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Engineering of chimeric catalase-Angiopep-2 for intracellular protection of brain endothelial cells against oxidative stress

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ABSTRACT

Blood–brain barrier (BBB) disruption and brain microvascular endothelial cells (BMVECs) death caused by excessive production of hydrogen peroxide (H_2O_2) have been implicated in several neurological conditions. To overcome this problem, H_2O_2-degrading enzyme with ability to enter the BMVECs is required. In the present study, genetic fusion of gene encoding human catalase and gene encoding Angiopep-2 (AP2), a brain targeting peptide, was performed. The fusion protein was successfully expressed in *Escherichia coli* and purified to homogeneity. The protein retained heme content and specific enzymatic activity in the same order of magnitude as that of native enzyme. Study of the BMVECs internalization showed that 0.1 μM of the fusion protein can enter the cell within 15 min, while internalization of the native protein was not observed at this condition. In addition, treatment of the BMVECs with 20 units of the fusion protein for 30 min showed protection against H_2O_2 up to 5.0 mM, whereas this protective effect was not observed from treatment with the native protein. Therefore, construction of chimeric human catalase and AP2 provides an insight into the development of potential therapeutic antioxidant with ability to penetrate the BBB for protection against neurodegenerative disorders.

1. Introduction

Brain microvascular endothelial cell (BMVEC) is a major component of blood brain barrier (BBB), a fundamental wall that strictly separates systemic blood circulation from central nervous system (CNS). The cell differs from endothelial cell of other organs in two aspects. First, the BMVEC lacks fenestrations and has very low endocytic activity, thereby, limiting transcellular transport. Second, these cells are tightly sealed by tight and adherens junctions, thus restricted paracellular transport [1]. Therefore, the presence of the BBB not only helps to maintain brain microvascular structure but their high integrity also protects the brain by limiting the passage of foreign molecules from peripheral blood into the brain [2]. Although the integrity of the BBB is extremely high, comparing with other endothelial barriers, it can be altered by various factors including oxidative stress [3–5], a condition when the production of oxidants exceeds the rate of their degradation. Reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), have been claimed to involve in many neurological diseases [6–8]. Among these oxidants, H_2O_2 has been shown to cause BMVECs death or destroy barrier's function under several experimental and neurological conditions [9–18]. Generally, H_2O_2 is detoxified to either water by glutathione peroxidase (GPx) or water and oxygen by catalase (CAT) [19]. However, reducing concentration of catalase has been found in various neurological disorders [20–22] indicating that production of catalase at physiological level is not enough to mitigate H_2O_2 produced under certain pathological conditions. Therefore, to protect BMVECs from H_2O_2-induced oxidative damage which could potentially increase vascular permeability and consequently lead to brain injury, exogenous administration of catalase is required. However, due to the lack of fenestration and low endocytic activity of BMVECs, transport of macromolecules, such as catalase into the cells is highly restricted [23]. To overcome this problem, the enzyme needs to be modified. Recently, based on the active protein domains that inhibit the function of protein degrading enzymes or domains of Kunitz-type protease inhibitors, Angiopep-2 (AP2), a BBB permeable peptide has been designed. This peptide composes of 19 amino acids (TFFYGGSRGKRNNFKTEEY) with net charge of +2 and molecular weight of 2.3 kDa. The peptide has been shown to enter and cross BMVECs via receptor-mediated transcytosis (RMT) by binding to low-density lipoprotein receptor-related protein-1

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(LRP-1), which abundantly expresses on the cell surface. Interestingly, in vivo and in vitro transcytoses across BBB of AP2 have been found to be more efficient than other brain targeting proteins e.g., transferrin and aprotinin [24,25]. Conjugations of AP2 with therapeutic drugs, such as paclitaxel, or with nanoparticle/liposome surface have been shown to increase brain accumulation of the cargo molecules [26–29]. Notably, application of AP2 as a fusion peptide that can mediate internalization of the fusion partner into BMVECs has never been reported.

Therefore, to investigate whether AP2 can be used as a fusion peptide that can transduce the fusion partner, catalase, into BMVECs and confer protection against H_2O_2-induced oxidative stress, herein, genetic fusion of catalase and AP2 has been performed. Gene encoding human catalase has been fused in-frame with AP2 gene, generating AP2 joining catalase at the C-terminus. In order to confirm transcytosis of AP2-tagged protein, catalase has also been fused with a protein transduction domain of HIV-1 Tat protein (amino acid 47-57), a well-known cell penetrating peptide that can penetrate into non-specific mammalian cell types [30]. This peptide composes of 11 amino acids (YGRKKRRQRRR) with net charge of +8 and molecular weight of 1.56 kDa. The native and fusion proteins have been expressed in *Escherichia coli* BL21(DE3), purified to homogeneity and characterized for enzymatic activity. The immortalized mouse brain endothelial cell line (bEnd.3) was used as an in vitro model to investigate transduction of the fusion proteins and protective effect against H_2O_2-induced oxidative stress.

