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Regulation of Choline Transport by Oxidative Stress at the Blood-Brain Barrier *In Vitro* Model

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Abstract – In the present study, we examined how the transport of choline is regulated at the blood-brain barrier (BBB) under the central nervous system (CNS) cellular damages by oxidative stress using a conditionally immortalized rat brain capillary endothelial cells (TR-BBB), *in vitro* the BBB model. It was also tested whether the choline uptake is influenced by membrane potential, extracellular pH, protonophore (FCCP) and amiloride in TR-BBB cells. In result, [^{3}H]choline uptake was inhibited by FCCP and dependent on extracellular pH. The treatment of TR-BBB cells with 20 ng/mL tumor necrosis factor-α (TNF-α), 10 ng/mL lipopolysaccharide (LPS), 100 μM diethyl maleate (DEM) and 100 μM glutamate resulted in 3.0-fold, 2.6-fold, 1.8-fold and 2.0-fold increases of [^{3}H]choline uptake at the respective peak time, respectively. In contrast, hydrogen peroxide and raffinose did not show any significant effects on choline uptake. In addition, choline efflux was significantly inhibited by TNF-α, LPS and DEM producing cell damage states. In conclusion, the influx and efflux transport system for choline existed in TR-BBB cell line and this process was affected by several oxidative stress inducing agents.

Keywords □ blood-brain barrier, immortalized rat brain capillary endothelial cells, choline transporter, tumor necrosis factor-α, lipopolysaccaride, dimethylmalate, glutamate

INTRODUCTION

It was understood generally that the blood-brain barrier (BBB) acts as a static wall protecting the brain parenchyma cells. However, it has appeared new concepts that the BBB acts as a dynamic regulatory interface in consequence of the development of recent advanced methodologies to study the BBB. Especially, many kinds of influx and efflux transporters exist at the BBB and these transporters play a role not only in supplying nutrients but also detoxifying any excess of neurotransmitters and their metabolites (Terasaki and Hosoya, 2001). Therefore, elucidating the BBB transporters and their mechanisms is very important to regulate the supply of nutrients and drugs to the brain.

The choline is an essential compound for the synthesis of membrane phospholipids and acetylcholine in the brain (Klein et al., 1993). However, there is very little de novo synthesis of choline in the brain, and a constant supply of choline from plasma to the brain is required (Crews *et al.*, 1980). Abnormal choline transport and metabolism have been implicated in Alzheimer's disease (Michel *et al.*, 2006). Therefore, it is important to study choline transport for elucidating the characteristics of choline transporters in the brain. Previous studies showed that choline is transported from blood to brain through the BBB via carrier-mediated mechanism that is dependent on membrane potential, and the characteristics of choline uptake across the BBB is similar to that of organic cation transporters (OCTs) system (Sawada *et al.*, 1999; Friedrich *et al.*, 2001). In addition, in our previous study, we suggested that transport of choline at BBB is membrane potential-dependent and has a saturable process through OCT2 in TR-BBB cells (Kang *et al.*, 2005). In addition, it has been shown that citicoline, the intermediate in the biosynthesis of phosphatidylcholine, which is started from choline, produces neuroprotective effect in a variety of CNS injury models (Olivia *et al.*, 2004). Recent report has also shown that choline deficiency appears to be one of the pathogenic mechanisms of mitochondrial and cellular oxidative damage (Ossani *et al.*, 2007). We supposed that choline may have neuroprotective effect in the brain and the

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change of choline transport activity at the BBB may be observed under the stress condition.

The purpose of this study is to clarify the driving force of choline transport through OCT2 using TR-BBB cells as an *in vitro* BBB model. Furthermore, we examined the regulatory mechanisms of choline transport under the oxidative stress condition induced by tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), diethyl maleate (DEM) and glutamate.

