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Original Article

Granulocyte-colony stimulating factor reduces striatal dopamine accumulation caused by cerebral ischemia

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ABSTRACT

Objectives: Granulocyte-colony stimulating factor (G-CSF) plays important roles in neuroprotection. Acute and massive increases in extracellular dopamine can cause damage to the striatal neurons during cerebral ischemia (CI). We hypothesized that G-CSF may exert neuroprotective effects by reducing dopamine accumulation.

Materials and Methods: CI was induced by infusing endothelin-1 (Et-1, 120 pmol in 10 μL saline) into the root of the middle cerebral artery in freely moving rats. G-CSF (200 μg/kg in 0.2 mL saline) was administered immediately after CI induction.

Results: G-CSF reduced dopamine accumulation in the ischemic striatum after CI was induced by Et-1. The total brain infarct volume was reduced by 67% ($p < 0.05$) at 24 hours after CI.

Conclusion: These data suggest that reduced dopamine accumulation may be one of the mechanisms underlying the neuroprotective effects of G-CSF.

1. Introduction

Granulocyte-colony stimulating factor (G-CSF) exerts neuroprotection and helps in tissue repair after cerebral ischemia (CI) [1–3]. The underlying mechanisms of its neuroprotection include mobilization of stem cells, neuronal differentiation, angiogenesis, and anti-inflammatory and antiapoptotic effects [1–3]. Moreover, G-CSF can exert neuroprotective effects by reducing excitotoxicity caused by glutamate overflow in the ischemic brain [4]. Overflow of generalized neurotransmitters after CI contributes to subsequent neuronal death. Dopamine is an important neurotransmitter. It is dramatically released or accumulated in the striatum [5,6] to cause significant damage to neurons during CI [7,8]. Acute and massive increases in extracellular dopamine and glutamate concentrations have been documented in the striatum after CI [9]. Reduction of dopamine accumulation alleviates cerebral damage [7]. Moreover, prior lesion of the *substantia nigra* exerts a strong protective effect against postischemic striatal neuronal death [8,10]. These results indicate the role of dopamine- and glutamate-induced excitotoxicity in ischemic neuronal cell death. However, sustained autooxidation of dopamine may lead to an excessive accumulation of quinones and oxygen free radicals [11]. Based on these findings, we hypothesized that G-CSF may also exert neuroprotective effect by reducing dopamine accumulation.

2. Materials and methods

2.1. Animals

The experimental animals were handled according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*, and experimental protocols were approved by the Animal Use Protocol Board of Tzu Chi Hospital (Approval No. 97-22). Animals were housed under a 12-hour light/dark cycle, with free access to food and water. Every effort was made to minimize the number of animals necessary to complete the study and their suffering, and the study was in compliance with the guidelines.

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2.2. Experimental protocol

Rats were assigned randomly to four experimental groups: sham + saline ($n = 3$), sham + G-CSF ($n = 6$), ischemia + saline ($n = 10$), and ischemia + G-CSF ($n = 11$) groups (Fig. 1A).

2.3. Cannula implantation

For guide cannula implantation, rats were anesthetized with 5% isoflurane for initiation and 2.5% for maintenance. Using standard stereotaxic techniques, two intracerebral guide cannulas, one for a microdialysis probe and the other for endothelin-1 (Et-1) delivery, were implanted. The coordinates of the microdialysis probe cannula were 0.0 mm posterior, 3.0 mm right, and 5.0 mm ventral to bregma at the skull surface, and those of the Et-1 delivery 27-gauge syringe cannula were 0.0 mm posterior, 5.2 mm right, and 8.0 mm ventral to bregma at the skull surface. These cannulas were implanted and fixed to the skull with dental cement. Animals were allowed to recover from surgery for at least 5 days (Fig. 1A) prior to CI induction.

