BH4 peptide derivative from Bcl-xL attenuates ischemia/reperfusion injury thorough anti-apoptotic mechanism in rat hearts

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Abstract

**Objective:** To prevent apoptosis is thought to be promising for myocardial protection in cardiac surgery. Recently, we showed that BH4 domain of Bcl-xL is essential for the prevention of apoptosis, and that BH4 fused to HIV TAT protein (TAT-BH4) prevented apoptotic cell death. Then, we hypothesized TAT-BH4 may attenuate ischemia/reperfusion injury in rat hearts.

**Methods:** The isolated rat hearts in the TAT-BH4 preconditioning group (BH4 group, \( n = 8 \)) or control group (C group, \( n = 8 \)) were subjected to warm ischemia (37 \( ^\circ \)C) for 30 min followed by 60 min of reperfusion using Langendorff perfusion system.

**Results:** Left ventricular developed pressure and maximum \( \frac{dP}{dt} \) after reperfusion were significantly improved in the BH4 group than those in the C group (\( P < 0.01 \)). Recovery of mitochondrial respiration was significantly better in the BH4 group than those in the C group (\( P < 0.05 \)). Moreover, expression of caspase-3 and TUNEL-positive myocardium were decreased in the BH4 group than those in the C group.

**Conclusions:** These results demonstrated that TAT-BH4 attenuates myocardial ischemia/reperfusion injury through preventing myocardial apoptosis. Thus, TAT-BH4 may be a novel therapeutic agent for myocardial protection in cardiac surgery.

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**Keywords:** Apoptosis; Cardiomyocytes; Ischemia/reperfusion injury; Heart surgery

1. Introduction

Recent advances in myocardial protection have improved the clinical results in open-heart surgery. However, severely critical cases associated with compromised heart, such as failing heart or post-ischemic conditions, still occur, and thus, further attempts to improve myocardial protection should be addressed.

Recently, a growing body of evidence have shown that apoptosis of myocardium is one of the major contributors to ischemic/reperfusion injury in experimental models \([1-5]\) and even in humans after open-heart surgery \([6,7]\). Therefore, many attempts through molecular mechanism to attenuate apoptosis of myocardium in ischemia/reperfusion injury have been reported \([8-15]\). However, no pharmacological strategy has been reported to attenuate apoptosis in the heart. Moreover, strategy using gene transfection during ischemia has limitations because it takes few ours to express proteins after reperfusion, which is not suitable for clinical application of myocardial protection against acute ischemic reperfusion injury.

We recently demonstrated that the biochemical role of the conserved N-terminal homology domain (BH4) of Bcl-xL is essential for the prevention of apoptosis, with respect to the regulation of mitochondrial membrane permeability and found that BH4 was required for Bcl-xL to prevent cytochrome c release. Using a newly developed TAT protein transduction system, which is the protein transduction domain of human immunodeficiency virus type 1 Tat protein (HIV TAT protein), we also showed that the BH4 domain fused to TAT protein (TAT-BH4), effectively prevented apoptotic cell death in vitro \([16]\), and showed feasibility of protein transduction into cells in vivo. Chen et al. demonstrated TAT-BH4 attenuated myocardial infarction in vivo \([17]\). Therefore, it is expected that preconditioning of TAT-BH4 may attenuate ischemia/reperfusion injury of the myocardium during open heart surgery.

In this study, we investigated whether the preconditioning of TAT-BH4 may attenuate ischemia/reperfusion injury in isolated rat heart model as a pre-clinical trial.
2. Method

2.1. Test compounds

TAT-BH4 protein and TAT mutant protein were provided by Shionogi Pharmacy Co., Ltd, Osaka, Japan. The proteins were dissolved in DMSO to the concentration of 5 μg/μl before use.

2.2. Pharmacological preconditioning and rat ischemia model

Sixteen Sprague-Dawley rats (300 g, male) were used for this study. Humane animal care complied with the 'Principle of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resource and published by the Institutes of Health (NIH Publication No. 86-23, revised 1985). The rats were divided into the control group (C group, n=8), and the TAT-BH4 group (BH4 group, n=8). All rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Following 10 min of the injection and anticoagulation with heparin (200 USP units, intra-peritoneally), the heart were quickly excised and perfused with modified Krebs-Henseleit buffer (120.0 mM NaCl, 4.5 mM KCl, 20.0 mM NaHCO3, 1.2 mM KH2PO4, 1.2 mM MgCl2, 2.5 mM CaCl2, and 10.0 mM glucose: gassed with 95% O2 + 5% CO2 to obtain pH 7.4 at 37 °C) at the pressure equal to 1 m H2O by means of a Langendorff apparatus. A thin-wall latex balloon was inserted into the left ventricle through the left atrium to monitor left ventricular pressure and to control left ventricular volume. After stabilization, heart rate (HR), left ventricular developed pressure (LVDP), maximum dP/dt (max dP/dt), and coronary flow (CF) were measured with LV diastolic pressure stabilized at 10 mmHg. Then, 100 μg (20 μl) of TAT-BH4 protein (BH4 group) or TAT mutant protein (C group), diluted by 5 ml of modified Krebs-Henseleit buffer, were administered through side port of apparatus at the speed of 1 ml/min. The hearts were then subjected to global ischemia at 37 °C for 30 min, followed by 60 min of reperfusion. The balloon was deflated during ischemia. The indices of cardiac function were continuously measured after reperfusion and analyzed using Polygraph System (Nihon Kouden, Japan). After 60 min of reperfusion, frozen sections of the hearts were made and stored at −80 °C for further assessment.