MATERIALS AND METHODS

Materials

Radiolabeled [methyl-^{3}H]choline (80 Ci/mmol) was purchased from Amersham Biosciences (Amersham, UK). *Lipopolysaccharide (LPS) from Salmonella Minnesota* R595 (Re) was obtained from List Biological Laboratories (Campbell, CA, USA). Tumor necrosis factor-α (TNF-α, human recombinant (Escherichia coli) solution, sterile, 10 μg/mL), diethyl maleate (DEM), glutamate, amiloride and carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) were purchased from Sigma Chemical (St. Louis, MO, USA). Donepezil hydrochloride, (±)-2-[(1-benzylpiperidin-4-yl) methyl]-5,6-dimethoxyindan-1-one monohydrochloride was kindly provided by Daewoong Co. (Seoul, Korea). All other chemicals were commercial products of reagent grade.

Cell culture

The TR-BBB cells were grown routinely in collagen type I coated tissue culture dishes (Iwaki, Tokyo, Japan) at 33°C in a humidified atmosphere of 5% CO_2/air. The cells were cultured in Dulbelcco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA), 15 μg/L endothelial cell growth factor (Roche, Mannheim, Germany), 100 U/mL penicillin and 100 ìg/mL streptomycin (Invitrogen, Grand Island, NY, USA). Hypertonic culture medium (380 mOsm/kg) was prepared by adding 100 mM raffinose to normal culture medium.

[^{3}H]Choline uptake study in the TR-BBB cells

TR-BBB cells (1×10^5 cells/well) were cultured on collagen type I coated 24 well plates (Iwaki, Tokyo, Japan) at 33°C for 2-3 days. Uptake experiments were performed in extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$ 10 mM D-Glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM HEPES) with pH 5.4 - pH 8.4. Uptake was initiated by applying 200 μL ECF buffer containing 1.0 μCi [^{3}H]choline to the cells at 37°C. To investigate the effect of FCCP on [^{3}H]choline uptake in TR-BBB cells, we used ECF buffer in the presence of 10 μM FCCP. When the influence of membrane potential on the [^{3}H]choline uptake process was studied, ECF buffer was changed to high K^+ - ECF buffer (122 mM KCl, 25 mM $KHCO_3$ 10 mM D-Glucose, 3 mM NaCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM Na_2HPO_4, 10 mM HEPES) with pH 5.4 - pH 8.4 or ECF buffer with Ba^{2+}, a K^+ channel blocker. To investigate the change of choline uptake under the oxidative stress condition, the TR-BBB cells were pretreated with 20 ng/mL TNF-α, 10 ng/mL LPS, 100 μM DEM or 100 μM glutamate for 3, 6, 12 and 24 h and the uptake study was performed as described above. Then, the cells were solubilized in 750 μL of 1 M NaOH-PBS buffer overnight. An aliquot (50 μL) was taken for protein assay using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The remaining solution (500 μL) was mixed with 4.5 mL of scintillation cocktail (Hionic-fluor, Packard, Meriden, CT, USA) for the measurement of radioactivity using a liquid scintillation counter (LS6500, Beckman Instruments Inc. Fullerton, CA, USA).

Measurements of [^{3}H]choline efflux in TR-BBB cells

After the cells were grown on collagen type I coated 24 well plates for 2-3 days, ECF buffer containing 1 μCi [^{3}H]choline was added to the TR-BBB cells for 60 min at 37°C. Then, the applied solution was removed from the cells and the cells were immersed in ice-cold ECF buffer. The ECF buffer at 37°C was added to the cells and incubated for the designated time periods, followed by the removal of the incubation medium from the cells. To test the change of choline efflux under the oxidative stress condition, the cells were pretreated with 20 ng/mL TNF-α, 10 ng/mL LPS, or 100 μM DEM for 3 h and the efflux study was performed as described above. The cells were then solubilized in 1 N NaOH overnight at room temperature and an aliquot was taken for the measurements of the amount of [^{3}H]choline remained in the cells.

Data analysis

Unless otherwise indicated, all data are given as mean ±SEM values. An unpaired, two-tailed Student's t-test was

used to determine the significance of differences between two group means and $p<0.05$ was considered statistically significant.

