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Research report

Molecules involve in the self-protection of neurons against glucose–oxygen–serum deprivation (GOSD)-induced cell damage

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ABSTRACT

Molecules involved in self-protection of neurons against glucose/oxygen/serum deprivation (GOSD) were investigated. Trypan blue dye exclusion assay, Western blotting, ELISA, cytokine antibody array and chemical blocking assay were applied in the study. Results showed that early induction (at 6 h of GOSD) of cyclooxygenase-2 (COX-2), leptin, transforming growth factor-β1 (TGF-β1), glial-cell-line-derived neurotrophic factor (GDNF) and neurotrophin-3 (NT-3) all played a compensatory role in the protection of neurons against GOSD. Decline of these molecules and peroxisome proliferators-activated receptor (PPAR)-γ and -α since 12 h of GOSD may lead to an irreversible neuronal death. Nitric oxide (NO) and superoxide at low concentrations were neuroprotective whereas at high concentrations were detrimental to neurons. Accumulation of NO and superoxide at late stage of GOSD should therefore be prevented. The study provided a useful platform for screening of potential anti-ischemic drugs and also explained why GOSD neuron derived conditioned medium (NCM) only exerted a time-restricted neuroprotection.

1. Introduction

Ischemia-induced neuronal damage has been related to brain inflammation, mitochondrial dysfunction, protease activation, altered gene expression and homeostasis of calcium, glutamate, nitric oxide (NO), reactive oxygen species (ROS) and other unidentified factors [24]. Search for optimal drug(s) against cerebral ischemia however is still ongoing. We have used a glucose/oxygen/serum deprivation (GOSD) model to mimic the condition of ischemia *in vitro*. Through this model, we discover that early exposure (6 h) to GOSD, glial cells can release growth factors including glial-cell-line-derived neurotrophic factor (GDNF), transforming growth factor-β1 (TGF-β1) and neurotrophin-3 (NT-3), to protect surrounding neurons and glial cells from GOSD-induced cell damage [14,17,16]. The protection however, is time limited. It was unclear whether neurons could also protect themselves against GOSD by releasing GDNF, TGF-β1 and NT-3 or other molecules such as peroxisome proliferators-activated receptor (PPAR)-γ, -α, cyclooxygenase-2 (COX-2), NO, superoxide and leptin.

Cyclooxygenase (COX) is a rate-limiting enzyme in prostanoids synthesis. Although COX-2 could lead to ischemic damage [12], other reports also reveal a neuroprotective role of COX-2 in cerebral ischemia [15]. PPARs including PPAR-α, PPAR-β/δ and PPAR-γ, are a superfamily of nuclear receptors. A variety of eicosanoids and prostaglandins (PGs: PGE_2 and/or PGJ_2) are ligands for PPARs [22]. The role of PPARs in ischemia as well as GOSD-induced neuronal damage is still controversial due to their anti-inflammatory and pro-inflammatory activities [3,22]. Ischemia-induced ROS and NO are known to cause severe damage of brain cells due to their oxidative and/or inflammatory nature [24]. Both factors however, are also beneficial to ischemic brain due to their roles in cell proliferation, angiogenesis or neurogenesis [23]. Leptin can either promote inflammation by stimulating activities of pro-inflammatory immunocytes or inhibit inflammation by promoting release of glucocorticoids from the hypothalamic pituitary adrenal (HPA) axis [29]. Although the protective effect of leptin against cerebral ischemia has been reported [32], its direct effect on ischemic neurons still required further confirmation. Overall, molecular mechanisms involved in self-protection of neurons against GOSD were investigated by focusing on temporal changes of PPAR-γ, -α, COX-2, TGF-β1, GDNF, NT-3, NO and superoxide in response to GOSD and their impacts on survival of neurons against GOSD.

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2. Materials and methods

2.1. Enrichment of neurons

Enriched neuron culture was prepared from day 18 Sprague–Dawley (SD) rat embryo brains as previously described [16]. Briefly, single cell suspension of neurons at a density of 4×10^5/ml/well or 6.4×10^6/dish/10 ml, was enriched in DMEM (D5648, Sigma) supplemented with 1% B-27 and 10 mM glutamate for 5 days before test. Purity of the enriched neurons was 95% that was confirmed by pre-staining cells with anti-MAP-2 antibodies. All the animal experiments were carried out in accordance with the NIH guideline for the care and use of laboratory animals.

