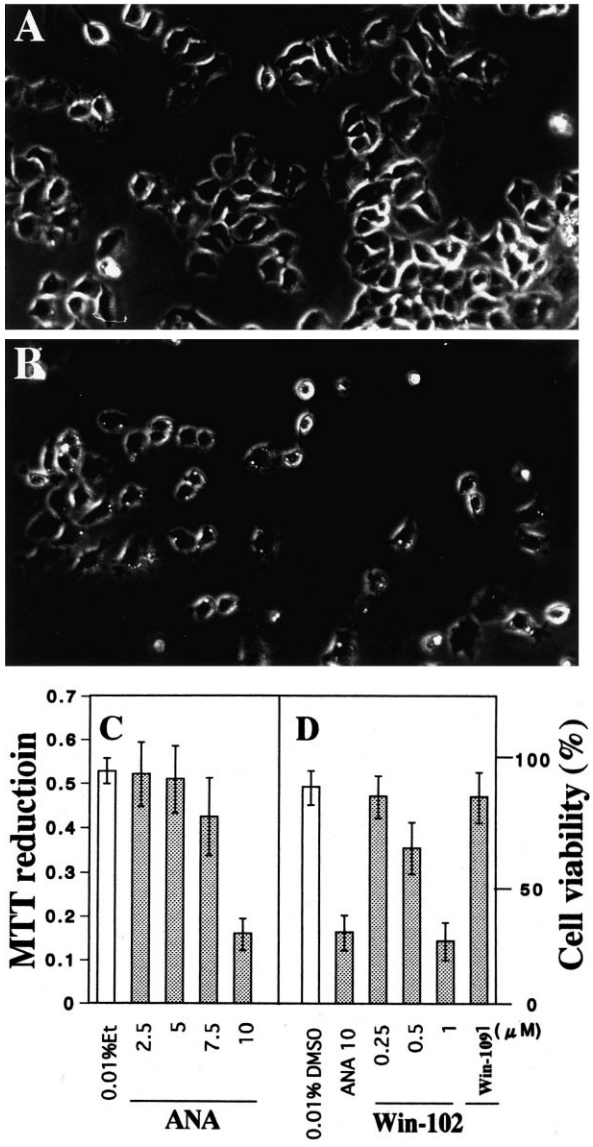

Fig. 1. Cytotoxic effects of anandamide (ANA) on PC-12 cells. Cells were plated in 6-well dishes at a density of 2×10^5/well. Following an 18 h attachment, cells were incubated in the presence or absence of ANA (10 μM) for 24 h. A: Control cells (0.01% ethanol). B: Anandamide-treated cells. Representative photographs were also taken at the same magnification. Original magnification, ×200. For determination of MTT reduction/cell viability, cells plated onto 96-well dishes were subjected to micromolar concentrations of anandamide for 24 h (C). D: Cells were incubated with micromolar concentrations of anandamide or Win-102 or Win-109 for 24 h. At the end of this incubation, percentage of viable cells was counted by MTT reduction assays as described in Section 2. Values are means ± standard deviations (S.D.) of three independent experiments, in triplicate.