RESULTS

Driving force of [^{3}H]choline uptake in TR-BBB cells

To investigate the influence of membrane potential on [^{3}H]choline uptake in TR-BBB cells, the uptake of [^{3}H]choline was measured under the conditions of increasing concentrations of K^+ at pH 7.4. The uptake of [^{3}H]choline in the cells was decreased in the presence of high K^+ concentration, while that was not decreased in the presence of Ba^{2+} that is a K^+ channel blocker (Fig. 1). The uptake of [^{3}H]choline in TR-BBB cells with ECF buffer at various pH levels is shown in Fig. 2. When the extracellular pH was changed from 8.4 to 5.4, [^{3}H]choline uptake was decreased gradually in TR-BBB cells. As shown in Table I, [^{3}H]choline uptake in the cells was decreased significantly in the presence of FCCP and amiloride at various pH levels.

Effect of oxidative stress on [^{3}H]choline uptake and efflux in TR-BBB cells

The effect of oxidative stress on [^{3}H]choline uptake was examined in TR-BBB cells and the results are summarized in Table II. Treatment with 20 ng/mL TNF-α, 10 ng/mL LPS, 100 μM DEM, or 100 μM glutamate for 24 h resulted in a significant increase in [^{3}H]choline uptake in TR-BBB cells, while hydrogen peroxide and hypertonic condition (100 mM raffinose; 380 mOsm/kg) had no significant effects on [^{3}H]choline uptake. As shown in Fig. 3, pre-treatment with TNF-α, LPS up to 3 h resulted in a dramatic increase in the [^{3}H]choline uptake and it fell continuously down. [^{3}H]Choline uptake was increased gradually up to 12 h after the pre-treatment with DEM in the TR-BBB cells and then, it was decreased slightly. Glutamate pre-treatment resulted in a steady increase of [^{3}H]choline uptake in TR-BBB cells for 24 h.

The effects of TNF-α, LPS, DEM on the efflux of [^{3}H]

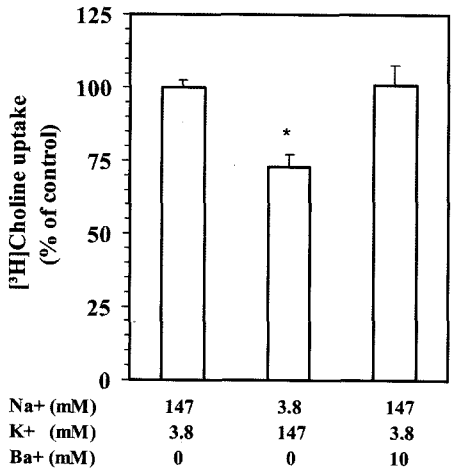

Fig. 1. Effect of membrane potential on [^{3}H]choline uptake in TR-BBB cells. The cells were incubated for 5 min at 37 °C with ECF buffer at the indicated ion concentrations containing [^{3}H]choline (62 nM, pH 7.4). Each column represents the mean±SEM (n=4).
*$p<0.01$, significantly different from control (the first column).

Table I. Effects of protonophore (FCCP, 10 μM) and Na^+/H^+ exchanger inhibitor (amiloride, 0.5 mM) at the various pH on [^{3}H]choline uptake in TR-BBB cells

Conditions	FCCP Cell/Medium Ratio (μL/mg protein)	Amiloride Cell/Medium Ratio (μL/mg protein)
Control	71.2±3.8	71.2±3.8
EtOH 0.025%	79.5±2.9	-
pH 5.4	38.7±3.9**	-
pH 6.4	42.8±1.8**	-
pH 7.4	46.0±6.1*	13.2±3.8†
pH 8.4	48.3±3.1*	13.6±1.0†

Cells were incubated for 5 min at 37°C with ECF buffer containing [^{3}H]choline (62 nM) at various pHs. The [^{3}H]choline uptake without FCCP at pH was represented as a control and the effect of EtOH (0.025%), solvent for FCCP, was also represented. Effect of Na^+/H^+ exchanger inhibitor, amiloride, on [^{3}H] choline uptake was investigated at pH 7.4 and 8.4. Each data point represents the mean±SEM (n=4).
*$p<0.05$; **$p<0.01$, significantly different from control.
†$p<0.001$, significantly different from control.

