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T-588 inhibits astrocyte apoptosis via mitogen-activated protein kinase signal pathway

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Abstract

The effect of (1*R*)-1-benzo[b]thiophen-5-yl-2-[2-(diethylamino)ethoxy]ethan-1-ol hydrochloride (T-588), a cognition enhancer, on reperfusion injury was studied in cultured rat astrocytes. T-588 at 1–10 μM partially protected astrocytes against reperfusion injury after exposure to Ca^{2+}-free medium or hydrogen peroxide. Nerve growth factor (NGF) had a similar protective effect. Addition of both T-588 and NGF resulted in complete protection against Ca^{2+} reperfusion injury. T-588 did not stimulate NGF production in astrocytes. The effect of T-588 on Ca^{2+} reperfusion injury including apoptosis was inhibited by the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase inhibitor 2′-amino-3′-methoxyflavone (PD98059), but not by the phosphoinositide 3-kinase inhibitor wortmannin. The effect of NGF was inhibited by PD98059 and wortmannin. T-588 stimulated rapidly the phosphorylation of ERK, but did not affect that of Akt in astrocytes. These findings suggest that the ERK MAP kinase pathway has a role in the protective effects of T-588 and NGF.

Keywords: Ca^{2+} reperfusion; T-588; NGF (nerve growth factor); PD98059; MAP (mitogen-activated protein) kinase; Astrocyte

1. Introduction

We previously showed that incubation of cultured rat astrocytes in Ca^{2+}-containing medium after exposure to Ca^{2+}-free medium caused an increase in intracellular Ca^{2+} followed by delayed cell death (Matsuda et al., 1996, 1997). This injury is considered to be an in vitro model of ischemia/reperfusion injury, because a similar paradoxical change in extracellular Ca^{2+} concentration is reported in ischemic brain tissue (Siemkowicz and Hansen, 1981; Silver and Erecinska, 1992; Kristian et al., 1994). We reported that Ca^{2+} reperfusion injury was mediated by excess Ca^{2+} influx via the Na^{+}–Ca^{2+} exchanger in the reverse mode, and was attenuated by Na^{+}–Ca^{2+} exchange inhibitors (Matsuda et al., 1996), heat shock proteins (Takuma et al., 1996a) and calcineurin inhibitors (Matsuda et al., 1998). Subsequently, we have found that Ca^{2+} reperfusion injury was mimicked by reperfusion after exposure to hydrogen peroxide (H_2O_2) (Takuma et al., 1999). The reperfusion injury models using Ca^{2+} depletion and H_2O_2 exposure may contribute to clarification of the mechanisms of drugs, which ameliorate ischemia/reperfusion-induced brain dysfunction.

(1*R*)-1-Benzo[b]thiophen-5-yl-2-[2-(diethylamino)ethoxy]ethan-1-ol hydrochloride (T-588) has been selected for development as a therapeutic agent for reversing the dementia associated with Alzheimer's disease and cerebrovascular disease. This compound has an anti-hypoxic effect in mice (Ono et al., 1993) and ameliorates memory and learning impairments in animal models including cere-

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bral embolization, basal forebrain lesion and transient forebrain ischemia (Ono et al., 1995). These pharmacological effects of T-588 are considered to be mediated at least partly by cholinergic and noradrenergic systems in the brain (Ono et al., 1995; Miyazaki et al., 1997; Maekawa et al., 1998), but the molecular mechanisms underlying the effect are not known. In this paper, we examined the effect of T-588 on Ca^{2+} reperfusion injury in cultured astrocytes. The effect of nerve growth factor (NGF) on astrocyte injury was also examined, since our preliminary experiment showed that the NGF-producing agent idebenone protected astrocytes against Ca^{2+} reperfusion injury. The present study demonstrates that T-588 and NGF protect astrocytes differentially against Ca^{2+} reperfusion injury in cultured rat astrocytes.

2. Materials and methods

2.1. Materials

Drugs were obtained from the following sources: fetal calf serum, mouse anti-glial fibrillary acidic protein antiserum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), NGF and anti-NGF antibody, Sigma (St. Louis, MO); platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), GIBCO BRL, Life Technologies (Rockville, MD); 2′-amino-3′-methoxyflavone (PD98059), Calbiochem (La Jolla, CA); wortmannin, Nacalai Tesque (Kyoto, Japan); Eagle's minimum essential medium, Nissui Pharmaceutical (Tokyo, Japan); tissue culture ware, Iwaki Glass (Tokyo, Japan); phospho-p44/42 MAP kinase antibody, p44/42 MAP kinase antibody and phospho-Akt antibody, New England Biolabs (Beverly, MA). T-588 was a gift from Toyama Chemical (Toyama, Japan). All other chemicals used were of the highest purity commercially available.

2.2. Astrocyte culture

Astrocytes were isolated from cerebral cortices of 1-day-old Wistar and Sprague–Dawley rats as previously reported (Takuma et al., 1994, 1995, 1996b). Briefly, tissue was dissociated with dispase and cultured in minimum essential medium containing 10% fetal calf serum and 2 mM of glutamine. Cells were plated in 75-ml tissue culture flasks, split once upon confluency, and plated in 24-well plastic tissue culture plates or 60-mm plastic tissue culture dishes. The second cultures were grown to confluence in all experiments. The cells consisted for more than 95% of flat polygonal astrocytes (type-1 astrocytes), as confirmed by phase-contrast microscopy and positive immunostaining with anti-glial fibrillary acidic protein antibody (Takuma et al., 1994).