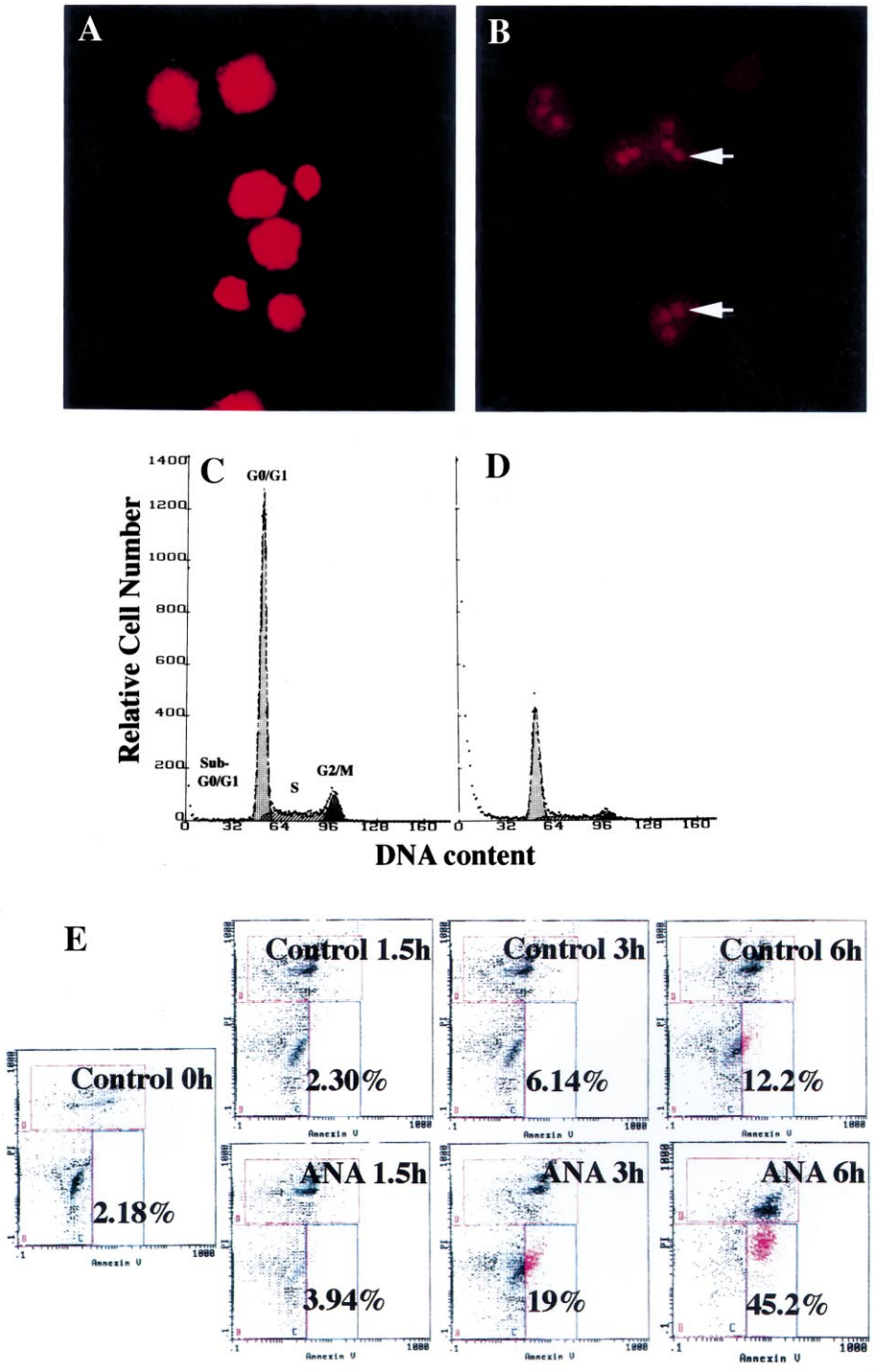

Fig. 2. Apoptotic features in anandamide-treated PC-12 cells. Cells were seeded onto Lab-Tek chambers treated with anandamide (10 μM) for 24 h. After this treatment, cells were stained with PI as described in Section 2. A: Control cells (0.01% ethanol). B: Anandamide-treated cells. Experiments were done in duplicate and photographs were taken at the same magnification. Original magnification, ×400. For analysis of cell cycles, cells were treated with anandamide (10 μM) for 24 h. Cell cycles were analyzed with FACS-can analyzer as outlined in Section 2. C: Control cells (0.01% ethanol). D: Anandamide (10 μM)-treated cells. E: Time course effect of anandamide on PS externalization. Cells were treated with anandamide (10 μM) for the indicated periods and PS externalization was determined with annexin V–FITC binding assay by flow cytometry (see Section 2). Similar experiments were done in duplicate.

at a relatively low concentration of anandamide (<7.5 μM). However, a massive cell death has occurred when cells were incubated with a higher concentration of anandamide. On incubating the cells with anandamide (10 μM), cell viability markedly decreased to $30 \pm 3.6\%$ of the control cultures (in the presence of 0.01% ethanol) at 24 h after anandamide treatment. Thus, it appears apparently that anandamide causes death of PC-12 cells relatively at a higher concentration. Anandamide (1 μM) vasodilates juxtamedullary afferent arterioles perfused in vitro [19]. Anandamide, at a concentration