Table II. The uptake of [^{3}H]choline in TR-BBB cells under the following different conditions

Treatment	Uptake of [^{3}H]choline (% of control)
Control	100±13
Tumor necrosis factor-α	146±4***
Lipopolysaccharide	79.0±7.1*
Diethyl maleate	160±7**
Glutamate	198±6***
H_2O_2	74.2±6.7
Raffinose	84.6±7.0

Cells were treated with tumor necrosis factor-α (20 ng/mL), lipopolysaccharide (10 ng/mL), diethyl maleate (100 μM), glutamate (100 μM), hydrogen peroxide(100 μM) or exposed to hypertonic condition (100 mM raffinose; 380 mOsm/kg) for 24 h, respectively. The cells were incubated for 5 min at 37°C with ECF buffer containing [^{3}H]choline (62 nM). Each data point represents the mean±SEM (n=3~4).
*$p<0.05$; **$p<0.01$; ***$p<0.001$, significantly different from control.

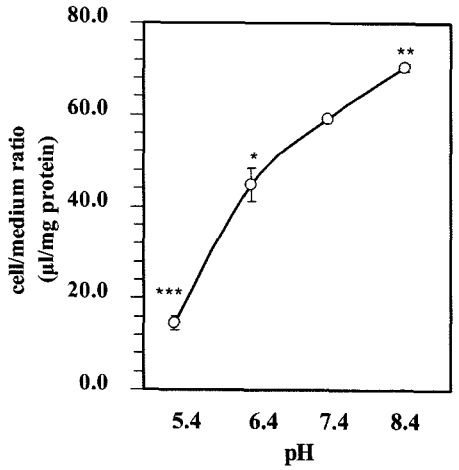

Fig. 2. Effect of extracellular pH on [^{3}H]choline uptake in TR-BBB cells. The cells were incubated for 5 min at 37°C with ECF buffer containing [^{3}H]choline (62 nM) at various pH. Each point represents the mean±SEM (n=4).
*p<0.05; ** p<0.01, significantly different from pH 7.4.

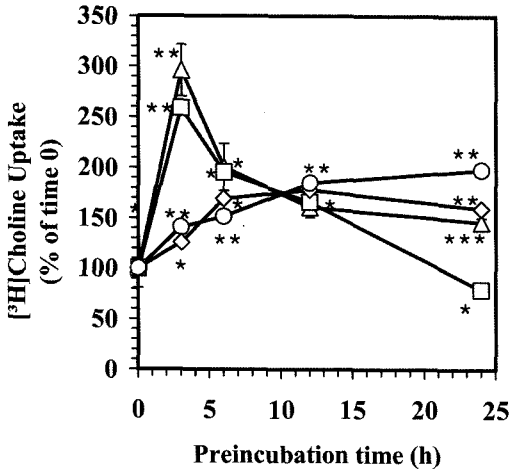

Fig. 3. Time-course of the effects by the oxidative stress inducing agents on [^{3}H]Choline uptake in TR-BBB cells. The cells were pre-incubated with 20 ng/mL TNF-α(△), 10 ng/mL LPS(□), 100 μM DEM(◇), and 100 μM glutamate (○) for the time periods in the figure. Then, the cells were incubated for 5 min at 37°C with ECF buffer containing [^{3}H] choline (62 nM). Each point represents the mean±SEM (n= 3).
*p<0.05; **p<0.01; ***p<0.001, significantly different from control (time=0).

choline were examined in TR-BBB cells and the results are illustrated in Fig. 4. [^{3}H]Choline efflux was reduced in the cells after the pre-treatment with TNF-α, LPS, and DEM for 3 h.