2.3. Reperfusion injury

The cells were washed and exposed to Earle's solution (control) and Ca^{2+}-free or H_2O_2-containing Earle's solution for 30 min. After two washes, the cells were incubated with Earle's solution for the indicated time (Matsuda et al., 1996; Takuma et al., 1999). The cells were gently washed twice with 500 μl of phosphate-buffered saline and MTT reduction activity was measured by a colorimetric assay (Matsuda et al., 1996). MTT reduction activity is expressed as a percentage of control.

2.4. Measurement of NGF

NGF protein level in cell-conditioned media was determined by a sensitive two-site ELISA according to the manufacturer's instructions (Promega, Madison, WI). In brief, 96-well, flat-bottomed Elisa plates (Nunc, Roskilde, Denmark) were coated with anti-NGF polyclonal antibody. The plates containing samples and standards were incubated at room temperature for 6 h on a plate shaker. NGF standards, ranging from 7.8 to 500 pg/ml, were prepared using recombinant human NGF. The captured NGF was reacted first with rat anti-NGF monoclonal antibody, and then with horseradish peroxidase-conjugated anti-rat immunoglobulin G antibody (1:5000). After the peroxidase reaction, the absorbance at 450 nm was recorded. Cell-derived NGF levels were determined by interpolation from standard curves assayed on individual plates.

2.5. Analysis of DNA ladder

Astrocytes were scraped off using a policeman, and collected by centrifugation at $1500 \times g$ for 10 min at 4°C. DNA was extracted and subjected to 1.8% agarose gel electrophoresis as reported previously (Takuma et al., 1999). DNA in the gel was stained with ethidium bromide and photographed with Polaroid instant films (type 667) under UV light.

2.6. Measurement of DNA fragmentation

The DNA fraction was mixed with an equal volume of 10% trichloroacetic acid and centrifuged at $15{,}000 \times g$ for 10 min at 4°C to separate intact DNA (pellet) from DNA fragments (supernatant), as reported previously (Takuma et al., 1999). Pellets were resuspended in the EDTA solution and 10% trichloroacetic acid (1:1). DNA was assayed using diphenylamine reagent (Burton, 1956). DNA fragmentation is expressed as a percentage of total DNA (intact plus fragmented).

2.7. Immunoblotting

The treated astrocytes were washed and harvested. Pellets were solubilized in sample buffer (3% sodium

dodecylsulfate (SDS), 62.5 mM Tris–HCl (pH 6.8), and 10% glycerol). The protein concentration in the sample was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). The sample was mixed with 0.1%(w/v) bromophenol blue and 0.05%(v/v) 2-mercaptoethanol, boiled for 5 min, and then loaded (equal amount of protein/lane) on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane and immunoblotting was carried out as reported previously (Matsuda et al., 1998), using phospho-p44/42 MAP kinase antibody, p44/42 MAP kinase antibody, phospho-Akt antibody and horseradish peroxidase-conjugated anti-rabbit antibody. Protein bands were detected with an enhanced chemiluminescence system. The densitometric analysis was carried out using FluoroImager 595 (Molecular Dynamics, USA).

2.8. Statistics

Statistical analysis of the experimental data was carried out by one-way analysis of variance (ANOVA) followed by post hoc Tukey's honestly significant difference (HSD) test, using SPSS 6.1 for Macintosh.

3. Results

3.1. Effect of T-588 on reperfusion injury

Reperfusion after exposure of astrocytes to Ca^{2+}-free medium resulted in a significant decrease in MTT reduction activity. T-588 attenuated the Ca^{2+} reperfusion-induced decrease in MTT reduction activity in a dose-dependent manner when administered up to 1 h after reperfusion (Fig. 1). The protective effect of T-588 was partial, but significant at concentrations higher than 1 μM. When astrocytes were exposed to H_2O_2 for 30 min and then incubated without H_2O_2, a significant decrease in MTT reduction activity was observed. T-588 had a protective effect on reperfusion-induced injury after H_2O_2 exposure in a dose-dependent manner, but the protective effect was not observed when the drug was added after reperfusion (Fig. 2).

3.2. Synergistic effect of T-588 and NGF

NGF protected cultured astrocytes against Ca^{2+} reperfusion injury in a dose-dependent manner, although the protective effect of NGF was partial (Fig. 3). NGF at 10 ng/ml alone showed a tendency to increase MTT reduction activity in control cells. When astrocytes were incubated with Earle's solution containing T-588 for 72 h, the content of NGF did not change: the levels (pg/ml, means ± S.E. for six wells obtained from two separate experiments) in control cells were 21.2 ± 1.6 (none) and 22.9 ± 1.9 (10 μM T-588), and those in cells exposed to Ca^{2+} depletion were 14.8 ± 2.0 (none) and 14.1 ± 2.1 (10 μM T-588). Simultaneous application of 10 μM T-588 and 10 ng/ml NGF resulted in almost complete protection against Ca^{2+} reperfusion injury (Fig. 4).

