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ORIGINAL PAPER

Cytoglobin Up-regulated by Hydrogen Peroxide Plays a Protective Role in Oxidative Stress

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Abstract Cytoglobin (Cygb) is a recently discovered intracellular respiratory globin, which exists in all types of cells. It has been suggested that Cygb has a role in protecting cells against oxidative stress. In the present study we have tested this hypothesis. The N2a neuroblastoma cells were exposed to various kinds of insults, including hydrogen peroxide (H_2O_2), hypoxia, kainic acid, high extracellular $CaCl_2$, high osmolarity, UV irradiation and heat shock. Among them, only H_2O_2-treatment induced a significant up-regulation of cytoglobin mRNA level. We stably transfected N2a cells with Cygb-siRNA vectors and successfully knocked down Cygb. The Cygb-siRNA could exacerbate cell death upon H_2O_2-treatment, as demonstrated by MTT cell viability assay. Thus, Cygb in neuronal cells might be specifically induced under oxidative stress to protect them from death.

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Introduction

Globins, well exemplified by hemoglobin (Hb) and myoglobin (Mb), are mostly responsible for respiratory functions in mammals. Cytoglobin (Cygb) and neuroglobin (Ngb) are two newly identified intracellular oxygen-binding proteins endowed with hexa-coordinated heme-Fe atoms in their ferrous and ferric forms [1–3]. In the hexa-coordinated heme, the distal HisE7 residue is directly coordinated to the heme-Fe atom. This is fundamentally different from the penta-coordinated heme in Hb and Mb, in which oxygen establishes a sixth coordination bond to the heme-Fe atom. The hexa-coordination makes oxygen difficult to be disassociated from Ngb and Cygb [4, 5]. Therefore, Ngb and Cygb are unlikely to play a role in facilitating oxygen transportation. Recently, Hamdane et al. [6] reported that the hexa-coordination structure is involved in protecting α-Hb from aggregation induced by H_2O_2, suggesting that the hexa-coordinated globins like Cygb might have distinct reaction with H_2O_2.

Cytoglobin is so called because of its wide distribution in all tissues and cells [2]. In the teleost fishes, the expression level of a Cygb homolog (*Cygb-2*) in neuronal tissues is 250-fold higher than that in other tissues [7]. In the adult mouse brain, Cygb is selectively expressed in oxidative stress-responsive regions such as hippocampus, thalamus, and hypothalamus [8]. The cellular distribution of Cygb in different cells is also divergent. In neurons, Cygb exists both in the nucleus and the cytoplasm while in fibroblast-like cells, Cygb is distributed exclusively in the cytoplasm [9, 10]. These data suggest differential functions of Cygb in different cell types.

The physiological function of Cygb is unclear. Several lines of evidence suggest that Cygb is hypoxia-inducible [8, 11, 12]. However, Li et al. [13] reported that the expression levels of Cygb mRNA and protein were not altered in the brain under sustained hypoxia or intermittent hypoxia [13]. Although the induction of Cygb by hypoxia is controversial, overexpression of Cygb in MIN6 cells and hepatic stellate cells is protective [14, 15]. More recently, Cygb is found to be associated with cancers [16, 17], which are also highly oxidative stress-related [18, 19]. Here, we attempted to explore the functional role of Cygb in neuronal cells by exposing N2a neuroblastoma cells to various insults and by using siRNA technique to control Cygb gene level [20, 21].

Experimental procedure

N2a neuroblastoma culture and stress induction

Mouse N2a neuroblastoma cells were cultured with Dulbecco's modified Eagle's medium (DMEM)/Opti-MEM (V/V=1:1)/5% (v/v) fetal bovine serum (FBS) (GIBCO BRL, Life Technologies, USA) in an incubator (Precision Scientific, Inc., USA) at 37°C with 95% air/5% CO_2 (v/v) and 95% humidity as reported previously [22]. To induce oxidative stress, cells were treated with 250 μM hydrogen peroxide (H_2O_2). To induce hypoxia, cells were placed in a humidified incubator (Water-Jacketed US Autoflow Automatic CO_2 Incubator, USA) at 37°C with an atmosphere of 95% N_2/5%CO_2. N2a cells were also exposed to various non-oxidative insults including kainic acid (300 μM), high extracellular $CaCl_2$ (5.4 mM), high osmolarity (0.3 M NaCl), short-wavelength (254 nm) UV irradiation (40 J/m^2) and heat shock (42°C, 1 h) inside the incubator as reported previously by others [23]. N2a cells under normal conditions (0 h) served as control.