DISCUSSION

Our previous study demonstrated that TR-BBB cells, an

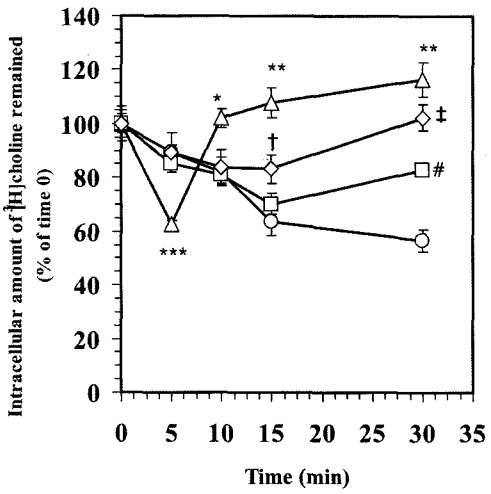

Fig. 4. Comparison of efflux effects by the oxidative stress inducing agents on [^{3}H]choline transport from TR-BBB cells. The cells were pre-treated with ECF buffer(O), 20 ng/mL TNF-α(△), 10 ng/mL LPS(□), and 100 μM DEM(◇) for 3 h. Samples were taken at the times indicated and the remaining amount of [^{3}H]choline in the cells was measured. Each point represents the mean±SEM (n=3).
*p<0.05; **p<0.01; ***p<0.001, significantly different from each time point of control efflux.
†p<0.05; ‡p<0.001, significantly different from each time point of control efflux.
#p<0.05, significantly different from each time point of control efflux.

in vitro model of the BBB, possess [^{3}H]choline transport activity (Kang *et al.*, 2005). In the present study, we aimed to search for the driving force of choline transport at the BBB. To investigate the influence of membrane potential on choline uptake, the uptake of [^{3}H]choline was measured in TR-BBB cells with increasing concentrations of K^+ in the uptake buffer which depolarized the cell membrane potential. The uptake of choline in the cells was decreased significantly in the presence of high concentration of K^+-ECF buffer at pH 7.4, while Ba^{2+} used as a K^+ channel blocker had no effect (Fig. 1). Our previous study indicated that [^{3}H]choline uptake was reduced by valinomycin, the ionophore of potassium (Kang *et al.*, 2005), suggesting that membrane potential differences may provide the driving force for choline uptake to the brain. This result was similar to the finding of Friedrich *et al.* regarding choline uptake to the brain using rat brain microvessel endothelial cell line (RBE4). However, our present results show that the choline uptake in TR-BBB cells is a sodium-dependent transport. We also examined the effect of extracellular pH on the uptake of [^{3}H]choline in TR-BBB cells (Fig. 2). The uptake of [^{3}H]choline was inhibited significantly by lower

pH than extracellular pH 7.4, indicating that an extracellular low concentration of proton enhanced the choline uptake significantly. This result suggests that choline may be transported by an organic cation (OC)/ H^+ exchanger in TR-BBB cells. The pH dependence of choline uptake in TR-BBB cells similarly corresponds with the results examined in MBEC4 cells (Kamath *et al.*, 2003), human keratinocytes HaCaT cells (Hoffmann *et al.*, 2002) and BeWo cells (Muller *et al.*, 2005). Therefore, we could suppose that choline uptake in TR-BBB cells could function as an OC/H^+ exchanger, pH-dependent decrease on uptake of [^{3}H]choline. In addition, we examined the effect of proton concentration on choline uptake using protonophore, FCCP in TR-BBB cells to confirm the present finding. FCCP is expected to increase the inside-negative H^+ diffusion potential (Miyamoto *et al.*, 1988). As shown in Table I, [^{3}H]choline uptake was recovered to the level of extracellular pH 7.4 by FCCP at all four pHs. According to these findings, we could suppose that the uptake of [^{3}H]choline is affected by the inside-negative H^+ diffusion-potential. It was reported that choline is transported by an electroneutral OC/ H^+ antiport and OC is then driven by a Na^+/ H^+ antiport coupled to the Na^+ pump (Kleinzeller et al., 1994). Amiloride is an effective inhibitor of Na^+-H^+ exchanger (Miyamoto *et al.*, 1988). In our study, amiloride inhibited [^{3}H]choline uptake significantly (Table I). Therefore, our results suggest that driving force of choline transport in TR-BBB cells may be related with Na^+/ H^+ antiport.