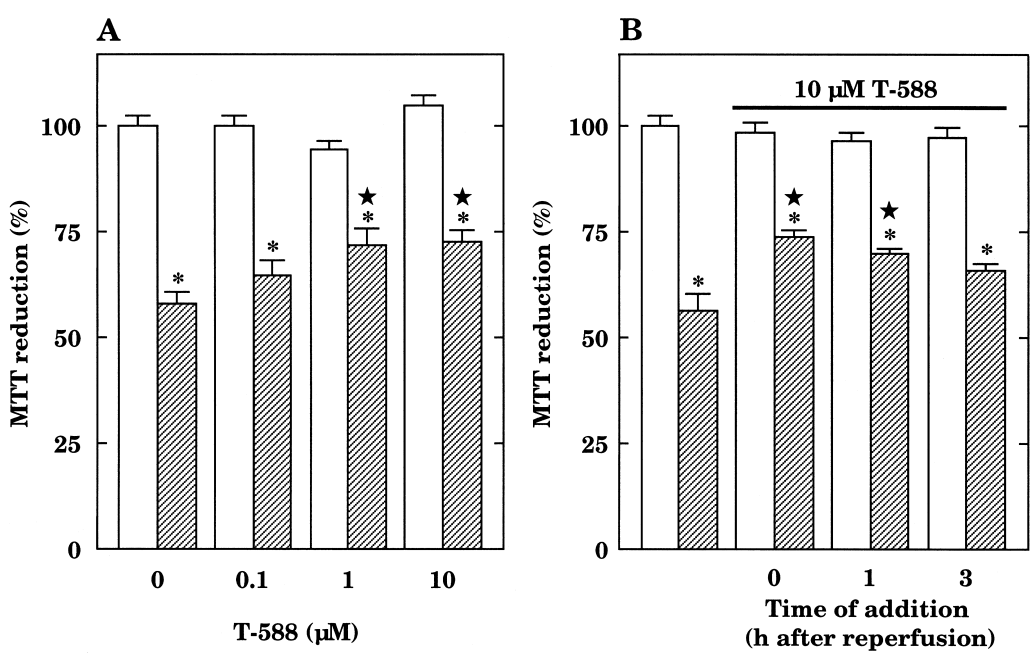

Fig. 1. Effect of T-588 on Ca^{2+} reperfusion injury in cultured rat astrocytes. Cells were exposed to normal (open column, control) or Ca^{2+}-free Earle's solution (hatched column) for 30 min, and then incubated with Earle's solution for 72 h. Cell injury was determined by MTT assay. (A) Dose dependence. The indicated concentrations of T-588 were present during Ca^{2+} reperfusion. (B) Post-treatment effects. T-588 (10 μM) was added at the indicated time and was present until assay. Results are means ± S.E.M. for 9–15 wells and were obtained from three to five separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$, significantly different from the values without T-588 (Tukey-HSD analysis).

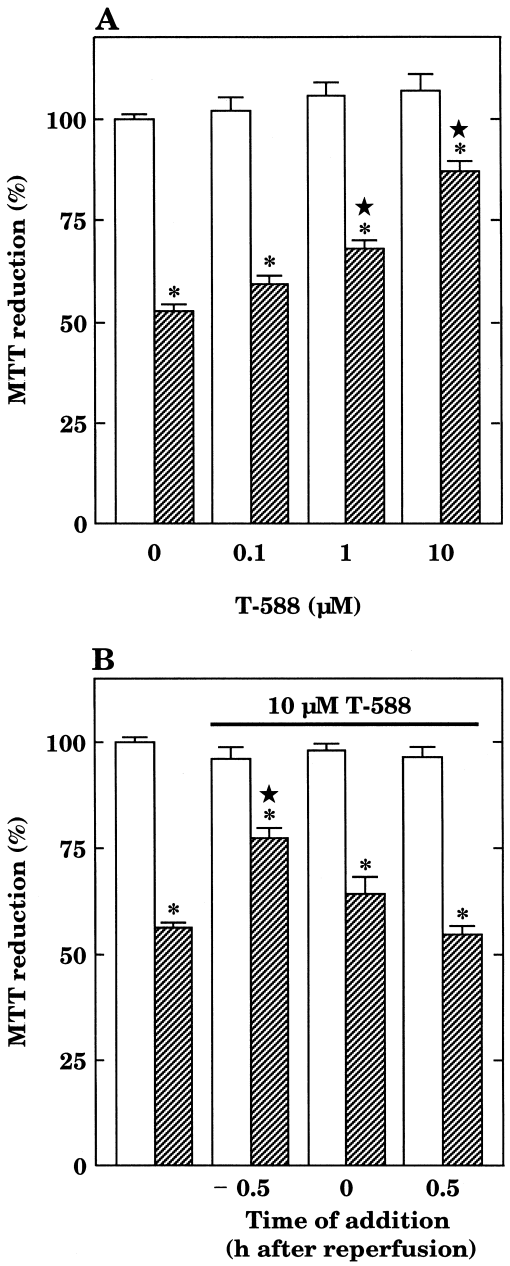

Fig. 2. Effect of T-588 on H_2O_2 exposure-induced cell injury in cultured rat astrocytes. Cells were exposed to normal (open column, control) or 100 μM H_2O_2 (hatched column) for 30 min, and then incubated with Earle's solution for 23.5 h. Cell injury was determined by MTT assay. (A) Dose dependence. The indicated concentrations of T-588 were added 30 min before H_2O_2 exposure and were present until assay. (B) Post-treatment effects. T-588 (10 μM) was added at the indicated time and was present until assay. Results are means ± S.E.M. for 9–15 wells and were obtained from three to five separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$, significantly different from the values without T-588 (Tukey-HSD analysis).

