原 著

Effect of Acidosis on Apoptosis and Necrosis Induced by Hypoxia in Neuronal Cells

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Abstract

Severe cerebral acidosis has been reported to worsen cerebral ischemic injury. Recent works have suggested that apoptosis as well as necrosis can be observed following cerebral ischemia. Acidosis was reported to activate some kind of endonuclease, which may lead cells to apoptosis. Accordingly, cerebral acidosis may worsen cerebral ischemic injury by increasing apoptotic cell death. In this study, we evaluated whether the ratio of apoptotic cell death increased in proportion as the degree of acidosis progressed after hypoxic insult in neuronal cells. We utilized PC 12 cells, which are derived from rat pheochromocytoma. The cells were incubated in N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] -buffered medium, the pH of which was adjusted at 7.4, 6.8, 6.2, 5.6 or 5.0. Hypoxic insult was induced by filling the chamber with N_2. Eight, 24, 48 or 72 hours after incubation, cells were stained with Hoechst 33342 and propidium iodide and analyzed under a nonconfocal fluorescence microscope to distinguish whether cells were apoptotic or necrotic. The percentage of apoptosis at pH 6.2 was 9.4 ± 2.9 and 19.9 ± 7.5 % at 8 and 24 hours, which was slightly but significantly higher compared to pH 7.4 (3.1 ± 1.5 and 12.7 ± 5.1 %, respectively). After that, apoptosis was not enhanced by any degrees of acidosis. Acidosis below pH 5.6 increased the percentage of necrosis to more than 80 % during the entire period. Contrary to our expectation, apoptosis was slightly enhanced by moderate acidosis only in the early phase of hypoxic insult, while severe acidosis increased necrotic cell death during the total period of hypoxic insult. Accordingly, we propose that the main cause of aggravation of hypoxic neuronal damage by acidosis is enhancement of necrosis, whereas apoptosis may play only a small role.

Key words : Acidosis, Cell death; apoptosis, necrosis, Cerebral ischemia; hypoxia, Cell culture; PC 12 cells

Various degrees of cerebral acidosis were observed during cerebral ischemia. It has been widely accepted that severe acidosis worsens cerebral ischemic injury[1]. However, this mechanism remains uncertain. Acidosis activates some kind of endonuclease, which may lead cells to apoptosis[2]. Apoptosis has been detected following focal and forebrain ischemia[3,4]. Accordingly, one of the possible mechanisms of deterioration of the cerebral ischemic injury by acidosis is the enhancement of apoptosis. In this study, we used a neuronal model (PC 12 cells) in which apoptosis as well as necrosis was induced by hypoxia and evaluated the effects of various degrees of acidosis on the ratio of apoptotic and necrotic cells over time. We hy-

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pothesized that the ratio of apoptotic cell death increased in proportion as the degree of acidosis progressed.

Materials and methods

Neither Institutional Review Board approval nor informed consent was required, because this study involved cell cultures and did not involve animal or human subjects.

PC12 cells, which were derived from rat pheochromocytoma, were originally provided by Riken Gene Bank, Tsukuba Science City, Japan. Cells having undergone up to ten passages from the original cell line were used in the experiments. PC12 cells were maintained on collagen-coated dishes (Biocoat Cellware, Bedford, MA) in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY), supplemented with 5% heat-inactivated fetal bovine serum (MoreGate, Melbourne, Australia) and 5% heat-inactivated horse serum (GIBCO). They were kept at 37℃ in a 100% humidified atmosphere containing 5% CO_2 / 95% air.

The cells (4 x 10^5) were subcultured to some of 35-mm collagen-coated dishes as a sister culture. Two days after normal culture at 37℃, the dishes in the same sister culture were exposed to pH arranged N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES)-buffered medium after washing twice with the same medium. HEPES-buffered medium consisted of NaCl 145 mmol L^{-1}, KCl 4 mmol L^{-1}, 2 mmol L^{-1}, HEPES 6 mmol L^{-1} and glucose 10 mmol L^{-1}. The pH of the medium was adjusted to pH 7.4, 6.8, 6.2, 5.6 or 5.0 by titration with HCl or NaOH (pHM-83, Radiometer, Copenhagen, Denmark).

