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Stimulatory Effects of Prostaglandin E2 on Neurogenesis in the Dentate Gyrus of the Adult Rat

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ABSTRACT—Neurogenesis in the dentate gyrus of adult rodents is elicited by transient global ischemia. Cyclooxygenase (COX) -2, a rate-limiting enzyme for prostanoid synthesis, is also induced by ischemia. We recently found that the administration of a non-selective COX inhibitor to ischemic animals suppressed cell proliferation in the subgranular zone (SGZ) at the dentate gyrus of the hippocampus. To clarify whether prostaglandin E2 (PGE2) synthesis by COX's is involved in neurogenesis, sulprostone, an analogue of PGE2, was injected into the rat hippocampus. Sulprostone injection increased the number of 5-bromo-2'-deoxyuridine (BrdU)-positive cells in the SGZ. BrdU-positive cells also expressed polysialylated isoforms of neural cell adhesion molecule and neuronal nuclear antigen. These results suggest that PGE2 plays an important role in the proliferation of cells in the SGZ.

Key words: neurogenesis, cyclooxygenase-2, prostaglandin, dentate gyrus, hippocampus

INTRODUCTION

Prostaglandin E2 (PGE2), which is synthesized by cyclooxygenases (COX's), has been shown to be involved in several important functions of the nervous system, such as pain modulation (Ferreira, 1972) and generation of fever (Milton and Wendlandt, 1970). COX exists as two isoforms, COX-1 and COX-2. Generally, COX-1 is constitutively expressed in many tissues, while COX-2 is mitogen-inducible (Smith *et al*., 1996). The level of PGE2 in the central nervous system is increased in various pathological conditions (Montine *et al*., 1999; Paoletti *et al*., 1998). For example, cerebral ischemia in rodents caused an increase in the expression of COX-2 mRNA and concentration of PGE2 in the hippocampus (Ohtsuki *et al*., 1996; Nakayama *et al*., 1998). Furthermore, cerebral ischemia induced not only the expression of COX-2 mRNA but also neurogenesis in the subgranular zone (SGZ) within one to two weeks after ischemic operation (Liu *et al*., 1998). It has been suggested that ischemia-induced neurogenesis is involved in the release of glutamate by ischemic insult, because cellular proliferation in the SGZ is regulated by stimulation of N-methy-D-aspartate (NMDA) receptors stimulus (Cameron *et al*., 1995; Gould *et al*., 1997). It has also been reported that release of adrenal steroids under stress conditions also affects cellular proliferation and reduces neurogensis in the SGZ of adult rodents (Gould *et al*., 1992; Cameron and Gould, 1994; Gould *et al*., 1997). These factors participate in the regulation of COX-2 mRNA such that NMDA receptor stimulation initiates expression of COX-2 mRNA (Yamagata *et al*., 1993; Miettinen *et al*., 1997), and that glucocorticoid represses COX-2 both via the transcriptional and posttranscriptional mechanisms (Newton *et al*., 1998). Therefore, COX-2 and PGE2 may possibly be involved in neurogenesis in adult rodents after ischemia. We have recently reported that acetylsalicylic acid (ASA), a non-selective COX inhibitor, inhibited the ischemia-induced increase in the BrdU-positive cells (Kumihashi *et al*., 2001). The aim of the present study is to determine whether PGE2 is involved in the neurogenesis in the dentate gyrus (DG) of the hippocampus. Since, EP3, a PGE2 receptor subtype, has been reported to be expressed in the DG of rodents (Nakamura *et al*., 2000; Monica *et al*., 2000), we examined the effects of sulprostone, an EP3 agonist, on the number of newly synthesized cells in the DG.