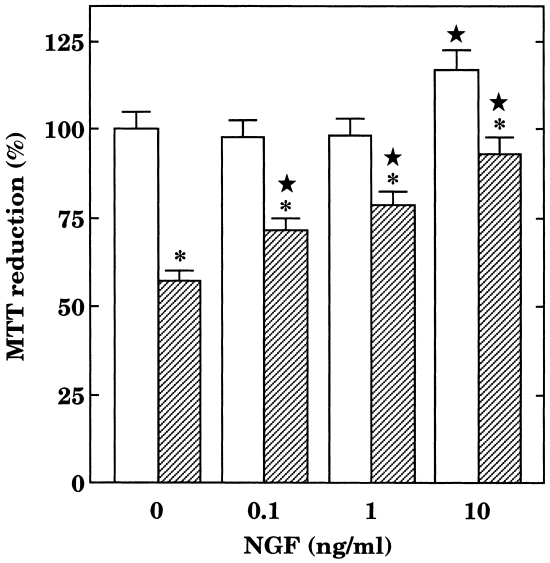

Fig. 3. Effect of NGF on Ca^{2+} reperfusion injury in cultured rat astrocytes. Cells were exposed to normal (open column) or Ca^{2+}-free Earle's solution (hatched column) for 30 min, and then incubated with Earle's solution for 72 h. Cell injury was determined by MTT assay. The indicated concentrations of NGF were present during Ca^{2+} reperfusion. Results are means ± S.E.M. for 15 wells and were obtained from five separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$, significantly different from the values without NGF (Tukey-HSD analysis).

3.3. Effects of PD98059 and wortmannin

Fig. 5 shows the effects of T-588 and NGF on Ca^{2+} reperfusion injury in astrocytes treated with the MAP ERK kinase (MEK) inhibitor PD98059 (100 μM) and the phosphoinositide-3 kinase inhibitor wortmannin (0.1 μM). The

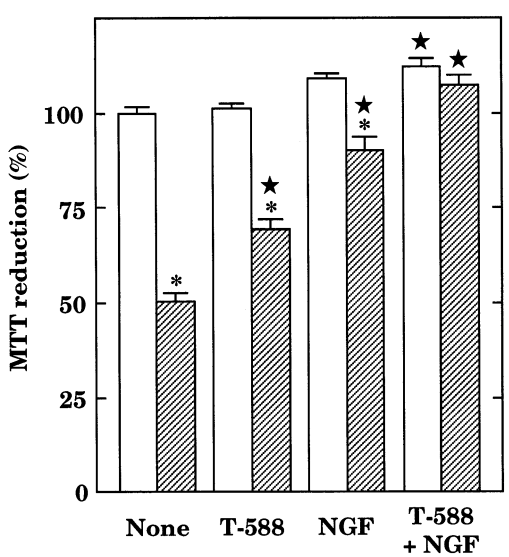

Fig. 4. Synergistic effect of T-588 and NGF on Ca^{2+} reperfusion injury in cultured rat astrocytes. Cells were exposed to normal (open column, control) or Ca^{2+}-free Earle's solution (hatched column) for 30 min, and then incubated with Earle's solution for 72 h. T-588 (10 μM) and NGF (10 ng/ml) were present during Ca^{2+} reperfusion. Results are means ± S.E.M. for nine wells and were obtained from three separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$, significantly different from the values without drugs (Tukey-HSD analysis).

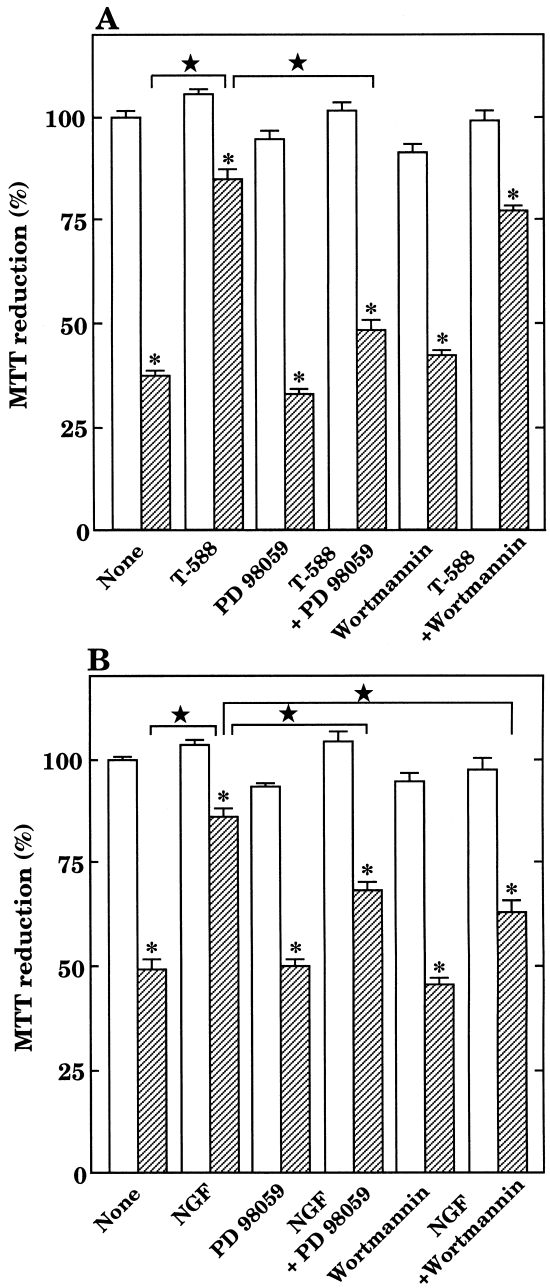

Fig. 5. Effects of PD98059 and wortmannin on the protection provided by T-588 and NGF against Ca^{2+} reperfusion injury in cultured rat astrocytes. Cells were exposed to normal (open column, control) or Ca^{2+}-free Earle's solution (hatched column) for 30 min, and then incubated with Earle's solution for 72 h. T-588 (10 μM; A), NGF (10 ng/ml; B) and wortmannin (100 nM) were present during Ca^{2+} reperfusion. PD98059 (100 μM) was added 30 min before Ca^{2+} depletion and was present until assay. Results are means ± S.E.M. for 9–24 wells and were obtained from three to eight separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$ (Tukey-HSD analysis).