Hypoxic insult was induced by filling the chamber with N_2. The oxygen concentration was strictly maintained between 1 and 2 % using an oxygen electrode (JKO-25S, Jiko, Tokyo, Japan). Eight, 24, 48 or 72 hours after insult under various pH conditions, the cells were harvested and the relative frequencies of necrotic and apoptotic cells were examined. For this, cells were stained for 30 min at 37℃ with Hoechst 33342 and propidium iodide and analyzed under a nonconfocal fluorescence microscope (Axiophoto, Zeiss, Jena, Germany) with excitation at 360 nm. The method used for the analysis was the same as described by Shimizu, S, et al.[5]. Briefly, all nuclei were stained by Hochest 33342 (blue) and only nuclei of cells with a disrupted plasma membrane were stained by propidium iodide (red). Accordingly, viable cells had round blue nuclei while necrotic cells had round red nuclei (Fig. 1). Apoptotic cells had fragmented nuclei regardless of whether they were stained with Hochest 33342 only (early apoptosis) or propidium iodide (terminal apoptosis) (Fig. 1).

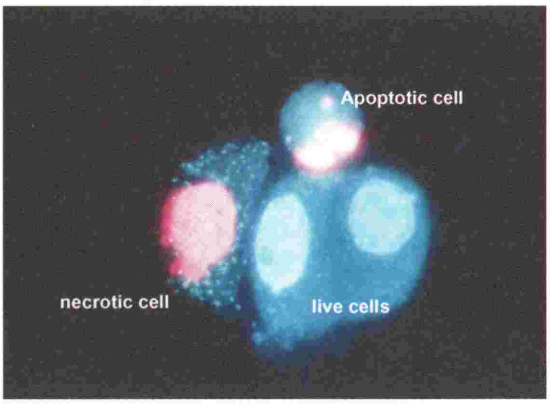

Fig. 1 Representative morphology of PC 12 cells after hypoxic insult
The cells were stained with Hoechst 33342 (blue) and propidium iodide (red) and analyzed under a non-confocal fluorescence microscope.

Quantitative analysis was performed by counting more than 1000 cells in each examination by the same blinded investigator. Values are percentages of apoptotic and necrotic cells and their amounts (dead cells) among total cells are expressed as mean ± standard deviation (SD). To compare the percentage of apoptotic, necrotic and dead cells in various acidotic conditions, one-factor analysis of variance (ANOVA) was used. When a significant difference was seen, post hoc analysis was performed with the Newman-Keuls test. Statistical significance was assumed when $p < 0.05$.

Results

Fig. 2 shows the time courses of percentages of the

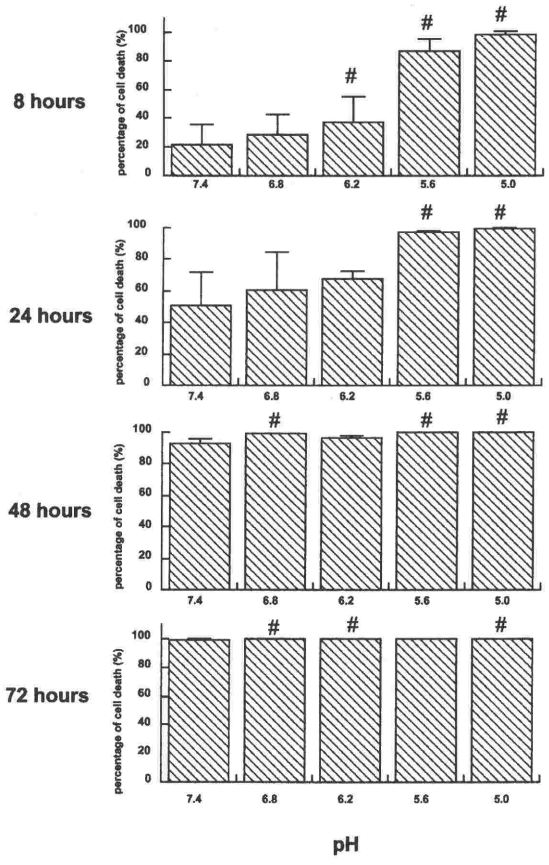

Fig. 2 Time courses of percentages of the amount of apoptotic and necrotic cells (dead cells) after hypoxic insult

Experiments were done 8 times.