2.4. CI induction

The CI procedure was modified from a previously published procedure [12]. CI was created by infusion of Et-1 (120 pmol in 10 μL saline at 1 μL/min; Sigma, St Louis, MO, USA) through a 27-gauge syringe positioned in proximity to the root of the middle cerebral artery [5]. The needle was kept in place for another 10 minutes to prevent backflow. G-CSF (200 μg/kg in 0.5 mL saline; Kirin Pharma Co., Ltd, Tokyo, Japan) was administered (subcutaneously) immediately after the infusion of Et-1. Vehicle control for G-CSF was 0.5 mL saline (Fig. 1A).

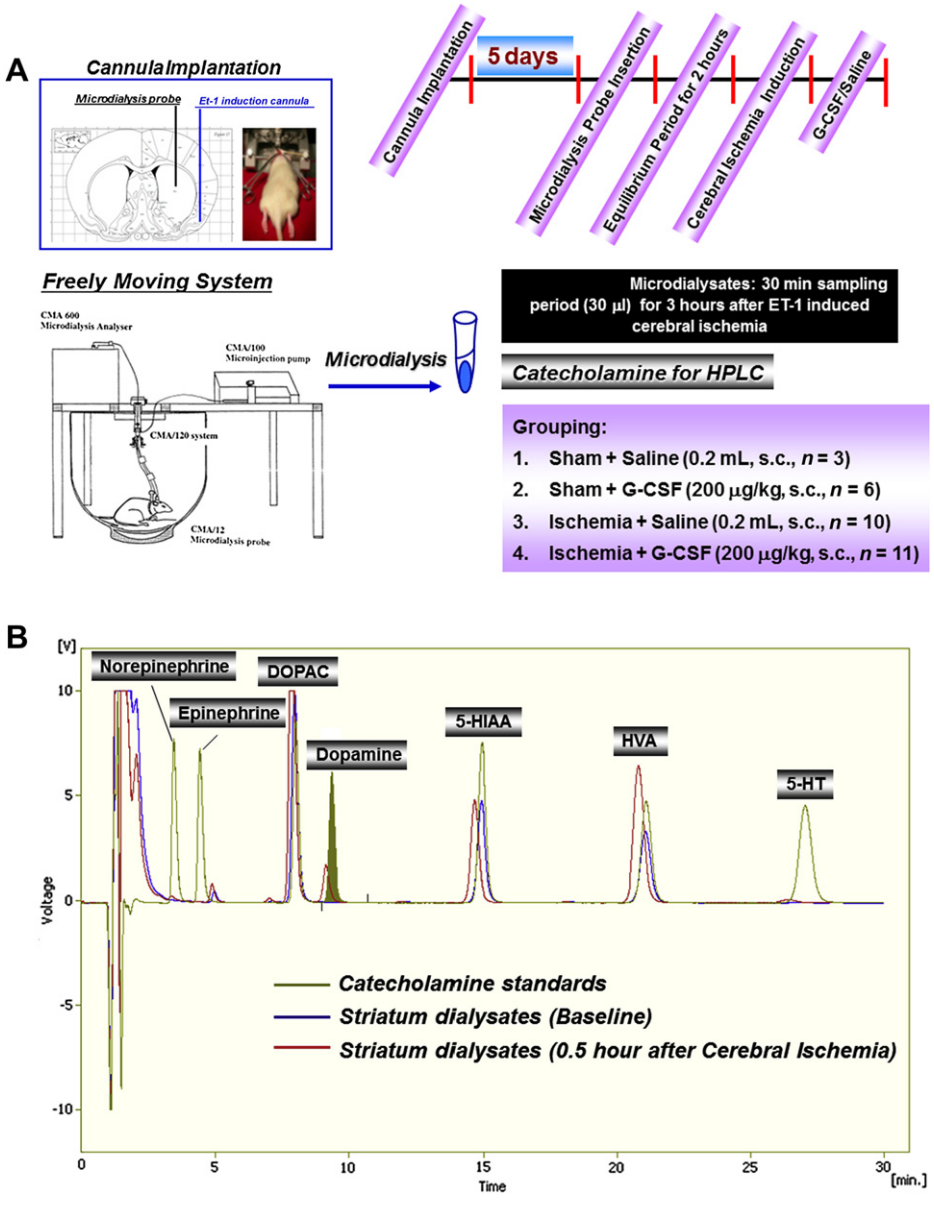

Fig. 1. (A) Schematic diagrams of microdialysis in freely moving rats. (B) A profile of the representative catecholamines in striatal tissues. DOPAC = 3,4-dihydroxyphenylacetic acid; Et-1 = endothelin-1; G-CSF = granulocyte-colony stimulating factor; 5-HIAA = 5-hydroxyindoleacetic acid; HPLC = high-performance liquid chromatography; 5-HT = 5-hydroxytryptamine; HVA = homovanillic acid.

2.5. Microdialysis in freely moving rats

To avoid interference by anesthetic drugs on the striatal neurotransmitter profile, microdialysis was conducted in freely moving rats (Fig. 1A). Briefly, on the day of the experiment, a microdialysis probe (CMA/12, cut-off 20 kDa and membrane length 3 mm; Carnegie Medicine, Stockholm, Sweden) and a 27-gauge syringe for induction of CI were inserted. The striatal tissue were perfused with an aseptic modified artificial cerebrospinal fluid (pH = 7.4), consisting of 125 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1.14 mM $MgSO_4 \cdot 7H_2O$, 1.29 mM KH_2PO_4, 25 mM $NaHCO_3$, and 0.1 mM ascorbic acid,. The perfusion rate was set at 1 μL/min. After a stabilizing period of 1 hour, microdialysates (30 μL) from each 30-minute period were collected in sample tubes containing 2.5 μL 3.3% perchloric acid, mixed vigorously, and centrifuged, and the supernatant was analyzed immediately by high-performance liquid chromatography (HPLC) using an MF-1020 (Bioanalytical Systems, West Lafayette, IN, USA) electrode chemical detector (ECD).