of 10 μM hyperpolarizes the membrane potential of the smooth muscle cells of endothelium-intact arteries [10]. Venance et al. has reported that anandamide (10 μM) regulates intercellular communication in astrocytes and non-glial cells [8]. A question arises as to whether the used concentration of anandamide in the present study is relevant to in vivo or not. As anandamide is very unstable in vivo, the exact level of anandamide in plasma/tissue remains to be defined. Although anandamide level in brain is lower than in either of adrenal, lung, kidney or plasma but it reaches to the brain within a minute [20]. Following endotoxin shock anandamide level in plasma is elevated to 7.8-fold the normal concentration [21]. Anandamide level in serum has been found to be ~500 nM of a septic shock patient (our unpublished observation). Hence, it might be considered as micromolar concentration of anandamide in pathologic state(s).

Anandamide is an endogenous cannabinoid receptor ligand and most of its cellular effects are mediated through CB1 receptor activation in brain [6]. Recently, it has been shown that delta 9-tetrahydrocannabinol (THC), the exogenous cannabinoid receptor ligand, causes apoptosis of hippocampal neurons. At the same time, that THC-induced cell death has been blocked by the CB1 receptor antagonist [22]. Therefore, it is thought that cannabinoid receptor activation induces apoptotic cell death. Hence, we sought whether cannabinoid receptor activation could mimic anandamide's effect in PC-12 cells, which express CB1 receptor [23]. In parallel cultures, we treated PC-12 cells with CB1 receptor agonist Win-102 (functionally active) and Win-109 (functionally inactive). CB1 receptor activation pronounced significant cell death. Fig. 1D shows that Win-102 dose-dependently evoked cytotoxic effect, resulting in marked cell death. Win-102, at a dose of 1 μM, induced loss of cell viability to 29 ± 5.8%, however, Win-109, at the same concentration (1 μM), aborted to exhibit the cytotoxic effect on PC-12 cells, indicating that the cytotoxic effect of anandamide is mediated through CB1 receptor activation. The finding that CB1 receptor antagonist inhibits apoptosis of hippocampal neurons induced by THC definitely favors our result [22].

3.2. Anandamide causes apoptosis of PC-12 cells

Cells die either following necrosis or apoptosis. Necrotic cell death is regarded as accidental cell death, in which first membrane is raptured, followed by the release of cytosolic organelles, resulting in inflammation. In contrast, apoptotic cell death rather occurs in a controlled manner, where morphological and biochemical changes are obvious. The morphological changes, the first defined apoptotic features, occur in three phases [24]. In the first, there is condensation of chromatin into crescentic caps at the nuclear periphery, nucleolar disintegration, and reduction of nuclear size. There is shrinkage of total cell volume, increase in cell density, compaction of cytoplasmic organelles, and dilation of endoplasmic reticulum but mitochondria remain morphologically normal. In phase 2 (which may overlap phase 1) there is budding and separation of nucleus and cytoplasm into multiple, small, membrane-bound apoptotic bodies, which may be shed from epithelial surfaces or phagocytosed by neighboring cells or macrophages [25]. In phase 3 there is progressive degeneration of residual nuclear and cytoplasmic structures. Morphological analysis of the anandamide-treated cells with PI staining displayed marked DNA condensation and fragmentation (Fig. 2B), demonstrating that anandamide leads to the PC-12 cells undergoing apoptosis.

On the other hand, the biochemical features include appearance of sub-diploid DNA (accumulation of cells at sub-G_0/G_1) and redistribution of membrane phosphatidyl serine, a phospholipid normally restricted to the inner leaflet of the plasma membrane. It is conceived that phosphatidyl serine is a membrane 'flag mark' of apoptosis, appears on outer membrane during early step of apoptosis, and helps macrophage or neighboring cells engulf apoptotic cell [26–29]. Thus, the exposed membrane PS plays pivotal role in the elimination of apoptotic cell before it exerts inflammation. In agreement with the DNA fragmentation result (Fig. 1B), cells having diploid DNA gradually decreased, and subsequently fragmented DNA containing cells appeared notably at sub-G_0/G_1. DNA content analysis indicated that about 56 and 19% of the total cell population were at sub-G_0/G_1 (Fig. 2C,D) at 24 h in anandamide and control (0.01% ethanol) cultures, respectively. These results indicate that anandamide causes apoptosis of PC-12 cells.