P values by one factor ANOVA were below 0.01 at 8 hours and 24 hours. They were 0.01 and 0.03 at 48 and 72 hours respectively.

# indicates significant difference against pH 7. 4 with the Newman-Keuls test.

dead cells. Below pH 5.6, almost all the cells died within 8 hours after hypoxia. In the groups above pH 6.2, the cells died gradually up to 72 hours after hypoxia.

Fig. 3 demonstrates the time courses of percentages of apoptotic and necrotic cells. At pH 6.2, the percentage of apoptotic cells was significantly high compared to pH 7.4 at 8 and 24 hours after hypoxia, although the actual differences between pH 7.4 and 6.2 were small. After 48 hours, the significant increase at pH 6.2 disappeared. This indicated that acidosis around pH 6.2 slightly enhanced apoptosis induced by hypoxia only in the early phase of hypoxic cell death.

More than 80 % of cells had already died due to necrosis below pH 5.6 eight hours after hypoxia (Fig. 3). This suggested that acidosis below pH 5.6 generated acute cell death due to necrosis during hypoxia. After 48 hours, the ratio of apoptotic to necrotic cells was about 1:2 above pH 6.2.

The percentage of apoptotic cells was significantly lower below pH 5.6 compared to pH 7.4 after 48 hours (Fig. 3). This was presumably because the number of apoptotic cells could not increase due to the large cell population that underwent necrotic cell death in these pH ranges.

Discussion

PC 12 cells share a number of properties with sympathetic neurons and their precursors and they also represent a widely accepted model of neuronal system[6,7]. By using a cell line such as PC 12 cells, we can obtain a large number of uniform cells compared to primary neuronal culture, which decreases statistical errors. Moreover, a method to discriminate between necrosis and apoptosis induced by hypoxia has been established in PC 12 cells[5,8,9]. This is because apoptotic and necrotic features indicated by fluorescence microscopy with Hoechst 33342 and propidium iodide, respectively coincide with findings indicated by electron microscopy for hypoxic PC 12 cell death[5]. Therefore, we used PC 12 cells in this study.

It is a well-established fact that acidosis aggravates cerebral ischemic damage[1]. Compromised cell calcium metabolism and/or enhanced production of free radicals have been suggested as the mechanism for the long-term[1]. However, evidence these processes has not been obtained yet in vivo. Another proposed mechanism is alteration of nuclear events, including changes in gene expression and activation of an endonuclease[1]. Some kind of endonuclease such as DNase II is activated by acidosis below 6.5[2]. Apop