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MATERIALS AND METHODS

Animals and agents used for injection

Fisher 344 male rats were housed individually on a 12 hr light/dark cycle with free access to water and food. Animals (240–260 g in body weight) were chronically catheterized in the hippocampus under anesthesia with sodium pentobarbital (40 mg/kg, Nembutal; Abott Lab., North Chicago, IL). Intracerebral catheters (C313G; Plastics One INC. Roanoke, VA) were inserted into the right DG of the hippocampus of each rat. The catheters were implanted stereotaxically according to the atlas of Paxinos and Watson (Paxinos and Watoson, 1997) at A-P, –3.6 mm from the bregma; L, 2.0 mm from the midline; and H, 6.8 mm above the interaural line. The catheters were placed onto the skull with acrylic dental cement and screws. Animals were allowed to recover for 1 week after the operation. Thereafter, each animal was injected with 0.1 nmol (n=3), 0.5 nmol (n=4), 1 nmol (n=10) or 2.5 nmol sulprostone (n=3) or phosphate-buffered saline (PBS, n=7) every 12 hr for 3 consecutive days. Sulprostone dissolved in 1 μl of vehicle was injected into the hippocampus over a period of 10 min. The period of injection of BrdU was determined on the basis of result of preliminary tests. Briefly, injections of BrdU were given intraperitoneally at a dose of 100 mg/kg once daily for 3 consecutive days from the second day after the last