2.2. Glucose–oxygen–serum deprivation (GOSD)

To mimic the ischemic condition *in vitro*, neuronal cultures were placed into an anoxic chamber (37 °C, 5% CO_2 and 95% N_2) and cultivated in glucose- and serum-free DMEM (D5030, Sigma) supplemented with 1% non-essential amino acids and antibiotics (P/S) for various times (0, 6, 12, 24, and 48 h). The GOSD-treated neurons are then viewed as ischemic neurons.

2.3. Pharmacological treatment

Drugs including COX-2 inhibitor NS-398 (N194, Sigma), PPAR-γ antagonist GW9662 (M6191, Sigma), PPAR-α inhibitor MK-886 (M2692, Sigma), leptin (598-LP, R&D), 2-aminopurine (2-AP; A2380, Sigma), NO scavenger carboxy-PTIO (c-PTIO; C221, Sigma), superoxide scavenger Tiron (33724, Sigma) and double-stranded RNA-activated protein kinase (PKR) inhibitor (527450, Calbiochem) were individually added to neurons and incubated under the condition of GOSD for various times (0, 6, 12, 24, and 48 h). NS-398, GW9662, MK-886, PKR inhibitor and 2-AP were added 30 min and leptin overnight (20 h) before GOSD. Tiron and c-PTIO were given at same time when GOSD was delivered. The incubation time for drug treatment was selected based on previous reports of others [6,7,10,18,26,30,33,34]. We had pre-tested and found that 30 min or shorter was long enough for all inhibitors to show their effects on GOSD neurons whereas leptin required a longer incubation period to protect GOSD neurons. Leptin-mediated neuronal protection against 6 h GOSD was time-dependent, required at least 6 h or longer to see its protection (data not shown). In the study, we therefore pre-treated neurons with leptin overnight (about 20 h) before subjecting to the GOSD treatment.

2.4. Immunoblocking assay with anti-Ob-R antibody

Neurons were pre-treated with 10 μg/ml anti-Ob-R antibody (sc-1834-R, Santa Cruz) or non-specific IgG antibody (ab6721, Abcam) for 1 h before subjecting to GOSD treatment for various times (0, 6, 12, 24, and 48 h).

2.5. Survival rate (%) determination

Effect of GOSD with or without supplemental inhibitor and individual effect of glucose-, oxygen- and serum-deprivation and leptin on survival of neurons were determined by trypan blue dye exclusion assay. Survival rate (%) of the treated neurons was calculated by dividing the surviving number of treated neurons by number of normal neurons at indicated times.

2.6. Neuronal conditioned media (NCM) preparation and blocking

Conditioned media from neuron culture (at a density of 6.4×10^6/dish) was collected at the end of each GOSD intervals (0, 0–6, 0–12, 0–24, 0–48, 7–12, 7–24, and 7–48 h) and was individually incubated with another batch of neurons (4×10^5/well) under GOSD condition for 6 h. The survival rate was calculated accordingly.

In another set of experiments, NCM (collected at 12 h after GOSD) was pre-treated with 50 μM NS-398, 3 μM Tiron, 3 μM c-PTIO or anti-leptin antibody (10 μg/ml) for 1 h, before adding it to neurons under GOSD condition for 6 h. The survival rate of neurons was determined at the end of GOSD treatment by trypan blue dye exclusion assay.

2.7. Western blot analysis

Protein expression levels of PPAR-γ, PPAR-α, COX-2, inducible nitric oxide synthase (iNOS) and leptin were determined in neurons (6.4×10^6/dish) after GOSD for various times as indicated by Western blot analysis. The dilution factor for anti-PPAR-γ (P0744, Sigma), anti-PPAR-α (P0369, Sigma) and anti-actin (A5060, Sigma) was 1:2000; for anti-COX-2 (sc-7951, Santa Cruz) and anti-iNOS (sc-650, Santa Cruz) was 1:500 and for anti-leptin (ab3583, Abcam) was 1:4000. The secondary antibody, horseradish-peroxidase-conjugated anti-rabbit IgG (NA 934, Amersham Bioscience), was used at 1:20,000. The amount of protein expression was semi-quantified by using MCID image analysis system (Imaging Research Inc., St. Catherines, Canada).

2.8. Nitrite release assay

The release of nitrite (or NO) from GOSD-treated neurons was determined at indicated times based on nitrite release assay as described previously [5]. The absorbance of each sample was measured at 545 nm, with an UV MAX kinetic microplate reader (2100 Series, Awareness Technology, Plam City, FL). The standard curve of sodium nitrite was prepared in a glucose- and serum-free DMEM.