inhibitors did not affect the viability of astrocytes reperfused after exposure to control and Ca^{2+}-free medium at the concentrations used here. The protective effect of T-588 against the decrease in MTT reduction activity was antagonized by PD98059, but not by wortmannin. A significant effect of PD98059 was observed at 10 μM (data not shown). The protective effect of NGF was partially

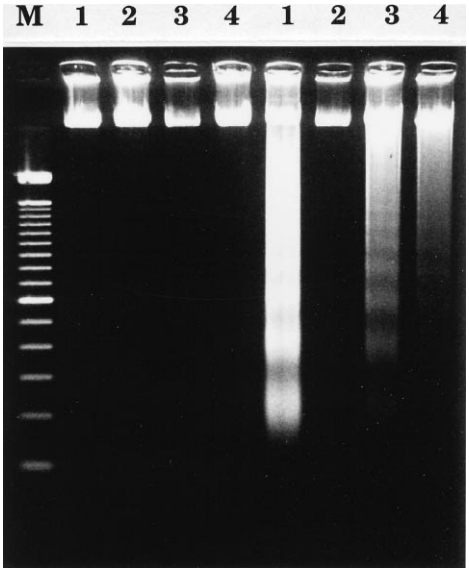

Fig. 6. Effects of T-588 and PD98059 on DNA ladder formation induced by Ca^{2+} reperfusion in cultured rat astrocytes. Cells were exposed to normal (lanes 1–4) and Ca^{2+}-free (lanes 5–8) Earle's solution for 30 min, and then incubated with Earle's solution for 7 days. T-588 (10 μM; lanes 2, 4, 6 and 8) was present during Ca^{2+} reperfusion. PD98059 (100 μM; lanes 3, 4, 7 and 8) was added 30 min before Ca^{2+} depletion and was present until assay. A typical result is shown (M: 100 bp marker).

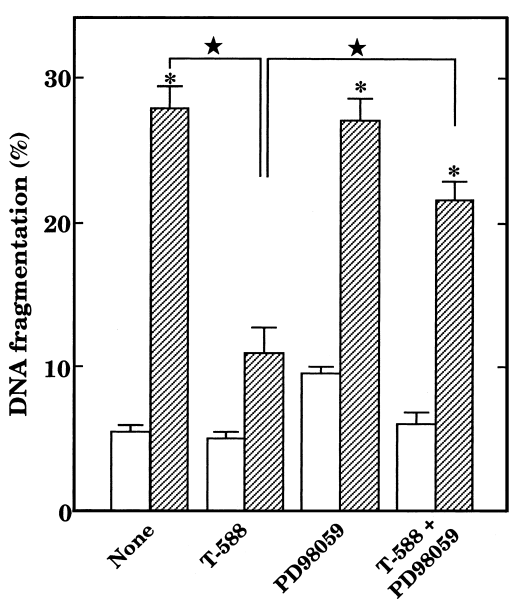

Fig. 7. Effects of T-588 and PD98059 on the increase in fragmented DNA induced by Ca^{2+} reperfusion in cultured rat astrocytes. Cells were exposed to normal (open column, control) or Ca^{2+}-free Earle's solution (hatched column) for 30 min, and then incubated with Earle's solution for 7 days. T-588 (10 μM) was present during Ca^{2+} reperfusion. PD98059 (100 μM) was added 30 min before Ca^{2+} depletion and was present until assay. Results are means ± S.E.M. for 6–10 wells and were obtained from three to five separate experiments. $^{*}P < 0.05$, significantly different from control; $^{\star}P < 0.05$ (Tukey-HSD analysis).

antagonized by PD98059 and wortmannin. T-588 inhibited the Ca^{2+} reperfusion-induced formation of a DNA ladder and the increase in the amount of fragmented DNA, and these effects were antagonized by PD98059 (Figs. 6 and 7).

3.4. Effect of T-588 on ERK phosphorylation

Although T-588 did not change non-phospho-ERK (Fig. 8A), it increased phospho-ERK in a dose-dependent manner (Fig. 8B). The effect seemed to be significant at concentrations higher than 0.1 μM and reached a plateau at 10 μM. The two proteins recognized by the antibody were also increased by 0.2 μg/ml EGF (data not shown). The effect of T-588 on ERK phosphorylation was blocked by PD98059 (Fig. 8C). T-588 did not affect Akt phosphorylation in cultured astrocytes (Fig. 8D). The effect of T-588 on ERK phosphorylation in astrocytes was rapid: the effect was observed 2 min after the treatment (data not shown).