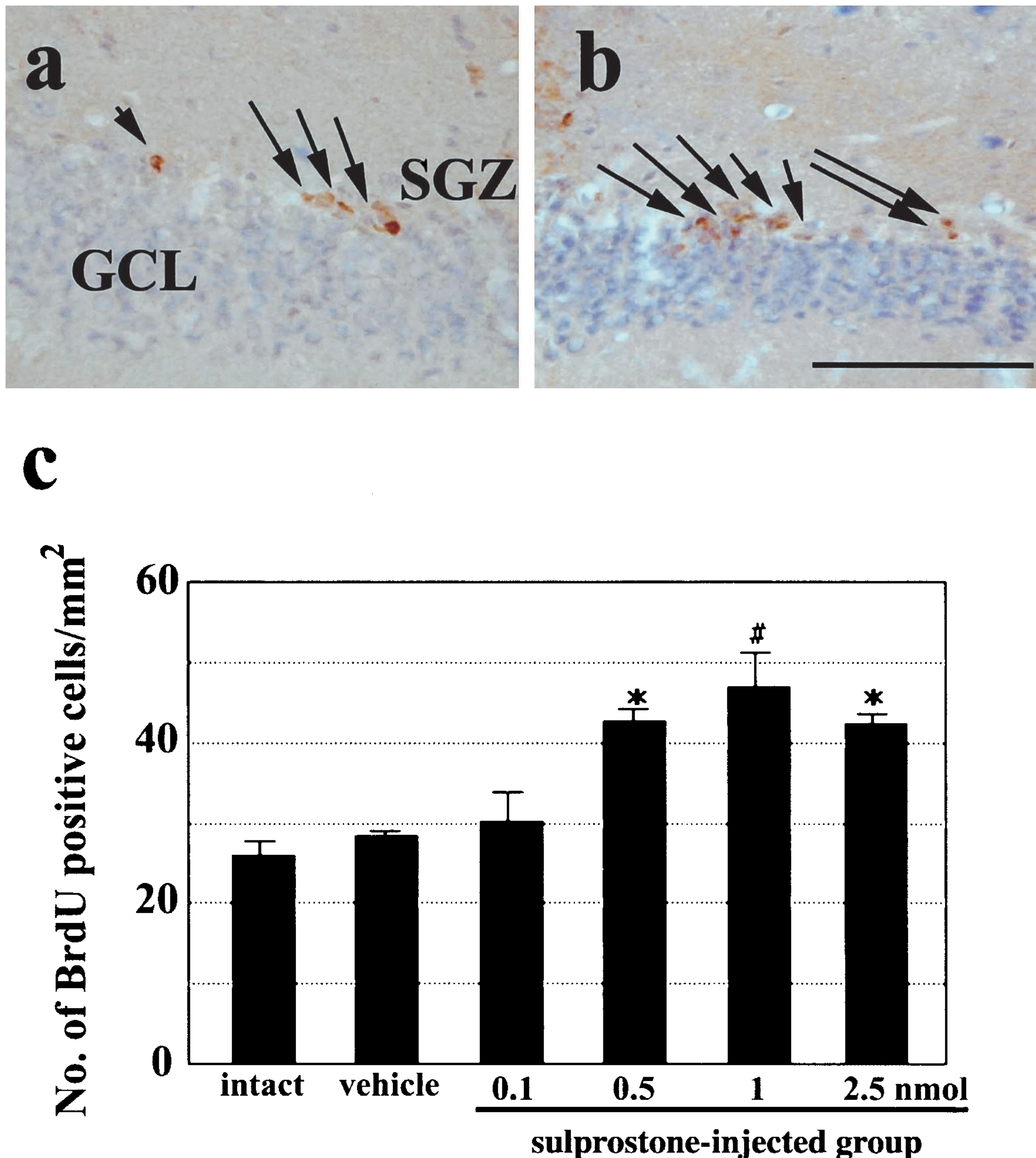

Fig. 1. (a and b) Photomicrographs of BrdU immunoreactivity in coronal sections of the DG of a vehicle-injected rat (a) and a 1 nmol suplostone-injected rat (b). On the first day after the last injection of BrdU, sections were stained with anti-BrdU antibodies and cresyl violet. Arrows indicate BrdU-positive cells. Scale bar=100 μm. (c) The dose-dependent effect of sulprostone injection on cell proliferation in the SGZ of the DG. Columns indicate the mean (± SEM) number of BrdU-positive cells/mm^2. *, #: significantly different from vehicle-injected rats (*, $p<0.05$; #, $p<0.01$).

intracerebral administration of sulprostone.

Another set of experimental groups was prepared for assessment of the phenotype of BrdU-positive cells in the granular cell layer (GCL). Two weeks after the last injection of BrdU, the animals were anesthetized with pentbarbital, and the brain was removed from each animals (n=3 for vehicle, n=5 for 1 nmol sulprostone).

Immunohistochemical study

(1) Detection of dividing cells on the SGZ following stimulating by sulprostone

Twenty-four hours after the last injection of BrdU, the animals (n=4 for intact, n=4 for vehicle, n=3, 4, 5, 3 for 0.1, 0.5, 1, 2.5 nmol sulprostone, respectively) were anesthetized with excess doses of sodium pentobarbital (60–80 mg/kg) and perfused transcardially with physiological saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed from each animal, post-fixed overnight in 4% PFA, and stored in 30% sucrose for 2 days. The brain was then frozen by liquid nitrogen and cut coronally on a cryostat at 16 μm. Sections were placed on 3-aminopropyl-triethoxysilane-coated slide glasses and processed for