Reverse transcription (RT)-PCR

Total RNA from cultures was isolated using TRI$^{®}_{ZOL}$ Reagent according to the manufacturer's protocol (Invitrogen Life Technology, USA). Total RNA (2 μg) was used to perform reverse transcription by using M-MLV transcriptase (Promega, USA), oligo (dT_{15}) primer (Promega, USA) in a total volume of 25 μl. Semi-quantitative PCR, 28 cycles of 94°C, 45 s; 60°C, 45 s; 72°C, 45 s plus final extension at 72°C for 10 min, was performed in a volume of 10 μl containing 1X PCR buffer, 2 μl cDNA, 200 μM dNTPs, 400 nM primers, and 1 U Taq DNA polymerase using β-actin or GAPDH as internal control as previously reported [24]. The primers used in PCR were mouse Cygb, 5′-cctcaagcacaaggtggaac-3′ and 5′-ggcacccagaaatggaaggt-3′; mouse Ngb, 5′-ctctggaacatggcactgtc-3′ and 5′-gcactggctcgtctcttact-3′; mouse GAPDH, 5′-tgatgacatcaagaaggtggtgaag-3′ and 5′-tccttggaggccatgtaggccat-3′; mouse β-actin, 5′-cagccttccttcttgggtat-3′ and 5′-gctcagtaacagtccgccta-3′. All PCR products were confirmed by DNA sequencing.

Plasmid construction and transfection of N2a cells

The coding region, without the stop codon, of mouse *Cygb* gene was isolated from mouse brain and inserted into the pDsRed-N1 vector (named Cygb-Red) (Clontech, USA). Cygb siRNA expression vectors (named Cygb-siRNA) targeting to different regions of mouse Cygb cDNA (gi:32822762) were constructed using a GFP-expressing siRNA vector (p-Genesil-1) according to the manufacturer's instruction (Wuhan Genesil Biotechnology Co., Ltd., China). The efficiency of Cygb-siRNA plasmids was confirmed by co-transfecting Cygb-siRNA and Cygb-Red plasmids at a ratio of 4:1 into N2a cells using LipofectAMINE^{TM} 2000 reagent (Invitrogen Life Technology, USA). All constructs were confirmed by DNA sequencing.

Stable N2a cell line expressing Cygb-siRNA vectors

N2a cells were transfected with Cygb-siRNA plasmids or control vectors (pGenesil-1) and stable cell lines were screened using neomycin antibiotic (Sigma, USA) starting at a concentration of 800 μg/ml. The concentration of neomycin antibiotic was gradually reduced to a final concentration of 200 μg/ml within two weeks. Stable N2a cell lines expressing neomycin-resistant Cygb-siRNA (N2a/Cygb-siRNA) or control plasmids (N2a/pGenesil-1) were obtained by sub-cloning. The cells were maintained in N2a media plus neomycin antibiotic (200 μg/ml) and the media was changed every 2 or 3 days.

Cell viability assay

Cell viability was determined by spectroscopic measurement of the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT). N2a/Cygb-siRNA or N2a/pGenesil-1 cells were seeded in 96-well-plates at a density of 50,000 cells/100 μl/well. 24 h after incubation, the cells were exposed to H_2O_2 (500 μM) in fresh N2a media for 4 h. 10 μl of MTT (5 mg/ml) was added to the cultures and incubated for 4 h. Then the media was discarded and the cells were dissolved with 100 μl of 10% SDS-0.01N HCl at 37°C for overnight. The absorbance of the wells was determined at 570 nm with a microtiter plate reader (Sunrise-Basic Tecan, Switzerland).

Statistical analysis

All data were presented as values of mean ± SEM of at least three individual experiments. RT-PCR results were quantified by measuring the band intensity of the PCR products using a densitometer. The expression levels of Cygb was normalized to those of β-actin or GAPDH and compared to controls. Statistical analysis was performed using Student's unpaired *t*-test at a confidence interval of 95% (i.e. $P \leq 0.05$)

Results

Cygb was selectively up-regulated by H_2O_2-treatment

N2a neuroblastoma cells were exposed to 250 μM H_2O_2 at various times and the expression of Cygb and Ngb was measured by semi-quantitative RT-PCR. The results showed that the expression levels of Cygb evidently increased after 3, 6, 12 and 24 h of H_2O_2-treatment (Fig. 1-A). Statistical analysis demonstrated that the expression levels of Cygb increased 2–4 folds upon H_2O_2-treatment (Fig. 1-B). Under similar conditions, however, the expression level of Ngb gene was not altered evidently (Fig. 1-A).