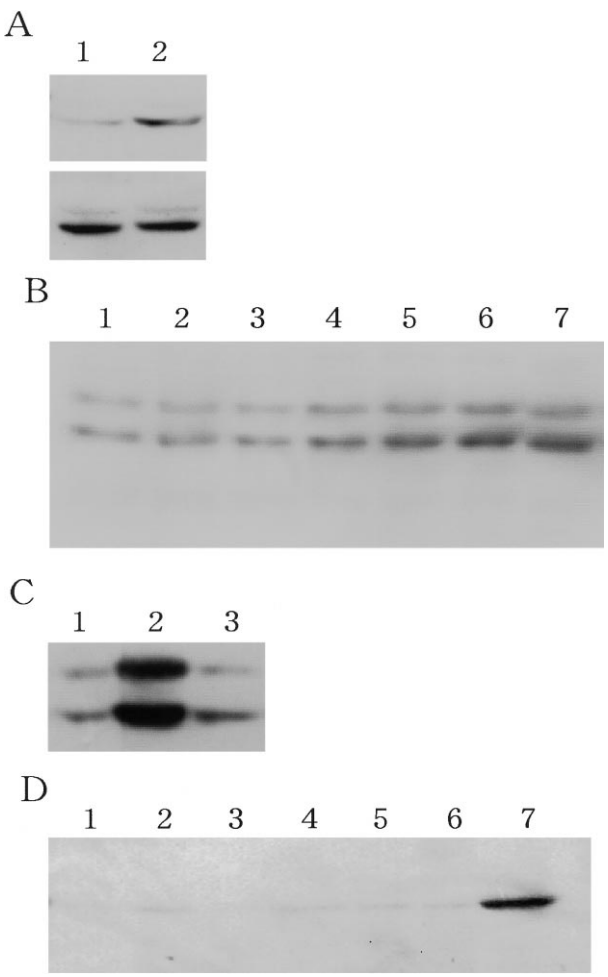

Fig. 8. Effect of T-588 on phosphorylation of ERK and Akt in cultured rat astrocytes. A typical experiment is shown. (A) The cells were treated with 10 μM T-588 for 10 min, and the cell extract (20 μg) was used for immunoblotting using phospho-ERK (upper) and non-phospho-ERK (lower) antibodies. (B) The cells were treated with T-588 at different concentrations (lane 1, none; 2, 10^{-9} M; 3, 10^{-8} M; 4, 10^{-7} M; 5, 10^{-6} M; 6, 10^{-5} M; 7, 10^{-4} M) for 10 min, and the cell extract (50 μg) was used for immunoblotting using phospho-specific ERK antibody. (C) The cells were treated with 10 μM T-588 in the presence and absence of 50 μM PD98059 for 10 min, and the cell extract (50 μg) was used for immunoblotting. Lane 1, control; lane 2, T-588; lane 3, T-588 + PD98059. (D) The cells were treated with T-588 at 10 μM for the different times (lane 1, 0 time; 2, 15 min; 3, 30 min; 4, 1 h; 5, 3 h; 6, 6h) for 10 min, and the cell extract (50 μg) was used for immunoblotting using phospho-Akt antibody. Lane 7 shows the effect of 50 ng/ml PDGF on phospho-Akt as positive control.

4. Discussion

The present study demonstrates that T-588 and NGF, which are reported to have a beneficial effect on ischemia/reperfusion-induced brain dysfunction (Ono et al., 1995; Holtzman et al., 1996; Guegan et al., 1998; Ishimaru et al., 1998), protect cultured astrocytes against Ca^{2+} reperfusion injury, and examines the cellular mechanisms underlying the protective effects of these compounds. We used two reperfusion systems using Ca^{2+} depletion and H_2O_2 exposure, as reported previously (Takuma et al., 1999). These systems differed in the effect of T-588 on the injury when the drug was administered after reperfusion: the protection against H_2O_2 exposure-induced cell injury required pretreatment before H_2O_2 exposure, while the drug was effective in reducing Ca^{2+} reperfusion injury even when it was added after reperfusion. This may be due to the difference in onset of the toxic effect between the two experiments: H_2O_2 rapidly activates the signal cascade, resulting in a decrease in MTT reduction activity, whereas paradoxical Ca^{2+} challenge causes more delayed cell injury, as reported previously (Matsuda et al., 1996; Takuma et al., 1999).

It is proposed that stress-activated protein kinase/c-Jun NH_2-terminal kinase (SAPK/JNK) and p38 kinase mediate apoptosis, and that the ERK signaling pathway plays a pivotal role in suppressing apoptosis (Xia et al., 1995; Sheng et al., 1997; Bergmann et al., 1998; Yujiri et al., 1998). Furthermore, phosphoinositide-3 kinase also has a role in cell survival (Jackson et al., 1996; Bartlett et al., 1997) and NGF-induced neuroprotection (Boniece and Wagner, 1993; Deckwerth and Johnson, 1993). The present study examined whether ERK and phosphoinositide 3-kinase signaling pathways are involved in the protective effects of T-588 and NGF, using the MEK inhibitor PD98059 (Alessi et al., 1995; Dudley et al., 1995) and the phosphoinositide-3 kinase inhibitor wortmannin. We found that the protective effect of T-588 was almost completely blocked by PD98059, while that of NGF was partially blocked by PD98059 and wortmannin. These findings suggest that the protective effect of T-588 is mediated by the ERK kinase signal pathway whereas that of NGF is mediated by both ERK kinase and phosphoinositide-3

kinase signal pathways. The involvement of the ERK kinase signal in the protective effects of T-588 was also demonstrated in the DNA fragmentation experiment. T-588 blocked Ca^{2+} reperfusion-induced DNA fragmentation, and this effect was significantly attenuated by PD98059. Galve Roperh et al. (1997) reported that astrocytes synthesized NGF in response to pro-inflammatory cytokines. But, the present study showed that Ca^{2+} reperfusion did not affect the NGF level in cultured astrocytes. In addition, T-588 did not stimulate NGF production in astrocytes. It should be noted that the simultaneous application of T-588 and NGF provided complete protection, although the protective effect of these drugs individually was partial. That is, the effects of T-588 and NGF were synergistic. These findings indicate that T-588 and NGF affect astrocytes differently.