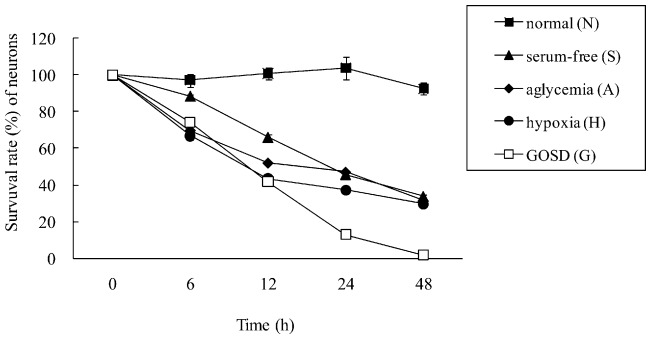

Fig. 1. GOSD and the related stresses caused significant damage of neurons in a time-dependent manner. Survival rate of neurons at indicated times after various treatments (serum-free, aglycemia, hypoxia and GOSD) were determined by trypan blue dye exclusion assay. The two-way ANOVA followed by a Fisher test, with $\alpha \leq 0.05$, were performed to see the group differences at indicated and cross times. Significant differences existed between N vs. S, A, H, and G group, respectively, at all time points except 0 h; S6 (S group at 6 h) vs. A6, H6 and G6, respectively; S12 vs. H12 and G12, respectively; H12 vs. G12; S24 vs. H24 and G24, respectively; A24 vs. H24 and G24, respectively; H24 vs. G 24; and G48 vs. S48, A48 and H48, respectively, with $\alpha \leq 0.05$. The experiment was repeated 3 times.

2.9. Cytochrome c reduction assay

The release of superoxide from GOSD-treated neurons was determined at indicated times by cytochrome *c* reduction assay as described previously [20]. Ferricytochrome C (50 μM; C2506, Sigma) with or without SOD (800 IU/ml; S2515, Sigma) was added to 1 ml of supernatants collected from GOSD neurons. The absorbance at 550 nm was read with Beckman DU640b UV–visible wavelength spectrophotometer. The amount of SOD inhibiting superoxide was calculated by using a molar extinction coefficient of $2.11 \times 10^4\ M^{-1}\ cm^{-1}$ for cytochrome *c* at 550 nm [19].

2.10. ELISA for TGF-β1, GDNF, and NT-3

The amounts of TGF-β1, GDNF, and NT-3 released by GOSD-treated neurons at indicated times were determined by ELISA kits (KAC1688, CHC2423, Biosource; and CYT 302, Chemicon). The measuring range for TGF-β1 and NT-3 was between 0 and 250 pg/ml; for GDNF was 0 and 62.5 pg/ml. All samples were measured within the ranges of the standard curve.

2.11. Cytokine antibody array assay

The amounts of leptin released by neurons under the GOSD condition (for 0, 6 and 24 h) were determined by using a cytokine antibody array kit (H0128003, RayBiotech). The procedures used were based on the protocols provided by the company. The reason to choose 0, 6 and 24 h for the measurements was based on the results from Western blotting showing that leptin expression was significantly increased by GOSD since 6 h then returned to basal level at 24 h (Fig. 3A). In addition, NCM collected at 6 h but not 24 h after GOSD appeared to be neuronal protective (Fig. 2).

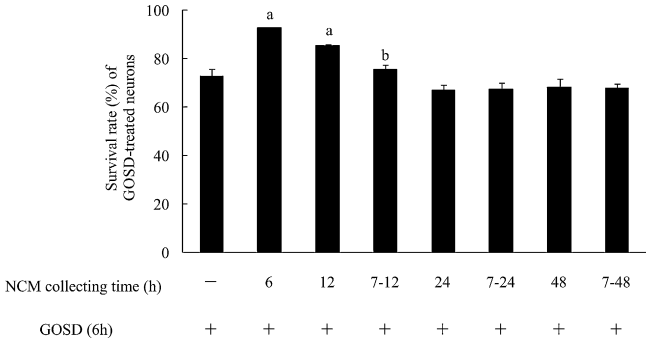

Fig. 2. The NCM-mediated protection of GOSD neurons was time limited. Survival rate of each group was calculated based on number of surviving neurons at 6 h after GOSD divided by number of normal neurons at 6 h. The one-way ANOVA followed by a Fisher test, with $\alpha \leq 0.05$, was performed in the test. [a] $p < 0.05$ vs. column 1; [b] $p < 0.05$ vs. column 3.