To test whether the up-regulation of Cygb mRNA is specific to H_2O_2, N2a cells were exposed to hypoxia ($5\%O_2$) and kainic acid (300 μM). The expression levels of Cygb gene were not altered at various time points (1, 3, 6 and 9 h) although the morphological change of cells upon hypoxia was evident (Fig. 1-C). Similar results were obtained by treating N2a cells with high extracellular Ca^{2+} concentration (5.4 mM $CaCl_2$), high osmolarity (0.3 M NaCl) and heat shock (42°C, 1 h) (data not shown). Exposing N2a cells to short-wavelength UV irradiation (40 J/m^2) for 0.5, 1 and 2 h decreased the expression levels of Cygb significantly (Fig. 1-D). These results together supported that the Cygb was up-regulated by H_2O_2-treatment.

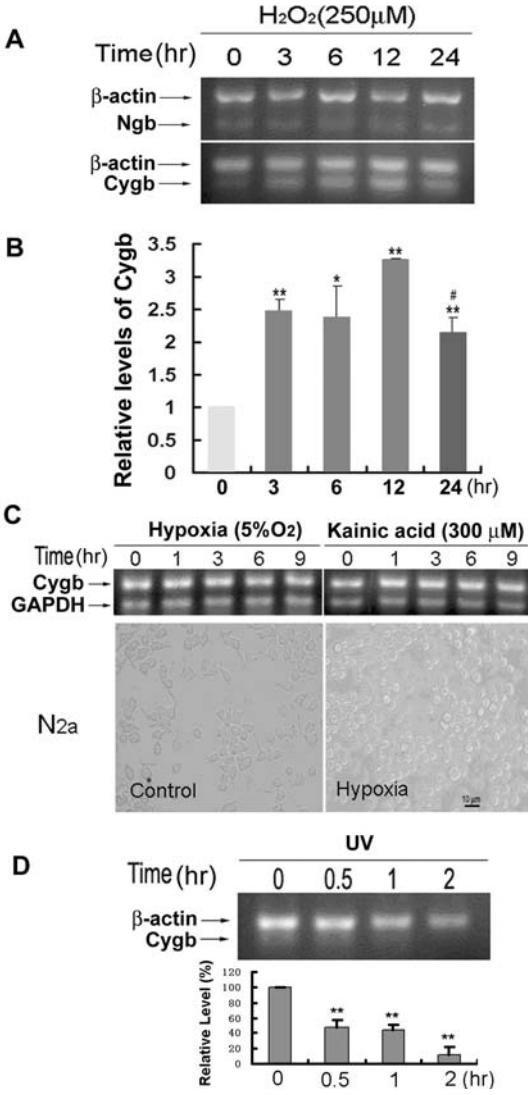

Fig. 1 Selective up-regulation of Cygb by H_2O_2. (**A**) N2a cells were treated with 250 μM H_2O_2 for 3, 6, 12 and 24 h. Representative RT-PCR result showed that Cygb, not Ngb, increased evidently. β-actin was amplified simultaneously as internal control. (**B**) statistical analysis demonstrated that the relative expression levels of Cygb (Cygb/actin) were significantly increased upon H_2O_2-treatment. Data represents the mean±SEM from at least three independent results; **P*<0.05 and ***P*<0.01 vs. 0 h, #*P*<0.05 vs. 12 h. (**C**) upper panel showed the expression of Cygb exposed to hypoxia (5% O_2) and 300 μM kainic acid; lower panel showed the morphological changes of N2a cells upon 9 h of hypoxia. (**D**), representative RT-PCR result showed that the expression levels of Cygb decreased upon UV irradiation. Statical analysis demonstrated that Cygb significantly decreased upon UV irradiation, data represent the mean±SEM from at least three independent results, ***P*<0.01 vs. 0 h