2.3. The recovery of mitochondrial respiration

Mitochondria were isolated from the hearts after reperfusion in 0.3 M mannitol/10 mM potassium Hepes, pH 7.4/0.2 mM EGTA, pH 7.4/0.1% fatty acid-free BSA by centrifugation at 2500×g for 10 min. The mitochondria were washed twice with this medium without EGTA to which 5 mM potassium phosphate was added and then suspended in it. Mitochondrial respiration was measured with an O2 electrode, and the recovery of respiration was defined as the ratio of mitochondrial respiration in the hearts after reperfusion to the hearts before ischemia.

2.4. Western blotting analysis of active caspase

To evaluate the activation of apoptotic cascade after reperfusion, western blot analysis for detection of active caspase-3 was performed using the frozen section samples after 60 min of reperfusion. We used active caspase-3 rabbit polyclonal IgG antibody, and anti-rabbit secondary antibody conjugated to horseradish peroxidase and Phototope-HRP Western detection kit. The degree of the protein expression was semi-quantitatively evaluated with computed densitometry (Scion Image: Windows; Microsoft Corporation).

2.5. Histological analysis of apoptosis

The frozen section samples after 60 min of reperfusion were prepared for histological analysis. TUNEL staining was performed using Terminal Deoxynucleotidyl Transferase Mediated UTP-Biotin In Situ Nick-End Labeling (Tunnel Intergen Kit) according to the manufacturer’s instruction. Quantitative assessment was calculated as a percentage of TUNEL-positive nuclei.

2.6. Statistical analysis

All data are expressed as mean±standard error of the means (SEM). Scores were compared using an unpaired Student’s t test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Recovery of cardiac function after global ischemia

To evaluate the efficacy of TAT-BH4 protein, we firstly analyzed cardiac function after global warm ischemia and reperfusion. The time course of percent recovery of LVDP after global ischemia (37 °C, 30 min) was shown in Fig. 1A. In comparison with the C group, a significant improvement of the percent recovery of LVDP was observed in the BH4 group at 10, 20, 30, 40, 50, and 60 min after reperfusion. The value after 60 min of reperfusion in the C group was 36±9%, and was significantly improved to 72±4% in the BH4 group (P<0.01).

The time course of percent recovery of max dP/dt after global ischemia was shown in Fig. 1B. In comparison with the C group, a significant improvement of the index was observed in the BH4 group at 20, 30, 40, 50, and 60 min after reperfusion. The value after 60 min of reperfusion in the C group was 36±9%, and was significantly improved to 71±3% in the BH4 group (P<0.01). CF after 60 min reperfusion was also significantly higher in the BH4 group compared with the C group (13.8±0.9 vs. 8.9±1.2 ml/min; P<0.01) (Fig. 1C).

3.2. Mitochondrial function by the recovery of respiration

Consistently, mitochondrial function assessed by the recovery of respiration markedly increased (C; 30±5, B; 77±10%, P<0.05) (Fig. 2).
3.3. Evaluation of apoptosis by active caspase-3 expression and TUNEL staining

Then, we evaluated the apoptosis of the myocardium by Western blotting of active caspase-3 and TUNEL staining of the myocardium 60 min after reperfusion. The western blotting analysis for caspase-3 showed lower expression of active caspase-3 in the BH4 group compared with the C group (Fig. 3A). According to semi-quantitative analysis with computed densitometry, the BH4 group showed 1/3–1/4 less caspase-3 expression than the C group. The TUNEL staining of the heart section showed none of TUNEL-positive cells in the BH4 group (Fig. 3C), but significantly positive in the C group (Fig. 3B). The percent TUNEL-positive cells in the C group was 2.2\% of all cardiomyocytes, whereas TUNEL-positive cells could not be seen in BH4 group (P<0.01).

