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Research Report

Mild hypothermia enhanced the protective effect of protein therapy with transductive anti-death FNK protein using a rat focal transient cerebral ischemia model

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ABSTRACT

We previously reported that the protein transduction domain fused FNK (PTD-FNK) protein, which was derived from anti-apoptotic Bcl-xL protein and thereby gained higher anti-cell death activity, has a strong neuroprotective effect on rat focal brain ischemia models. The aim of this study was to investigate the effect of PTD-FNK protein and hypothermia combined therapy on cerebral infarction. Male SD rats were subjected to 120 min middle cerebral artery occlusion (MCAO) with intraluminal thread. Rats were divided into 4 groups: 1) 37 °C vehicle administration (37V); 2) 37 °C PTD-FNK administration (37F); 3) 35 °C vehicle administration (35V); and 4) 35 °C PTD-FNK administration (35F). PTD-FNK protein was intravenously administered 60 min after the induction of MCAO. Hypothermia (35 °C) was applied during 120 min MCAO. Rats were sacrificed 24 h later; infarct volumes were measured, and Bax, Bcl-2, TUNEL and caspase-12 immunostaining was evaluated. There was significant infarct volume reduction in 37F, 35V, and 35F groups compared to 37V. There was also a significant difference between 37F and 35F. This suggests that hypothermia enhanced the effect of PTD-FNK. Similar results were found in neurological symptoms. Caspase-12 and TUNEL staining showed a significant difference between 37F and 35F; however, Bax and Bcl-2 staining failed to show a difference. In this study we showed an additive protective effect of hypothermia on PTD-FNK treatment, and immunohistological results showed that the protective mechanisms might involve the inhibition of apoptotic pathways through caspase-12, but not through Bcl-2.

1. Introduction

Many pharmacological agents have been shown to have neuroprotective abilities in animal experimental studies; however, most of them failed to show effectiveness in clinical trials. Therefore, exploring new therapeutic strategies is still an important issue.

Proteins of the Bcl-2 family have pro- and anti-apoptotic abilities and their role in the sequence of neuronal death is shown to be important. Bax promote apoptosis, whereas Bcl-

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2 and Bcl-xL block the translocation of cytochrome c and thereby prevent neurons from apoptosis; therefore, a potential neuroprotectant will be an anti-apoptotic protein such as Bcl-2 or Bcl-xL. Introducing proteins into brain cells by intravenous injection is known to be difficult because of the function of the blood–brain barrier. Protein transduction domain (PTD) from HIV Tat protein has been proposed to deliver therapeutic proteins directly into ischemic brain tissue by intravenous injection (Katsura et al., 2008; Wadia and Dowdy, 2002).

FNK protein is artificially derived from anti-apoptotic protein Bcl-xL by substituting three amino acids and thereby gains higher anti-cell death activity (Asoh et al., 2000). FNK protein was fused with PTD (protein transduction domain) of HIV/Tat protein to be able to pass through cell membranes. It was shown to be transduced into neuronal cells rapidly (Katsura et al., 2008).

PTD-FNK protein has been shown to have cytoprotective effects in various cells and various conditions (Arakawa et al., 2007; Asoh et al., 2005; Chen et al., 2007). PTD-FNK protected human neuroblastoma cells and rat neocortical neurons against staurosporine-induced apoptosis and glutamate-induced excitotoxicity (necrosis). It was also shown to have anti-necrotic and anti-apoptotic activity in cultured neuronal cells (Asoh et al., 2002).

We have previously shown that PTD-FNK had a strong neuroprotective effect using 90-min middle cerebral artery occlusion (MCAO) and reperfusion model (Katsura et al., 2008). The cortical infarct volume decreased to about 50% in that study.

On the other hand, hypothermia is known to have strong neuroprotective effects via many mechanisms (such as decreasing the metabolic rate, reducing glutamate release, reducing reactive oxygen species, preventing blood–brain barrier breakdown, modifying the gene expression of inflammation, apoptotic proteins etc.) (Yenari et al., 2008). However, clinical application of hypothermia to cerebral ischemia had been difficult because of several severe side effects. Therefore, we chose the very mild hypothermia of 35 °C which may have much less side effects even in clinical application. We have published several studies in which very mild hypothermia enhanced the protective abilities, and elongated the therapeutic time windows of several drugs (Nito et al., 2003, 2004).