2.6. HPLC-ECD analysis

The first and second microdialysate samples were pooled for quantification of the basal catecholamine concentration prior to CI. The microdialysates (20 μL) were separated and quantified by HPLC-ECD. The mobile phase (100 mM NaH_2PO_4, 0.75 mM sodium octane sulfonate, 0.027 mM EDTA·$2H_2O$, and 10% methanol; pH = 3.0, adjusted with phosphoric acid) was filtered through a 0.2 μm cellulose nitrate filter (Gelman Sciences, Ann Arbor, MI, USA) and degassed under vacuum prior to use. This mobile phase was set at a flow rate of 0.2 mL/min (pump: PM-92E; BAS Instruments, West Lafayette, IN, USA). A Vision HT Hypersil column (C18 3μ, 2.1 × 100 mM^2; Grace, Deerfield, IL, USA) was used for separation. The recovery rate of all microdialysis probes *in vitro* was in the range of 20% ± 5%, and the probes were calibrated with known amounts of standards. All standards [norepinephrine, epinephrine, 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA), and 5-hydroxytryptamine (5-HT)] were purchased from Sigma. The concentrations of all catecholamines were calculated using standard curves constructed with known amounts of standards (Fig. 1B). The minimum detectable level of the catecholamines was 5 nM.

2.7. Total infarction volume

Rats were decapitated under deep anesthesia at 24 hours after CI. The brain was removed, and the forebrain was sliced into 1-mm-thick coronal sections and stained with 2% 2,3,5-triphenyltetrazolium (Sigma) for 20 minutes at 37°C in the dark. The total infarction volume was determined using Image J software (NIH, Bethesda, MD, USA). These sections were also used for the verification of correct positions of the Et-1 infusion and microdialysis probes.