In many different cell types, SAPK/JNK and p38 MAP kinase family members are activated predominantly by cellular stress or inflammatory signals, whereas ERK MAP kinase is activated by mitogenic stimuli (Han et al., 1994; Rouse et al., 1994; Cano and Mahadevan, 1995). Since the ERK signal pathway is proposed to play a role in cell survival (Xia et al., 1995; Sheng et al., 1997; Bergmann et al., 1998; Yujiri et al., 1998), it is possible that the MAP signal pathway may be a target for drugs to ameliorate ischemia/reperfusion injury. Previous studies showed that H_2O_2 exposure resulted in activation of three MAP kinase subgroups (Guyton et al., 1996; Wang et al., 1998; Bhat and Zhang, 1999). We have not yet examined whether reperfusion after Ca^{2+} depletion and H_2O_2 exposure affects the MAP kinase pathway in astrocytes; however, using pharmacological inhibitors, we found that the protective effect of T-588 might be mediated by activation of the ERK MAP kinase pathway. Furthermore, the present study shows that T-588 stimulates rapidly the phosphorylation of ERK. In this study, ERK activity was assayed by immunoblotting analysis using anti-phospho ERK1/2 antibody. The antibody recognized two proteins in astrocytes, as reported previously (Tournier et al., 1994). T-588-induced phosphorylation of ERK was blocked by PD98059, but the compound did not affect the phosphorylation level of Akt, an important regulator of cell survival (Kaplan and Miller, 1997; Marte and Downward, 1997; Crowder and Freeman, 1998). These findings suggest that T-588 stimulates preferentially the ERK MAP kinase pathway. Previous studies showed that the anti-hypoxic effect of T-588 was completely inhibited by scopolamine (Ono et al., 1993) and that carbachol activated ERK phosphorylation (Duan et al., 1995). We did not observe any effect of atropine on T-588-induced activation of ERK phosphorylation (data not shown). The exact mechanism for the effect of T-588 on the ERK MAP kinase pathway is not known. We showed that PD98059 did not affect cell survival in astrocytes. In contrast, Bhat and Zhang (1999) reported that H_2O_2-mediated cytotoxicity was blocked by PD98059. Thus, the role of the ERK pathway seems to depend on the cell type. We speculate that the ERK pathway plays a role in the protective effect of T-588 against reperfusion injury in astrocytes, although it seems that the ability of a cell to die or survive may be dictated by a critical balance between the ERK pathway and the SAPK/JNK or p38 kinase pathway (Stadheim and Kucera, 1998). The present study implies that the ERK MAP kinase pathway is an important target for drugs used to ameliorate reperfusion injury.

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References

Alessi, D.R., Cuenda, A., Cohen, P., Dudley, D.T., Saltiel, A.R., 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J. Biol. Chem. 270, 27489–27494.

Bartlett, S.E., Reynolds, A.J., Weible, M., Heydon, K., Hendry, I.A., 1997. In sympathetic but not sensory neurones, phosphoinositide-3 kinase is important for NGF-dependent survival and the retrograde transport of ^{125}I-βNGF. Brain Res. 761, 257–262.

Bergmann, A., Agapite, J., McCall, K., Steller, H., 1998. The *Drosophila* gene hid is a direct molecular target of Ras-dependent survival signaling. Cell 95, 331–341.

Bhat, N.R., Zhang, P., 1999. Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. J. Neurochem. 72, 112–119.

Boniece, I.R., Wagner, J.A., 1993. Growth factors protect PC12 cells against ischemia by a mechanism that is independent of PKA, PKC, and protein synthesis. J. Neurosci. 13, 4220–4228.

Burton, K., 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62, 315–323.

Cano, E., Mahadevan, L.C., 1995. Parallel signal processing among mammalian MAPKs. Trends Biochem. Sci. 20, 117–122.

Crowder, R.J., Freeman, R.S., 1998. Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. J. Neurosci. 18, 2933–2943.

Deckwerth, T.L., Johnson, E.M. Jr., 1993. Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. J. Cell. Biol. 123, 1207–1222.

Duan, R.D., Zheng, C.F., Guan, K.L., Williams, J.A., 1995. Activation of MAP kinase (MEK) and Ras by cholecystokinin in rat pancreatic acini. Am. J. Physiol. 268, G1060–G1065.

Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., Saltiel, A.R., 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. U. S. A. 92, 7686–7689.

Galve Roperh, I., Haro, A., Diaz Laviada, I., 1997. Induction of nerve

growth factor synthesis by sphingomyelinase and ceramide in primary astrocytes cultures. Mol. Brain Res. 52, 90–97.

Guegan, C., Onteniente, B., Makiura, Y., Merad-Boudia, M., Ceballos Picot, I., Sola, B., 1998. Reduction of cortical infarction and impairment of apoptosis in NGF-transgenic mice subjected to permanent focal ischemia. Mol. Brain Res. 30, 133–140.

Guyton, K.Z., Liu, Y., Gorospe, M., Xu, Q., Holbrook, N.J., 1996. Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. J. Biol. Chem. 271, 4138–4142.

Han, J., Lee, J.D., Bibbs, L., Ulevitch, R.J., 1994. AMAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265, 808–811.

Holtzman, D.M., Sheldon, R.A., Jaffe, W., Cheng, Y., Ferriero, D.M., 1996. Nerve growth factor protects the neonatal brain against hypoxic–ischemic injury. Ann. Neurol. 39, 114–122.

Ishimaru, H., Takahashi, A., Ikarashi, Y., Maruyama, Y., 1998. NGF delays rather than prevents the cholinergic terminal damage and delayed neuronal death in the hippocampus after ischemia. Brain Res. 789, 194–200.

Jackson, T.R., Blader, I.J., Hammonds Odie, L.P., Burga, C.R., Cooke, F., Hawkins, P.T., Wolf, A.G., Heldman, K.A., Theibert, A.B., 1996. Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphoinositide 3-kinase. J. Cell. Sci. 109, 289–300.