Cygb-siRNA exacerbated cell death under oxidative stress

To explore the biological function of cytoglobin, three Cygb-siRNA expression vectors were constructed. The Cygb-siRNA vectors were co-transfected with Cygb-Red vectors into N2a cells to verify their inhibitory efficiency. Only the Cygb-siRNA vector targeting to GCAGTACTTCAGCCAGTTTAG of Cygb cDNA effectively inhibited the expression of Cygb-Red (Fig. 2-A). This effective Cygb-siRNA vector was then transfected into N2a cells to establish stable Cygb-siRNA-expressing cell line (N2a/Cygb-siRNA) by neomysin screening. Neomycin is an antibiotic used for selecting cells expressing plasmids, which are neomycin resistant. Results of RT-PCR (Fig. 2-B) showed that the expression level of Cygb in N2a/Cygb-siRNA (lane 3) decreased 73.1% as compared to that of

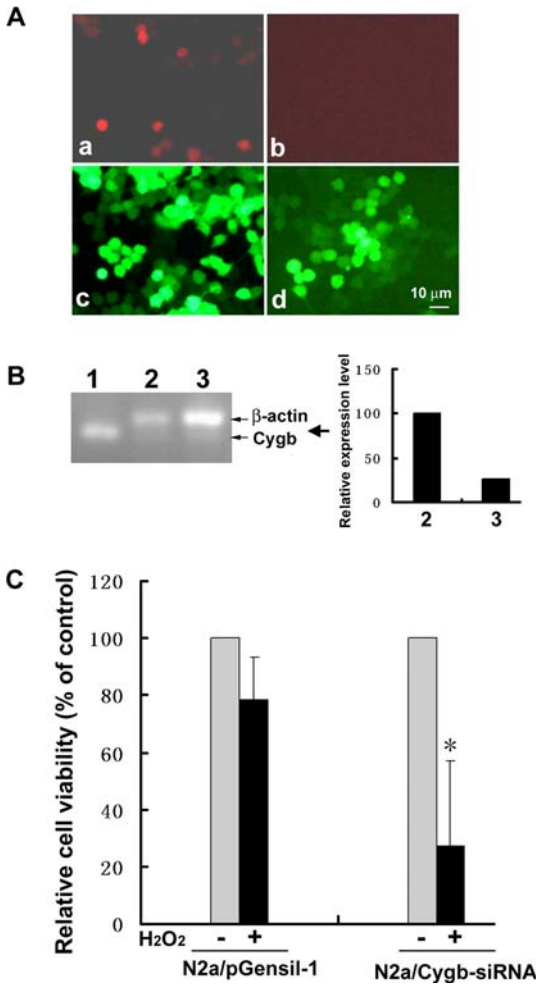

Fig. 2 Inhibition of Cygb by siRNA exacerbates cell death. (**A**) N2a cells were co-transfected with Cygb-Red (red, a and b) and Cygb-siRNA (green, d) or pGenesil-1 control vector (green, c) at a ratio of 1:4. Expression of Cygb-Red (b) was inhibited in Cygb-siRNA co-transfected cells. (**B**) result of RT-PCR showed that the relative expression level of Cygb (Cygb/actin) in N2a/Cygb-siRNA cells (lane 3) decreased as compared to that of N2a/pGenesil-1 control cells (lane 2). Lane 1 was the PCR positive control using Cygb-Red plasmids as templates. (**C**) MTT assay demonstrated that the cell viability of N2a / Cygb-siRNA cells significantly decreased as compared to that of N2a/ pGenesil-1 under 500 μM H_2O_2. Data represents the mean±SEM from four independent results, *P<0.05 vs. N2a/pGenesil-1 control NOTE: For interpretation of the references to color in this figure legend, the reader is referred to the online version of this article

N2a/pGenesil-1 (lane 2), further confirming the effectiveness of Cygb-siRNA.

The N2a/Cygb-siRNA and N2a/pGenesil-1 were exposed to 500 μM H_2O_2 for 4 h and the cytotoxicity was measured by MTT assay. Statistical analysis demonstrated that the cell viability of N2a/Cygb-siRNA cells was significantly decreased as compared to that of N2a/pGenesil-1 upon H_2O_2-treatment (Fig. 2-C). These results demonstrated that the up-regulation of Cygb in N2a cells by oxidative stress was protective.