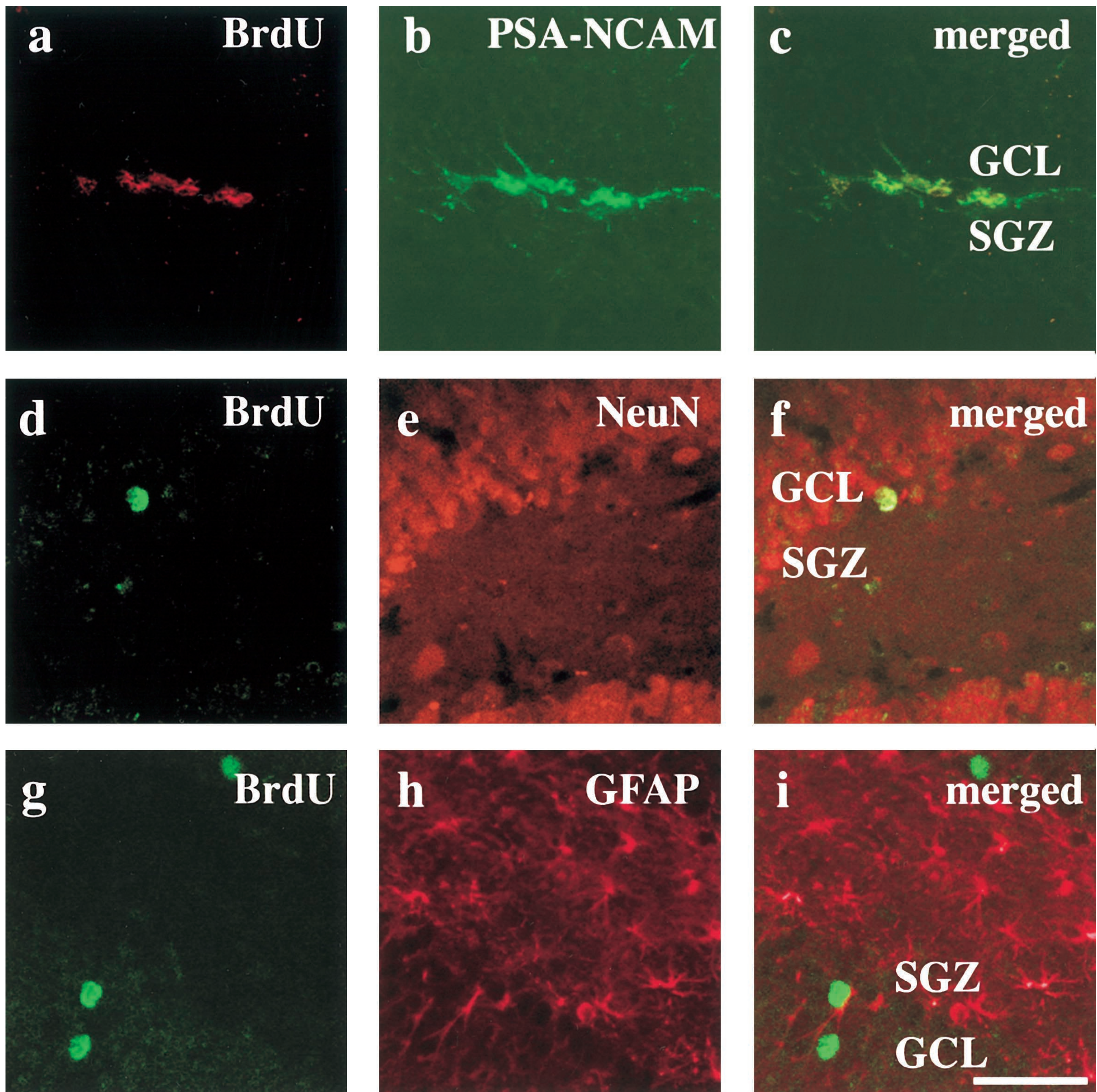

Fig. 2. Photomicrographs showing double immunostaining with anti-BrdU and a respective cell marker in coronal sections of the DG of sulprostone-injected rats. On the first day after the last injection of BrdU, sections were stained with anti-BrdU and anti-PSA-NCAM. A confocal microscopic image shows that BrdU-positive cells (red: a) in the SGZ colocalized with PSA-NCAM (green: b) as indicated by the yellow color (c). By 2 weeks after the last injection, BrdU-positive cells in the SGZ had migrated to the GCL. A confocal microscopic image of BrdU-positive cells (green: d, yellow: f) in the GCL was labeled for NeuN (red: e, yellow: f), but co-localizing of BrdU (green: g and i) with GFAP (red: h and i) was scarcely observed. Scale bar=50 μm.

Table 1. Rate of differentiation of BrdU positive cells into neuronal and glial cells 2 weeks after BrdU labeling in the GCL

	total BrdU positive cells/mm^2	survival rate (%)	NeuN+ BrdU+ cells (per total BrdU+ cells [%])	GFAP+ BrdU+ cells (per total BrdU+ cells [%])
vehicle (n=3)	21.32 ± 1.39	75.33	86.92 ± 1.24	9.24 ± 1.13
1 nmol sulprostone (n=5)	31.76 ± 3.40*	67.89	92.22 ± 0.73*	6.11 ± 0.93

Survival rate of BrdU-positive cells was determined as the number of BrdU-positive cells 2 weeks after BrdU injection divided by that on the following day after BrdU injection.
A double positive rate is indicated by the ratio of NeuN+ cells/BrdU+ cells or GFAP+ cells/BrdU+ cells to the total BrdU-positive cells. Data are presented as the mean ± SEM. * $P<0.05$ vs. vehicle.

further treatment. Sections were rinsed in PBS and incubated at 37°C for 30 min with 2N HCl. After basification with 0.1 M Tris-HCl (pH 8.5) for 10 min, sections were rinsed in PBS, incubated for 30 min with 5% normal horse serum diluted in PBS containing 0.1% Triton X-100 (PBST), and incubated overnight with mouse anti-BrdU (1:400; Sigma Chemical Co., St. Louis, MO). After being washed in PBST, sections were incubated for 30 min at room temperature with biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA). Sections were then incubated with avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 2 hr at room temperature, and BrdU-positive cells were visualized with 0.05% diaminobenzidine and 0.02% H_2O_2 in PBS. For the detection of Nissl bodies, sections were thereafter rinsed in water and stained with 0.5% cresyl violet (Wako Pure Chemical Industries, Ltd., Osaka).