2. Materials and methods

2.1. Chemicals and reagents

*Nco*I, *Xho*I, *Hind*III and T4 DNA ligase were from New England Biolabs Inc. (Ipswich, MA, USA). *Pfu* DNA polymerase was purchased from Promega (Madison, WI, USA). QuikChange site-directed mutagenesis kit was from Agilent Technologies, CA, USA. Protein assay dye reagent concentrate was from Bio-Rad laboratories Inc. (CA, USA). Catalase from bovine liver was ordered from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was from MERCK (Darmstadt, Germany). Alexa Flour® 488 protein labeling kit, Alexa Flour® 488 annexin V/dead cell apoptosis kit and fetal bovine serum (FBS) were ordered from Invitrogen (Eugene, OR, USA). Gentamycin was from VESCO pharmaceuticals LTD, (BKK, Thailand). Trypsin 0.25% (1×) solution was purchased from J R Scientific Inc. (Woodland, CA, USA). Hyclone® DMEM/High glucose was ordered from Hyclone Laboratories Inc. (South Logan, UT, USA).

2.2. Plasmids, bacterial strains and cell line

Novablue and BL21(DE3) strains of *E. coli*, pACYCDuet-1, pET46Ek/LIC plasmids and other components required for ligation independent cloning (LIC) procedure were purchased from Novagen, EMD Biosciences (Damstadt, Germany). Mouse brain endothelial cell line, bEnd.3, was ordered from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA).

2.3. Construction of chimeric genes encoding catalase-Angiopep-2 (CAT-AP2) and catalase-Tat (CAT-TAT)

Construction of pET46CAT, a plasmid expressing human catalase was performed by the aid of PCR and LIC technique [31]. The forward (5′-GACGACGACAAGATGGCTGACAGCC GGGATCCC-3′) and the reverse primers (5′-GAGGAGAAGCCCGGTTCATCACAGATTT GCCTTC-3′) for amplification of cDNA encoding human catalase were designed to contain adapters for the LIC vector (underlined text). The cloning procedure was performed according to manufacturer's protocol. To construct pACYC-CAT, another catalase expressing plasmid, the gene encoding human catalase was excised from pET46CAT by treating with *Nco*I and *Xho*I then inserted into pACYCDuet-1 treated with the same enzymes. Prior to construction of chimeric gene, the stop codon of catalase was changed to *Hind*III recognition site by site-directed mutagenesis. To construct pACYC-CAT-AP2 (a plasmid expressing chimeric catalase-Angiopep-2), gene encoding Angiopep-2 was synthesized to contain *Hind*III and *Xho*I at 5′ and 3′ ends, respectively. Then, the gene was fused in-frame with 3′ end of catalase gene in pACYC-CAT. In a similar manner, pACYC-CAT-TAT, a plasmid expressing chimeric catalase and HIV-1 Tat cell penetrating peptide, was also constructed.

2.4. Expression and purification of CAT-AP2 and CAT-TAT chimeras

E. coli BL21(DE3) transformed with pACYC-CAT, pACYC-CAT-AP2 and pACYC-CAT-TAT were grown in terrific broth (TB) containing 34 μg/ml chloramphenicol at 37 °C, 150 rpm for 6 h. To induce protein expression, cultures were supplemented with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid hydrochloride for enhancement of heme biosynthesis. The cultivation was continued for 16 h at 30 °C with shaking at 120 rpm. Cells were pelleted by centrifugation at 6000 rpm, 4 °C for 10 min. The cell pellets were resuspended and disrupted by ultrasonic disintegration in sonication buffer containing 20 mM Tris–HCl, 500 mM NaCl, pH 7.9. The supernatant and pellet fractions were separated by centrifugation at 4 °C, 15,000 rpm for 10 min. Prior to purification, the crude extract was filtered using 0.45 μm Minisart filter (Sartorius) to remove insoluble materials. Then, the crude extract was loaded onto Ni-nitrilotriacetic acid (Ni-NTA) agarose column (pre-equilibrated with sonication buffer) attached to ÄKTA prime protein purification system (GE healthcare life sciences, Sweden). The chimeric proteins were eluted with gradient imidazole in the same buffer. The proteins were further purified by 16/60 Sephacryl S-300 HR gel filtration chromatography column (GE healthcare life sciences, Sweden). Fractions containing target protein were pooled and concentrated by 100K Amicon® Ultra-15 centrifugal filter devices (EMD Millipore Corporation, MA, USA). Purified proteins were checked for purity and molecular weight under denaturing condition by SDS-PAGE analysis. Highly purified proteins were kept under −80 °C, in the presence of 10-20% glycerol until used.

2.5. Analysis of heme content of chimeric proteins via spectrophotometry

Spectral properties of native and chimeric catalases were studied using UV-visible spectrophotometer (UV-1601 SHIMADZU). Aliquots of protein sample (0.5 mg/ml) were prepared in 50 mM potassium phosphate buffer, pH 7.0 containing 1.25 M NaCl and the spectral absorbance was scanned from 250 to 700 nm.

2.6. Enzymatic activity and kinetic parameters measurement

Catalase activity was assayed spectrophotometrically as described by Hugo Aebi [32]. The assay was performed at 25 °C in 50 mM potassium phosphate buffer, pH 7.0. The reaction was initiated by adding 1 ml of 30 mM H_2O_2 into 2 ml of reaction mixture containing target enzyme. Phosphate buffer was substituted for hydrogen peroxide solution in the control reaction. The reduction of H_2O_2 was recorded by the decreasing of absorbance at 240 nm. An extinction coefficient of 43.6 M^{-1} cm^{-1} of H_2O_2 was applied for calculation of enzyme specific activity [33]. Catalase from bovine liver with known activity (U/mg protein) was used as control. One unit