Discussion

The discovery of new respiratory globins, namely Cygb and Ngb, in non-muscle cells is revolutionary to our knowledge in oxygen metabolism in these cells [2, 3]. The hypothesis that Cygb and Ngb might function as oxygen transporter and storage as myoglobin in muscles is a matter of debate because the amount of Cygb and Ngb in cells is much less that that of Mb in muscle cells and the hexa-coordination nature makes the dissociation of oxygen from Cygb and Ngb difficult [1–3]. Another prevailing hypothesis is that Cygb and Ngb might play critical roles in oxidative stress [8, 11, 12, 25]. We have previously reported that Ngb protected astrocytes from ischemia-induced apoptosis [25]. Here, we provide direct evidence supporting that Cygb is protective upon oxidative stress.

In this study, we systemically screened the response of Cygb upon injuries by exposing N2a cells to various kinds of insults. Previous reports mainly studied the expression of Cygb under hypoxia but the results are controversial [11–13]. Very recently, Fordel et al. [26] reported that SY-SH5Y cells under 24 h of 300 μM H_2O_2-treatment did not show any increase in Cygb protein. Unfortunately, the study was only performed at one time point and at one H_2O_2 concentration. Our results clearly demonstrated that the expression of Cygb in N2a cells was induced only by H_2O_2 in a time-dependent manner (maximal level at 12 h of treatment) but not by non-oxidative insults (i.e., kainic acid, high osmolarity, high extracellular calcium concentration, heat shock and UV irradiation) (Fig. 1). Hypoxia (5% O_2) did not alter the Cygb expression in our system (Fig. 1). Ito et al. [23] found that that only hydrogen peroxide and hypoxia induced stanniocalcin 2 gene expression while other treatments such as heat shock and UV irradiation did not. They considered H_2O_2 and hypoxia as ER stressors, which differ from other insults such as heat shock and UV irradiation. In this study, we found that only H_2O_2 induced effect suggesting that Cygb expression was sensitive to H_2O_2 but not hypoxia. This inducer might be free radicals. More recently, Hamdane et al. [6] reported that the hexa-coordination in α-Hb protects it from H_2O_2. This suggests that Cygb might be similar. To answer the question whether the specific up-regulation Cygb plays specific functional role, we utilized the Cygb-siRNA to directly harness Cygb-mRNA. This knock-down technique is specific and fundamentally different from Cygb-GFP overexpression technique used in previous studies [14, 15, 26]. Here, we demonstrated that the knock-down of Cygb expression by siRNA aggravated H_2O_2-induced cell death (Fig. 2). These results together support a critical role of Cygb in protecting cells from oxidative injury.

Oxidative stress is a fundamental pathological insult faced by all cells. Since Cygb is widely expressed in all

types of mammalian cells [2], our finding suggests that the induction of Cygb might be a general adaptive response to oxidative stress and this response is protective. This line of reasoning is in accordance with a recent study, in which over-expression of Cygb protected the hepatic stellate cells against ferric nitrilotriacetate- and arachidonic acid-induced cell death [15]. In the nervous system, oxidative stress is a critical pathological factor in causing neuronal death and is heavily involved in the pathogenesis of various neurological diseases such as neurodegenerative disorders and stroke [27, 28]. In the brain, Cygb is highly expressed in oxidative-responsive regions such as hippocampus [8], which are also vulnerable regions in neurological diseases. The finding that Cygb is inducible and protective under oxidative stress indicates that Cygb might be implicated in the pathogenesis of neurological diseases and injuries, such as stroke and Alzheimer's disease.

How Cygb is up-regulated by oxidative stress and exerts its protective role is unknown. Analysis of the transcription factor-binding sequences shows that many putative transcription factor binding sites such as c-Ets-1, Sp1 motif and hypoxia response element (HRE) exist in the cytoglobin promoter region, suggesting a complicated regulatory machinery of Cygb [29, 30]. Fordel et al. [12] reported that the expression of Cygb was affected in hypoxia inducible factor (HIF)-knockout mice, supporting an important role of HIF in Cygb expression. Whether the up-regulation of cytoglobin by H_2O_2-treatment is regulated through HIF-pathway or not deserves further study.

Taken together, this study first demonstrated that Cygb was specifically up-regulated by H_2O_2 and the knock-down of Cygb gene expression by siRNA has demonstrated the protective role of Cygb under oxidative stress. Our data implied that Cygb might play a critical role in neurological diseases in which oxidative stress plays a role in their pathogenesis.

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