Kaplan, D.R., Miller, F.D., 1997. Signal transduction by the neurotrophin receptors. Curr. Opin. Cell Biol. 9, 213–221.

Kristian, T., Katsura, K., Gido, G., Siesjö, B.K., 1994. The influence of pH on cellular calcium influx during ischemia. Brain Res. 641, 295–302.

Maekawa, M., Murayama, T., Ono, S., Narita, H., Nomura, Y., 1998. The effects of T-588, a novel cognitive enhancer, on noradrenaline uptake and release in rat cerebral cortical slices. Jpn. J. Pharmacol. 77, 155–160.

Marte, B.M., Downward, J., 1997. PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. Trends Biochem. Sci. 22, 355–358.

Matsuda, T., Takuma, K., Nishiguchi, E., Hashimoto, H., Azuma, J., Baba, A., 1996. Involvement of Na^+–Ca^{2+} exchanger in reperfusion-induced delayed cell death of cultured rat astrocytes. Eur. J. Neurosci. 8, 951–958.

Matsuda, T., Takuma, K., Baba, A., 1997. Na^+–Ca^{2+} exchanger: physiology and pharmacology. Jpn. J. Pharmacol. 74, 1–20.

Matsuda, T., Takuma, K., Asano, S., Kishida, Y., Nakamura, H., Mori, K., Maeda, S., Baba, A., 1998. Involvement of calcineurin in Ca^{2+} paradox-like injury of cultured rat astrocytes. J. Neurochem. 70, 2004–2011.

Miyazaki, H., Murayama, T., Ono, S., Narita, H., Nomura, Y., 1997. Effects of *R*(−)-1-(benzo[b]thiophen-5-yl)-2-[2-*N*,*N*-diethylamino]ethoxy]ethanol hydrochloride (T-588), a novel cognitive enhancer, on noradrenaline release in rat cerebral cortical slices. Biochem. Pharmacol. 53, 1263–1269.

Ono, S., Kitamura, K., Maekawa, M., Hirata, K., Ano, M., Ukai, W., Yamafuji, T., Narita, H., 1993. Protective effect of *R*(−)-1-(benzo[b]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino)ethoxy]ethanol hydrochloride (T-588), a novel cerebral activator, against experimental cerebral anoxia. Jpn. J. Pharmacol. 62, 81–86.

Ono, S., Yamafuji, T., Chaki, H., Todo, Y., Maekawa, M., Kitamura, K., Kimura, T., Nakada, Y., Mozumi, K., Narita, H., 1995. A new cognition-enhancing agent, (*R*)-(−)-1-(benzo[b]thiophen-5-yl)-2-[2-(*N*,*N*-diethylamino)ethoxy]ethanol hydrochloride. Effects on memory impairment in rats generated by cerebral embolization and basal forebrain lesions. Biol. Pharm. Bull. 18, 1779–1783.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso Llamazares, A., Zamanillo, D., Hunt, T., Nebreda, A.R., 1994. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78, 1027–1037.

Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J.H., Chien, K.R., 1997. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. J. Biol. Chem. 272, 5783–5791.

Siemkowicz, E., Hansen, A.J., 1981. Brain extracellular ion composition and EEG activity following 10 minutes ischemia in normo- and hyperglycemic rats. Stroke 12, 236–240.

Silver, I.A., Erecinska, M., 1992. Ion homeostasis in rat brain in vivo: intra- and extracellular Ca^{2+} and H^+ in the hippocampus during recovery from short-term, transient ischemia. J. Cereb. Blood Flow Metab. 12, 759–772.

Stadheim, T.A., Kucera, G.L., 1998. Extracellular signal-regulated kinase (ERK) activity is required for TPA-mediated inhibition of drug-induced apoptosis. Biochem. Biophys. Res. Commun. 245, 266–271.

Takuma, K., Matsuda, T., Hashimoto, H., Asano, S., Baba, A., 1994. Cultured rat astrocytes possess Na^+–Ca^{2+} exchanger. Glia 12, 336–342.

Takuma, K., Matsuda, T., Asano, S., Baba, A., 1995. Intracellular ascorbic acid inhibits the Na^+–Ca^{2+} exchanger in cultured rat astrocytes. J. Neurochem. 64, 1536–1540.

Takuma, K., Matsuda, T., Kishida, Y., Asano, S., Seong, Y.H., Baba, A., 1996a. Heat shock protects cultured rat astrocytes in a model of reperfusion injury. Brain Res. 735, 265–270.

Takuma, K., Matsuda, T., Hashimoto, H., Kitanaka, J., Asano, S., Kishida, Y., Baba, A., 1996b. Role of Na^+–Ca^{2+} exchanger in agonist-induced Ca^{2+} signaling in cultured rat astrocytes. J. Neurochem. 67, 1840–1845.

Takuma, K., Lee, E., Kidawara, M., Mori, K., Kimura, Y., Baba, A., Matsuda, T., 1999. Apoptosis in Ca^{2+} reperfusion injury of cultured astrocytes: roles of reactive oxygen species and NF-κB activation. Eur. J. Neurosci. 11, 4204–4212.

Tournier, C., Pomerance, M., Gavaret, J.M., Pierre, M., 1994. MAP kinase cascade in astrocytes. Glia 10, 81–88.

Wang, X., Martindale, J.L., Liu, Y., Holbrook, N.J., 1998. The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem. J. 333, 291–300.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., Greenberg, M.E., 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270, 1326–1331.

Yujiri, T., Sather, S., Fanger, G.R., Johnson, G.L., 1998. Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 282, 1911–1914.