4. Discussion

In the present report, we showed cardioprotective effect of TAT-BH4; a novel linkage of the protein transduction domain of HIV TAT to the functional domain of Bcl-xL. The recovery of cardiac function, mitochondrial respiration of the myocardium after ischemia/reperfusion was significantly better by TAT-BH4 administration. Moreover, TAT-BH4 attenuated caspase-3 expression and reduced apoptosis of cardiomyocytes.

Apoptosis is an actively regulated process of cellular self-destruction, thereby distinct from necrosis. It encloses mitochondrial changes with characteristic release of substances promoting apoptosis like cytochrome c. Downstream in the apoptotic program the caspase cascade such as caspase-3 is activated, followed by cytoskeletal alterations, chromatin condensation, and DNA fragmentation, culminating in cell death. Previous experimental studies have well shown that apoptosis of cardiomyocytes is induced from early stage of ischemia/reperfusion, playing an important role for the cardiac dysfunction [1-5]. Using isolated rat...
heart model, Scarabelli et al. [4] demonstrated that apoptosis (caspase-3 and TUNEL-positive nuclei) is seen in the very early stages (5 min of reperfusion) of ischemia/reperfusion in both endothelial cells and cardiomyocytes. Previous clinical studies also demonstrated apoptosis is evident early after ischemia/reperfusion during open-heart surgery. Schmitt et al. [6] showed that cytochrome c release and TUNEL-positive myocytes were increased even at the time of weaning from extracorporeal circulation (40 min of reperfusion), and apoptotic index showed a negative correlation with left ventricular function during surgery. Wu et al. [7] demonstrated percent TUNEL-positive myocytes was significantly increased even just after cross-clamping release (10 min of reperfusion). These findings are consistent with our result of increased caspase-3 activities and TUNEL-positive myocytes 60 min after reperfusion.

There are many reports to attenuate ischemia/reperfusion injury through inhibiting apoptosis [8–15]. Several reports demonstrated the effects of gene transfer of anti-apoptotic gene [8–12]. Huang et al. [11] showed the effects of Bcl-XL gene transfer in rat model, but gene transfection needs a couple of days to gene expression, so this method is not suitable clinically for cardio-protection during open heart surgery. Other reports [13–15] showed the effects of anti-apoptotic protein administration such as caspase inhibitor [13], but this method needs frequent or continuous administration of relatively high dose of drugs during ischemia and reperfusion, which might induce deleterious side effects in other organs. Compared with the previous methods, TAT-BH4 administration before ischemia has some advantages over previous approach, because administration of essential domain for Bcl-XL by TAT system enables effective protein transduction into the target cells and potentiate immediately after reperfusion.

Our results showed that BH4 administration suppressed apoptotic cascade, resulting in improvement of cardiac function from early stage of reperfusion. Caspase-3 expression, an important molecule in the cellular suicide cascade, was minimal and no TUNEL-positive cells were seen in the BH4 group. The mechanism that BH4 inhibits apoptosis has already been demonstrated in our previous report [16]. BH4 of Bcl-XL is essential for inhibition of apoptosis with respect to the regulation of mitochondrial membrane permeability and is able to inhibit both voltage-dependent anion channel activity even in the presence of Bax and apoptotic mitochondrial membrane permeability loss. The mechanisms of this rapid effect for cardiac function by inhibiting apoptosis is remain to be addressed. Cheng et al. [18] reported that even such a small number of cells affected by apoptosis may have a significant impact on cardiac contractility, because single-cell death impinges upon the force-generating ability of neighboring cells that are still viable but stunned. Relatively few apoptotic cells may substantially impair side-to-side slippage of myocytes, resulting in a disproportionate and much more severe cardiac dysfunction. As a result, TAT-BH4 administration before ischemia inhibited almost completely apoptotic cascade during ischemia/reperfusion, contributing improvement of cardiac function from the early stage of reperfusion.

The HIV TAT protein: the amino-acid transduction domain of TAT contains a domain that facilitates protein transduction across cellular membranes. Although the exact mechanism of protein transduction across cellular membranes remains unknown, TAT-mediated protein transduction has been shown to occur even at 4°C and is receptor independent [19]. These characteristics of TAT protein are also thought to be promising for protein transduction during open heart surgery.

These well known mechanisms of TAT-BH4 preventing mitochondrial function and apoptosis characterizes the promising possibility for clinical appreciation of this agent in cardiac surgery. Our data have strongly supported the importance of further investigation for clinical appreciation of this agent in future.

In conclusion, we obtained evidence that TAT-BH4 attenuates myocardial ischemia/reperfusion injury via inhibition of apoptosis of the myocardium. Thus, TAT-BH4 may be a novel therapeutic strategy for the protection of post-operative cardiac dysfunction in cardiovascular surgery.