Next, we looked for the appearance of phosphatidyl serine on the outer membrane by annexin V–FITC binding assay. Consistent with the results obtained from morphological study, exposure of PS has occurred noticeably in anandamide-treated cells. As shown in Fig. 2E, induction of PS ex-

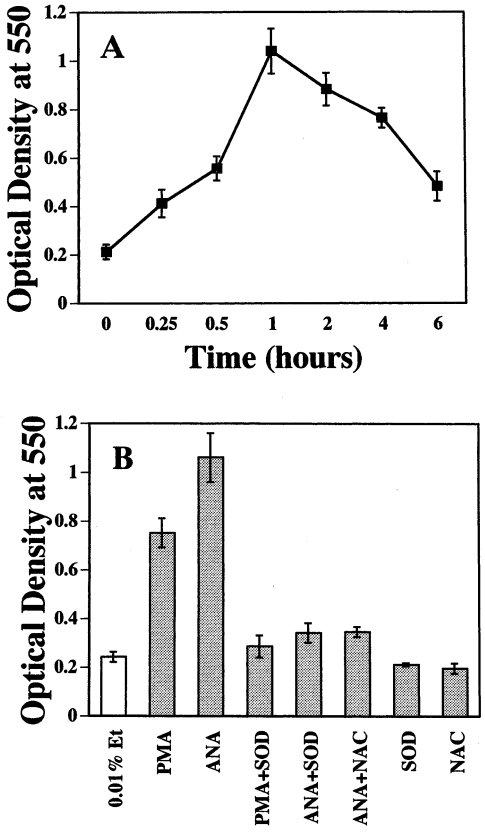

Fig. 3. Effects of anandamide on superoxide anion production in PC-12 cells. A: Time course effects of anandamide on O^{-2} formation. Cells were treated with anandamide (10 μM) for the indicated periods and superoxide was assayed as described in Section 2. B: Cells were incubated with anandamide in the presence or absence of antioxidant agents for 1 h. The results are means ± S.D. of three separate experiments.

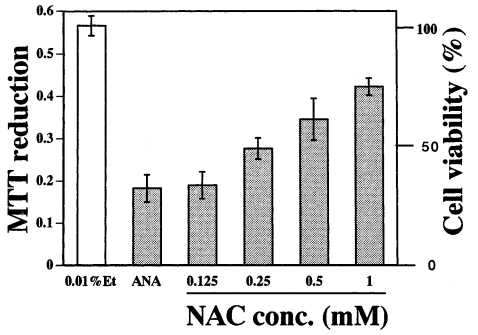

Fig. 4. Effects of NAC on anandamide-induced apoptosis of PC-12 cells. Cells were plated onto 96-well plates. Indicated concentrations of NAC were added 1 h prior to the addition of anandamide (10 μM) and incubation was continued for 24 h. MTT reduction assays were done as described in Section 2. Results are given as mean ± S.D. of the three independent experiments.

ternalization was started within 3 h that, in turn, significantly increased at 6 h after anandamide addition. Flow cytometric analysis indicates that annexin-positive cells were 45.2 and 12% in anandamide-treated and control (0.01% ethanol) cultures, respectively, at 6 h onset of treatment. Taken together, our results strongly suggest that anandamide induces apoptotic death of PC-12 cells. Our findings are in line with the previous report that anandamide causes inhibition of mitogen-activated lymphocyte proliferation, and induces apoptosis [30].

3.3. Anandamide causes superoxide anion formation in PC-12 cells

Enhanced production of cellular superoxide or resulting redox level takes part in the signal transduction pathway during apoptosis induced by a wide variety of agents. Increased level of superoxide anion (O^{-2}) or hydrogen peroxide (H_2O_2) has been implicated in TNFα- and thrombin-induced cell death [4,31]. Therefore, it tempted us to seek for the generation of superoxide anion in anandamide-treated PC-12 cells. As shown in Fig. 3A, treatment of the PC-12 cells with anandamide resulted in a flush of superoxide release in the extracellular milieu as noted by the reduction of ferricytochrome *c* to ferrocytochrome *c* by superoxide anion. The release of superoxide anion was initiated within 15 min and reached the peak at 1 h time point followed by progressive decrease. Cellular superoxide or free radical can be scavenged by superoxide anion scavenger, such as SOD, an enzyme that catalyzes the conversion of two of these radicals into hydrogen peroxide and molecular oxygen (O_2) ($O^{-2}+O^{-2} = H_2O_2+O_2$). Fig. 3B shows that anandamide caused generation of superoxide anion significantly, which, in turn, was abolished by the addition of SOD. Moreover, the superoxide production was inhibited by antioxidants like NAC, suggesting that superoxide is regulating the downstream events of apoptosis in anandamide-treated PC-12 cells.