(2) Double immunostaining using antibodies against BrdU and cell marker proteins

For detection of migrating cells from the SGZ to GCL, sections were incubated overnight with mouse anti-PSA-NCAM (1:500, a gift from Dr. Tatsunori Seki, Juntendo University, Tokyo). After being washed in PBST, sections were incubated with biotinylated anti-mouse IgM (Vector Laboratories, Burlingame, CA) and then visualized using a TSA fluorescence system (NEN Life Science Products, Boston, MA). Following detachment of antibodies by 0.1 M glycine buffer (pH 2.2) for 2 h at room temperature, sections were incubated overnight with mouse anti-BrdU. After being washed in PBST, sections were incubated for 30 min at room temperature with rhodamine-conjugated anti-mouse IgG (Chemicon International Inc., Temecula CA).

For detection of differentiation of progenitor cells in the GCL, sections were incubated with mouse anti-BrdU, then incubated with biotinylated anti-mouse IgG. Sections were visualized using a TSA fluorescence system. Following detachment of antibodies, sections were incubated with mouse anti-NeuN (1:500, Chemicon International Inc., Temecula CA) or mouse anti-glial fibrillary acidic protein (GFAP; 1:500, Sigma Chemical Co., St. Louis, MO). After being washed in PBST, sections were incubated with rhodamine-conjugated anti-mouse IgG. Fluorescence was detected using a confocal laser-scanning microscope (LSM410, Carl Zeiss, Jena, Germany).

Quantification

For each brain, 8–10 sections containing the central area of the DG were randomly selected, and the number of BrdU-labeled cells in the SGZ was counted. Microscopic images of these sections were processed using Adobe Photoshop 5.0 LE (Adobe Systems, San Jose, CA). The area of the DG including the hilus and SGZ in each section was measured using Scion Image for Windows (Scion Corp., Frederick, MD). The density of BrdU-positive nuclei in each section was calculated by dividing the number of BrdU-positive nuclei by the area of the DG according to the method of Liu *et al.* (1998). Data are expressed as means ± SEM. Statistical analysis was performed using ANOVA followed by post-hoc comparison using Fisher's PSLD. Differences were considered significant at $P<0.05$. To assess the phenotype of BrdU-positive cells in the GCL, we used NeuN and GFAP as cell markers. A double positive ratio was calculated as BrdU+/NeuN+ or BrdU+/GFAP+ cells divided by total BrdU-positive cells in the GCL. Data are expressed as means ± SEM. Comparisons were made by Student's *t*-test (unpaired). Differences were considered significant when $P<0.05$.

RESULTS

BrdU-labeled cells were found within the SGZ in all groups of rats. Direct injection of sulprostone into the hippocampus increased the number of BrdU-positive cells in the SGZ (Fig.1-a, b). The number of BrdU-labeled cells in the sulprostone-injected group (except for 0.1 nmol group) was significantly greater than that in the vehicle-injected group (Fig. 1-c). Treatment with 1 nmol sulprostone increased the number of BrdU-positive cells in the SGZ up to twofold. In the contralateral SGZ, no marked increases in the number of BrdU-positive cells were observed. Clustering of BrdU-positive cells was localized in the SGZ, but few BrdU-positive cells were observed in the GCL at the period 24 hr after the last injection of BrdU. Furthermore, a proportion of BrdU-positive cells in the SGZ were stained with mouse anti-PSA-NCAM (Fig. 2a-c), indicating that the dividing cells had apparently differentiated into neuronal cells.

On the other hand, BrdU-positive cells had migrated from the SGZ to GCL by 2 weeks after the last injection of BrdU. The majority of BrdU-positive cells in the GCL expressed NeuN, known as the marker for matured neurons (Fig. 2d-f). In contrast, coexpression of BrdU and GFAP was scarcely detected cells in this area (Fig. 2g-i). Survival rates for BrdU-positive cells 2 weeks after injection of BrdU were 75.33% and 67.89% in vehicle-injected and 1 nmol sulprostone-injected group, respectively. Therefore, the number of BrdU-positive cells was reduced by approximately 30% by 2 weeks after the last injection of BrdU in both groups. However, the ratio of BrdU+/NeuN+ cells to the number of total BrdU-positive cells in sulprostone-injected group was significantly greater than that in vehicle-injected group (Table. 1).