Phosphatidylcholine (PtdCho) is a major component of phospholipids in cell membranes, which is made of choline through the pathway of the de novo synthesis. Among the intermediate in the biosynthesis of PtdCho, CDP-choline only has neuroprotective effect with positive results in all randomized, double-blind trials (Davalos *et al.*, 2002). It has been shown that CDP-choline produces neuroprotective effects in a variety of CNS injury models, including cerebral ischemia. At the experimental level, it has been reported to decrease the infarcted volume and the size of edema, and/or to improve neurological deficits, either alone or in combination with other agents (Alkan *et al.*, 2001; Andersen *et al.*, 1988; Onal *et al.*, 1997; Schabitz *et al.*, 1996, 1999; Shuaib *et al.*, 2000). For several tissues, there are increasing evidences suggesting the involvement of the inhibition of PtdCho synthesis in the molecular mechanisms leading to cell death triggered by different insults, such as ischemia or glutamate excitotoxicity in brain, TNF-α, exogenous ceramides or antitumoural treatments (Sarri *et al.*, 2006). However, the regulation of choline transport at the BBB under CNS disorder such as ischemia still has been unknown. To evaluate the hypothesis that choline transport at the BBB is regulated by CNS cell damage, we examined the changes of choline uptake and efflux in TR-BBB cells through the pre-treatment with TNF-α that is a cytokine inducing cell damage, LPS that is a bacterial endotoxin, DEM that is a compound inducing oxidative stress, glutamate that is an excitatory neurotransmitter, hydrogen peroxide that is a compound inducing apoptosis, and raffinose that is a compound for producing hypertonic condition. TNF-α, LPS, DEM and glutamate induced [^{3}H]choline uptake in TR-BBB cells (Table II and Fig. 3). TNF-α is induced in the brain after cerebral ischemia and traumatic brain injury (Shohami *et al.*, 1994; Meistrell *et al.*, 1997). The extent of neuronal cell injury in rat brain was significantly increased by antenatal exposure to LPS (Larouche *et al.*, 2005). DEM induces oxidative stress through the depletion of intracellular reduced glutathione (GSH), which plays a role in protecting cells under the oxidative stress condition (Gilmont *et al.*, 1998). Activation of metabotropic glutamate receptors in the cortex can further augment the increase in BBB permeability caused by focal ischemia (Liu *et al.*, 2004). Therefore, disruption of BBB by TNF-α, LPS, DEM and glutamate in response to central nervous system (CNS) cell damage may produce one of factors responsible for the up-regulation of choline transport at the BBB. Although the mechanism of the neuroprotective effect of choline is still unclear, one possible physiological mechanism is an increasing choline level in the brain through the up-regulated BBB choline transport.

It has been reported that a small fraction of the intracellular choline was transported back into the external medium from cerebral capillary endothelial cells (Estrada *et al.*, 1990). In our previous report, we proved the efflux transport of choline from brain to blood using *in vivo* BEI method and by *in vitro* efflux study in TR-BBB cells (Lee and Kang, 2006). Choline efflux transport rate at the BBB was similar to blood-to-brain influx rate and this efflux process was saturable (Lee and Kang, 2006). This efflux system was thought to play an important role in the choline turnover in the brain. Therefore, we also examined whether the efflux of choline from brain to blood is regulated through CNS cell damage by TNF-α, LPS and DEM in TR-BBB cells. As a result, oxidative stress inducing agents reduced [^{3}H]choline efflux in a time-dependent

manner (Fig. 4), suggesting that these agents retain [^{3}H]choline in the brain. This result is consistent with the present finding that choline uptake was increased by TNF-α, LPS and DEM in TR-BBB cells (Fig. 3). Overall, the present results suggest that several oxidative stress inducing agents induce an increase of brain choline level, but, further studies are required to determine the relationship between increased brain choline level and brain neuroprotection.

In conclusion, choline is transported into TR-BBB cells through a sodium-dependent transport system and choline transport is inhibited by high concentration of K^+ and high extracellular H^+ concentration. Therefore, our results suggest that driving force of choline transport in TR-BBB cells may be related with Na^+/ H^+ antiport. In addition, influx and efflux transport of choline in TR-BBB cells can be regulated by TNF-α, LPS, DEM and glutamate, which may be one of mechanisms responsible for the neuroprotection in response to central nervous system (CNS) cell damage.

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