3.4. N-acetyl cysteine prevents apoptotic death of anandamide-induced PC-12 cells

Data presented in Fig. 3 support the speculation that intracellular superoxide plays a critical role in anandamide-treated cells. The possible involvement of superoxide or free radical in anandamide-induced cell death was examined on incubating the PC-12 cells with *N*-acetyl cysteine, which has been shown to protect apoptosis induced by oxidative stress [32,33]. Next, we extended our study to investigate if NAC could prevent anandamide-induced cell death or not. If intracellular superoxide mediates anandamide-induced apoptosis, then antioxidant NAC should block that cell death. As expected, NAC concentration-dependently abrogated the anandamide-induced cell death. Fig. 4 illustrates that a dose as low as 1 mM NAC that usually traps cellular H_2O_2, rescued the cells as the viable cells increased markedly to 74.5 ± 2% of the control cultures (without anandamide), establishing a definite link between anandamide-induced superoxide production and cell destruction. This result is in agreement with the finding that NAC abrogates thrombin-induced Neuro-2a cell death where intracellular superoxide or free radical has been attributed to the mediator of that cell death [4].

3.5. Anandamide induces CPP32-like protease activation in PC-12 cells

It has been demonstrated that apoptosis may occur independent of caspase activation [34,35], even though it is well documented that cysteine protease caspase functions as a converging point in apoptosis induced by different stimuli in various types of cells [36,37]. On the other hand, H_2O_2 causes the activation of the intracellular death signaling protease caspase-3 [38], conversely, it has been reported that caspase-3 induces the generation of hydrogen peroxide [39,40]. Hence, it was of interest to clarify if the CPP32-like protease was activated or not in anandamide-treated cells. Anandamide led to the activation of CPP32-like protease activation in a time-dependent manner in PC-12 cells. Fig. 5 shows that activation of CPP32-like protease was initiated within 3 h, which, in turn, was notably elevated (3.9-fold higher than basal level) at 6 h after anandamide treatment, indicating that CPP32-like protease is involved in anandamide-induced PC-12 cell death. Recently, it has been unearthed that the MCF-7 cell line lacks caspase-3 gene [41]. In order to reveal that anandamide-induced apoptosis is dependent of caspase activation, we took the advantage and treated the MCF-7 cells with anandamide. Consistent with the previous finding [42], we repeatedly observed that anandamide (10 μM) failed to exert loss of cell viability of MCF-7 cells (data not shown). Moreover, it is supported by the observation that sodium nitropruside, at a dose that exhibits cytotoxic effects in PC-12 cells, does not affect the viability of MCF-7 cells (Sarker,

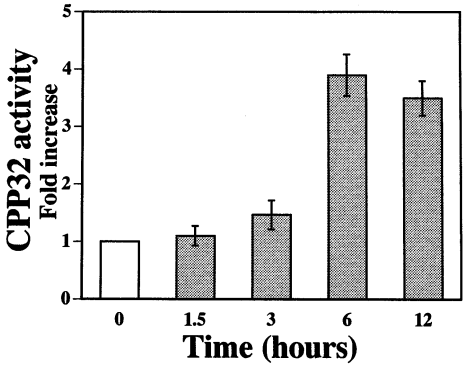

Fig. 5. Activation of CPP32-like protease in PC-12 cells induced by anandamide. Cells were plated onto 100 mm dishes (5×10^6/dish) and treated with anandamide (10 μM) for the indicated periods. CPP32-like protease activity was assayed as described in Section 2. The data are means ± S.D. of three individual experiments.

unpublished). Hence, the inability of anandamide to affect viability of MCF-7 cells is likely to be attributed to the caspase-3 deficiency in this cell line. Thus, our data conclude that anandamide-induced cell death signaling converges to the caspase-3 activation in PC-12 cells.

Acknowledgements: We thank Miss N. Uto for her help in flow cytometry. We are grateful to Toray Pharmaceutical Co. (Tokyo, Japan) for financial support for this project.

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