DISCUSSION

We demonstrated for the first time in this study that sulprostone, a PGE2 analogue, stimulated *de novo* neurogenesis at the DG of rat hippocampus. The number of BrdU-positive cells was increased dose-dependently following sulprostone injection. Furthermore, the majority of BrdU-positive cells developed to neuronal cells as was demonstrated by colocalization with neuron-specific cell makers.

It has been shown that neurons are produced in two limited regions of the brain in adulthood, i.e., the DG of the hippocampus and the olfactory bulb (Altman and Das, 1965; Kaplan and Hinds, 1977). More recently, the neurogenesis was reported to be stimulated markedly by transient global ischemia in the adult brain (Kee *et al*., 2001) despite the continuous generation of new neurons throughout the whole life time. It has been disclosed that transient induction of COX-2 accompanies global ischemia in rat DG (Ohtsuki *et al*., 1996). We also found recently that the administration of ASA suppressed cell proliferation in the SGZ of ischemic animals (Kumihashi *et al*., 2001). These findings suggest that PG's are involved in cell proliferation in the SGZ.

Since sulprostone is an agonist for EP3 receptors, the present result suggests that PG's may stimulate neurogenesis via EP3 receptors in the DG. Supporting this idea is the finding that the EP3 receptors are present in the DG (Nakamura *et al*., 2000; Monica *et al*., 2000). However, sulprostone is not a highly specific agonist for EP3, and therefore, the involvement of other receptor subclasses remains to be elucidated.

Sulprostone injection resulted in approximately 2-fold increase in the number of progenitor cells compared to those in intact and vehicle-injected animals in the present study. The degree of increase appears less marked in comparison with 3 to 8-fold increase following global brain ischemia (Kumihashi *et al*., 2001; Liu *et al*., 1998). Full increase in progenitor cells after ischemia may, therefore, require some other factors in addition to PG's. Noradrenaline, serotonin and estrogen are known to increase neurogenesis in the DG (Duman RS *et al*., 1999; Banasr M *et al*., 2001). Further studies are required to examine whether PG's stimulate neurogenesis in the DG in cooperation with these factors.

The majority (92%) of BrdU-positive cells coexisted with NeuN in the sulprostone-injected group, indicating that the major part of progenitor cells developed to neurons after stimulation by sulprostone. Expression of PSA-NCAM is known to be present as soon as the progenitors exit the cell cycle and start to migrate toward their final destination (Seki and Arai 1993). Its expression in the SGZ is also reported to parallel cellular proliferation (Kuhn *et al*., 1996). NeuN is a transcription factor that is expressed in the nucleus and cytoplasm of mature neurons. The double positive rate for NeuN and BrdU was slightly but significantly higher in the sulprostone-injected group than that in the control group, raising the possibility that sulprostone may drive differentiation of progenitor cells to neurons. It is intriguing that the rate of neuronal differentiation of BrdU-positive cells in the sulprostone-injected group in this study was comparable to that following the ischemic paradigm employed by previous investigators (Sharp *et al*., 2002; Takasawa *et al*., 2002). On the other hand, sulprostone injection did not affect the suvival rate of BrdU-positive cells in this study. Van pragg *et al*. (1999) demonstrated that hippocampus-dependent learning task had been necessary for the survival of newborn cells. Thus, it seems likely that a coordinated actions of neurons in the hippocampal formation is required for increasing the survival rate of progenitor cells. A small population of BrdU-positive cells also contained GFAP in both the sulprostone- and vehicle-injected groups. The GFAP+/BrdU+ ratio in the sulprostone-injected group was slightly lower than that in the vehicle-injected group, though statistically not significantly different. Since the number of double positive cells for GFAP and BrdU was very small, however, it is hard to discuss over the effect of sulprostone on production of glial cells.

In conclusion, the present study demonstrated that sulprostone promoted neurogenesis, supporting the proposal that PG's are involved in the process of neurogenesis in the hippocampus. At the present time, neither the mechanism(s) for sulprostone action nor target cells for primary sulprostone action is not clear. Cultured astrocytes have been also shown to express EP3 receptors (Kitanaka *et al*., 1996), thus, sulprostone could primarily act not only on neural progenitor cells but also on these glial cells. It remains to be clarified at which stage of neural development and to what cell population(s) sulprostone exerts its effects.

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