Our aim of the present study was to clarify whether combination therapy with PTD-FNK and very mild hypothermia is more effective than monotherapy, and to study the mechanisms of protection.

2. Results

2.1. Effect on physiological parameters

Table 1 shows physiological parameters, measured before, during and after focal ischemia. Although $PaCO_2$ 10 min after reperfusion in normothermic groups increased significantly compared to individual values before ischemia, there was no significant difference between vehicle-treated groups and PTD-FNK-treated groups. By two-factor ANOVA, there was no significant difference between normothermic and hypothermic parameters at individual time points.

Table 1 – Physiological parameters before, during and after ischemia.

		Before ischemia	During ischemia	After ischemia
Normothermia (37 °C)				
MABP	Vehicle (n=5)	98.0±10.4	108.0±5.7	94.0±13.9
(mmHg)	FNK(n=5)	94.0±8.9	109.0±18.5	96.0±21.6
Blood glucose	Vehicle (n=5)	102.4±21.1	89.4±20.3	90.4±21.8
(mg/dl)	FNK (n=5)	97.4±12.5	84.2±10.1	91.8±14.5
pH	Vehicle (n=5)	7.42±0.03	7.40±0.02	7.38±0.02
	FNK (n=5)	7.41±0.02	7.39±0.02	7.37±0.03
$PaCO_2$	Vehicle (n=5)	39.8±2.6	43.0±1.4	45.6±0.9*
(mmHg)	FNK (n=5)	42.4±2.3	44.6±3.7	48.6±4.2*
PaO_2 (mmHg)	Vehicle (n=5)	99.8±10.4	95.0±11.2	92.2±2.6
	FNK (n=5)	100.0±9.2	96.2±10.6	97.8±7.2
Hypothermia (35 °C)				
MABP	Vehicle (n=6)	103.3±12.1	117.5±12.9	107.5±14.8
(mmHg)	FNK (n=8)	101.3±7.4	121.3±7.4	104.4±9.4
Blood glucose	Vehicle (n=6)	98.7±15.2	82.0±5.7	86.3±6.8
(mg/dl)	FNK (n=8)	101.8±13.6	87.6±11.3	89.5±15.9
pH	Vehicle (n=6)	7.40±0.03	7.40±0.02	7.38±0.02
	FNK (n=8)	7.40±0.02	7.39±0.02	7.37±0.02
$PaCO_2$	Vehicle (n=6)	41.5±2.3	45.5±2.7	44.8±2.9
(mmHg)	FNK (n=8)	42.1±2.3	46.0±1.9	46.4±2.6
PaO_2 (mmHg)	Vehicle (n=6)	110.2±6.5	102.5±12.1	104.3±5.1
	FNK (n=8)	114.9±8.4	106.8±4.7	106.4±5.0

* $p<0.05$ compared to before ischemia.

2.2. Effect of combination therapy on infarct volume

Two-factor ANOVA showed a significant influence of PTD-FNK treatment or temperature change on infarct volume (n=5–8 for each group). Total infarct volume of 37F, 35V, or 35F was significantly reduced compared to that of 37V ($p<0.01$, each), as shown in Fig. 2. Hypothermia and PTD-FNK combination therapy showed significant additional reduction of total infarct volume compared to the hypothermia-alone group. Although there was a similar tendency in cortex infarct volume, there was no significant difference between 35V and 35F. Striatum infarct volume was decreased only in 35F, not 37V or 37F.

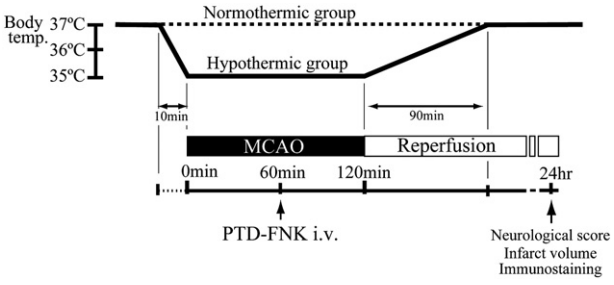

Fig. 1 – Experimental protocol including time course of temperature change, injection of PTD-FNK, evaluation of infarct volume, neurological score and immunostaining.