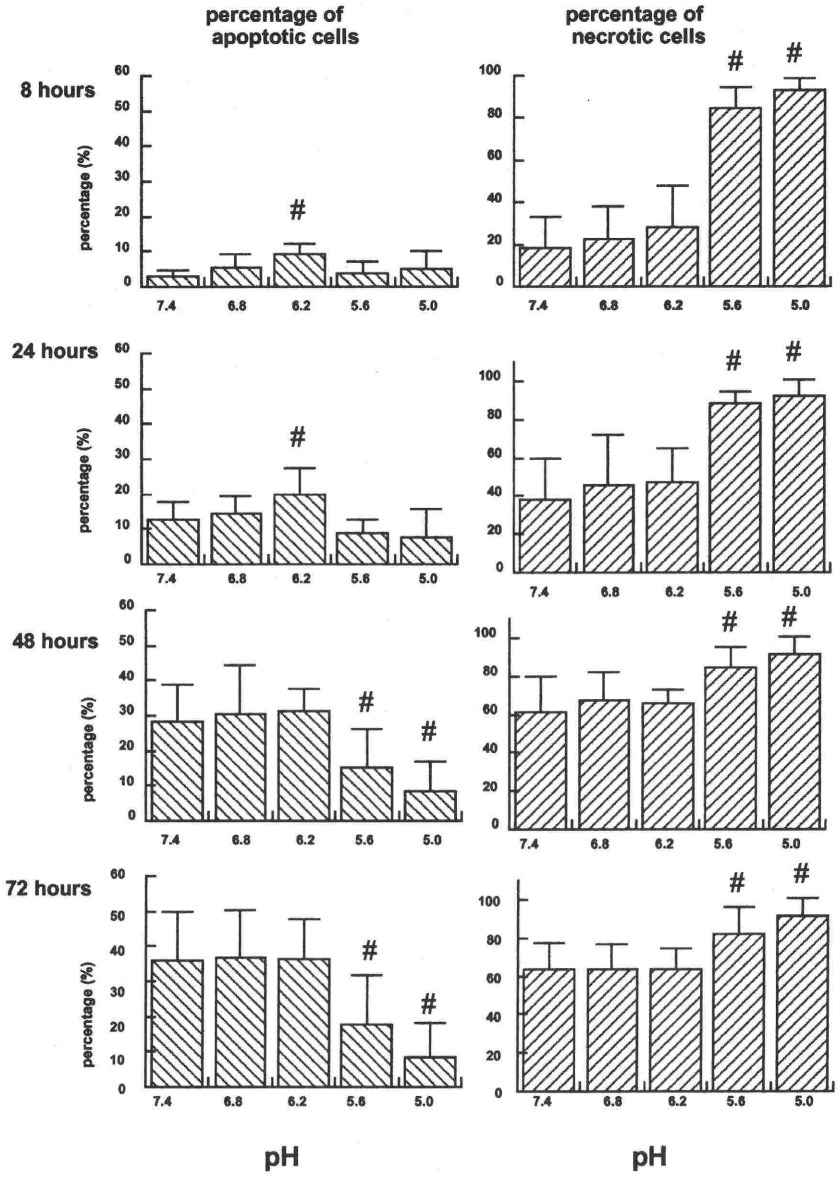

Fig. 3 Time courses of percentages of apoptotic and necrotic cells, respectively after hypoxic insult
Experiments were done 8 times
All the p values by one factor ANOVA were below 0.01.
# indicates significant difference against pH 7. 4 with the Newman-Keuls test.

tosis was detected following focal cerebral ischemia, especially in the penumbral region[4,10]. It has also been reported that delayed neuronal death following global ischemia may be partly attributable to apoptosis[3,11]. These findings suggest the possibility that cerebral acidosis activates some endonuclease and enhances apoptotic cell death during or after the cerebral ischemia. A previous report, however, demonstrated that acidosis at pH 6.8 reduced apoptosis induced by 42 hours of hypoxia in mouse embryo fibroblasts[12]. In contrast, acidosis at pH 6.6 by itself induced apoptosis as well as necrosis in cultured hippocampal slices[13].

Our study revealed that acidosis at pH 6.2 slightly enhanced apoptosis only in the early phase of hypoxic cell death. Instead, necrosis was excessively accelerated below pH 5.6.

After 15 min of incomplete forebrain ischemia, cerebral pH decreased to 6.37 and 5.95 in hypo- and hyperglycemic rats[14]. After 30 min of global ischemia, cerebral pH decreased to 6.18 and 5.95 in normo- and surviving hyperglycemic dogs[15]. Moreover, cerebral pH dropped to 5.27 in the hyperglycemic dogs who had consequent cerebral death. Therefore, it is possible that cerebral pH decreases below 5.6 in vivo under special conditions such as preischemic hyperglycemia, which is well known to enhance cerebral ischemic damage[1].

It has been reported that hypoxic cell death is blocked by antioxidants such as reduced glutathione and N-acetylcystein in PC 12 cells[9]. Even under 1% O_2, an increase in reactive oxygen species was demonstrated in PC 12 cells[16]. Therefore, in our study, it is possible that acidosis enhanced production of reactive oxygen species and that necrotic cell death was exaggerated.

In conclusion, apoptosis was slightly enhanced by moderate acidosis (around pH 6.2) only in the early phase of hypoxic insult, while severe acidosis (below pH 5.6) increased necrotic cell death during the total period of hypoxic insult. Accordingly, we propose that the main cause of aggravation of hypoxic neuronal damage by acidosis is enhancement of necrosis, whereas apoptosis may play only a small role.

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