2.8. Statistical analysis

Data were statistically analyzed using Prism software (GraphPad Software, Inc., La Jolla, CA, USA) for the Student *t* test and were presented as mean ± standard error of the mean (SEM). Statistical comparisons among multiple groups were made using one-way analysis of variance (ANOVA), and among multiple time points using two-way ANOVA followed by the Bonferroni correction. In all instances, *n* referred to the number of animals in a particular group. A *p* value of <0.05 was considered statistically significant.

3. Results

The systemic blood pressure and heart rate were not affected in all rats subjected to infusion of Et-1 or subcutaneous administration of G-CSF. The basal extracellular concentration of the striatal dopamine was 14.7 ± 2.0 nM. The extracellular concentration of dopamine in the striatum increased to a maximum (240 ± 83-folds) at 1 hour after CI (Fig. 2A, ischemia + saline group vs. sham + saline group). G-CSF treatment significantly suppressed the increase in dopamine to 41 ± 20-fold (Fig. 2A, ischemia + G-CSF group vs. sham + saline group), while G-CSF or saline alone did not affect the basal dopamine level (Fig. 2A). The metabolites of dopamine (DOPAC and HVA) and serotonin (5-HIAA) were significantly decreased after CI, but were not affected by G-CSF administration (Fig. 2B–D) compared with the saline-treated group (ischemia + saline group) (Fig. 2B–D).

The average cerebral infarction volume in the rats of the ischemia + saline group was 171 mm^3, whereas the cerebral infarction volume of the G-CSF-treated CI rats was reduced to 57 mm^3 (67% reduction) (Fig. 3).

4. Discussion

This study demonstrated for the first time that G-CSF treatment significantly reduces dopamine accumulation, which in turn reduces the total cerebral infarction volume in animals with Et-1-induced CI.

Striatal tissues are perceived to be affected differentially by ischemia, with a more vulnerable ischemic core and a compromised but relatively more salvageable peri-infarct zone [13,14]. However, salvageability decreases with time, making speedy and effective therapeutic intervention critical. In various ischemic models, the susceptibility of the striatum to ischemia has been found to be associated with dopamine accumulation [8,10,15–18]. Dopamine is widely recognized as a potential neurotoxic transmitter that contributes to subsequent neuronal damage after ischemia–reperfusion [7,8,19]. A rapid accumulation of extracellular dopamine, rather than the slower glutamate overflow, is likely the primary event initiating a cascade that ultimately leads to cell death and neurological deficits [5]. In contrast, dopamine depletion, either by pharmacological or by surgical means, improves the ischemic outcome in the striatum [7,8]. In agreement with those findings, our results demonstrated that G-CSF reduces dopamine accumulation and contributes, at least in part, to the reduction of the total cerebral infarction volume in rats with Et-1-induced CI. The changes in dopamine metabolites and serotonin were less profound.

G-CSF can penetrate the blood–brain barrier even in the intact brain [20]. The underlying mechanisms contributing to the reduction of dopamine accumulation *in vivo* by G-CSF are unclear. The excessive increase in extracellular dopamine, which occurs during ischemia, is probably a result of cell destruction rather than of *secretion*. However, secretion of dopamine may also increase cerebral glucose utilization, which in turn contributes to an increase in cerebral energy demand during ischemia. As a consequence, brain tissues are affected by both stresses during CI, which may lead to greater secondary damage. Therefore, reduction in dopamine accumulation by G-CSF may be one of the mechanisms by which G-CSF can reduce infarct volume.

In our CI model, the total infarction volume covered both the cortex and the striatum (Fig. 3). However, the specific mechanism that caused reduced cortical infarction is unknown. A plausible explanation is that the reduction in the cortical infarction volume is due to G-CSF potentials in anti-inflammation, antiapoptotic cell death, and stem cell activation/recruitment [21,22].

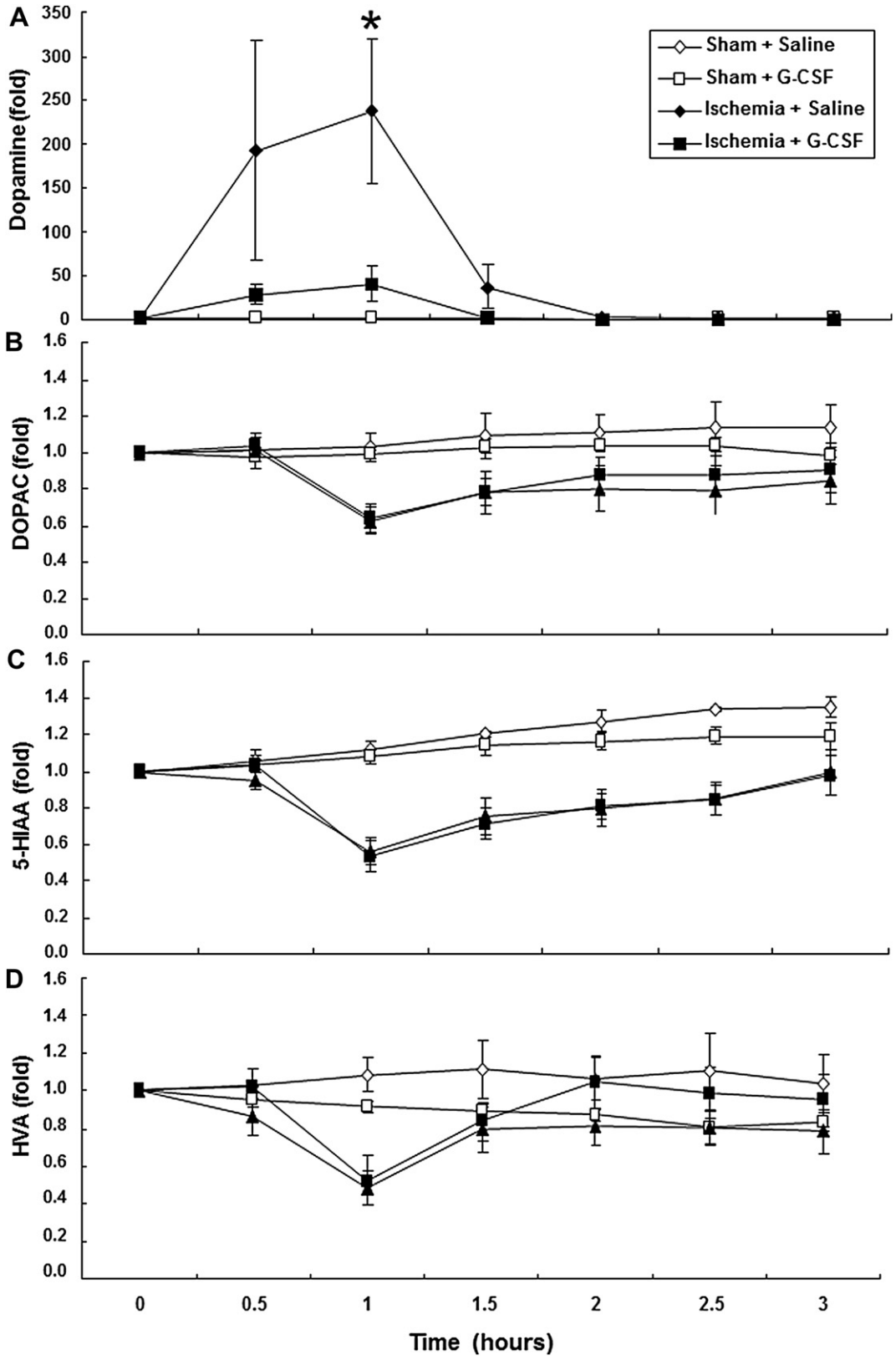

Fig. 2. Effects of G-CSF on catecholamine concentrations in microdialysates collected from the striatum subjected to CI: (A) dopamine; (B) DOPAC; (C) 5-HIAA; (D) HVA. Time 0 refers to Et-1 infusion. The value at time 0 indicates the baseline concentration. $^{*}p<0.05$ for the ischemia + saline group compared with sham + saline group. Data are expressed as fold increase compared with the baseline concentration (mean ± SEM). CI = cerebral ischemia; DOPAC = 3,4-dihydroxyphenylacetic acid; Et-1 = endothelin-1; G-CSF = granulocyte-colony stimulating factor; 5-HIAA = 5-hydroxyindoleacetic acid; HVA = homovanillic acid.

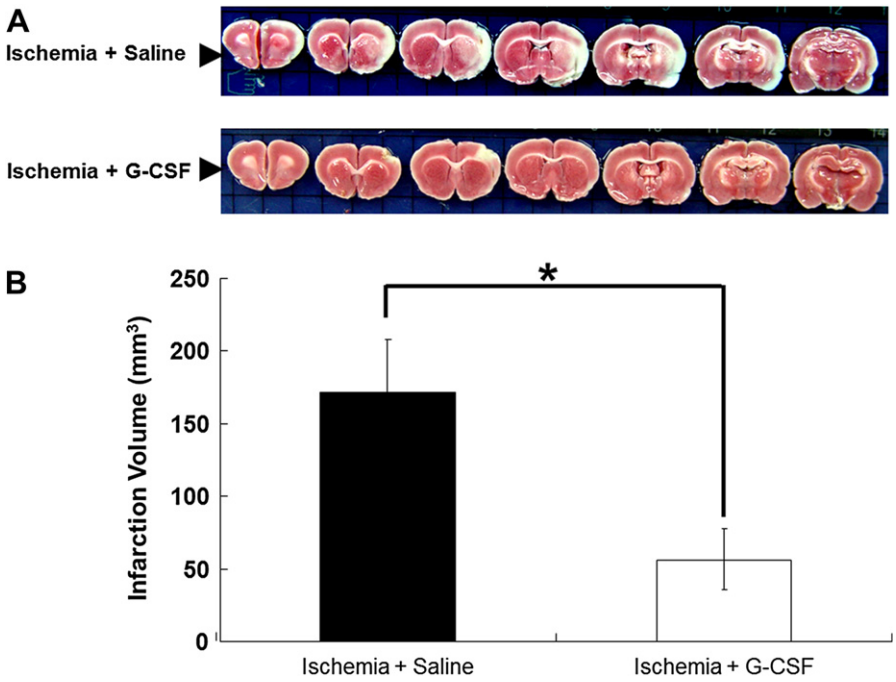

Fig. 3. (A) Effects of G-CSF (200 μg/kg, subcutaneous) on the infarct volume caused by CI. (B) Rats were killed 24 hours after the infusion of Et-1. The infarct volume was determined using Image J software on the seven predetermined coronal planes through the brain as shown in (A). Statistical analysis showed significant reduction of infarct volume in G-CSF treated brains: $^{*}p<0.05$ between the two groups. Data are presented as mean ± SEM. CI = cerebral ischemia; Et-1 = endothelin-1; G-CSF = granulocyte-colony stimulating factor.

5. Conclusion

The actual neuroprotective mechanism(s) of G-CSF awaits further investigation. However, the major advantage of this protein is that it has a well-known pharmacological profile and safety record, and thus many clinical trials have been carried out using G-CSF as a therapeutic agent (please search "G-CSF" in http://www.clinicaltrials.gov for details). Because G-CSF exerts neuroprotective effects through reduction of excitotoxicity caused by glutamate overflow in the ischemic brain [4], we conclude that G-CSF can also reduce dopamine overflow and exert a neuroprotective effect on striatal neuronal cells after CI.

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