Cellular protection from oxidative DNA damage by over-expression of the novel globin cytoglobin in vitro

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Cytoglobin is a recently identified member of the mammalian globin family that is expressed in neuronal cells in the central and peripheral nervous system where its physiological role remains to be determined. In the current study, we demonstrate that a cytoglobin–green fluorescent protein (GFP) fusion protein when expressed in the human neuronal cell line TE671 has a nuclear localization in a subpopulation of transfected cells (~15%). Furthermore, the cytoglobin–GFP fusion protein but not GFP alone significantly reduced the induction of intracellular reactive oxygen species as assessed by oxidation of the redox-sensitive probe dichlorofluorescein following treatment with non-cytotoxic concentrations of the pro-oxidant Ro19-8022. In addition, expression of cytoglobin–GFP also afforded cytoprotection from Ro19-8022-induced oxidative DNA damage as assessed by the Fpg-modified comet assay. In conclusion, the current study provides evidence supportive of a role for cytoglobin in cytoprotection of neuronal cells from oxidative-related damage, for example, during ischaemic reperfusion injury following hypoxia.

Introduction

Haemoglobin and myoglobin are among the best studied and understood of all proteins. Recently, cytoglobin and neuroglobin, two new members of the mammalian globin family have been identified (1–3). Since their identification, the physiological role of these two new members of the globin family has remained elusive and is the focus of considerable debate [see (4–6) for recent discussions]. Neuroglobin is a monomeric globin that can reversibly bind to oxygen (7,8), is expressed in the neurons of the central and peripheral nervous system and it is also found at particularly high concentrations in the retina where a role in cellular respiration has been suggested (9,10). Neuroglobin has also been reported to be up-regulated following hypoxia (11–14) and appears to offer cytoprotection from ischaemic reperfusion injury (15–20) potentially through detoxification of reactive oxygen species (ROS) and nitrogen species (21,22).

In contrast, cytoglobin was initially identified as stellate cell activation-associated protein during a proteomic screen of differentially expressed transcripts in fibrotic rodent liver where it was found to be expressed specifically in hepatic stellate cells (23). Subsequently, cytoglobin has been found to be expressed widely throughout many tissues including heart, liver, lung, spleen, stomach and muscle where expression is limited to cells of a fibroblast origin and appears to be cytoplasmic in location (24–26). Although it was initially assumed that cytoglobin functioned in an analogous manner to myoglobin facilitating intracellular transportation of oxygen to the mitochondria, the fact that its expression is limited mainly to cells of a fibroblast lineage and that it is expressed at relatively low levels do not substantiate this hypothesis. Rather a role in the fibrotic process, where it may function as an oxygen donor during the synthesis and cross-linking of collagen has been suggested (26). A role for cytoglobin in tissue fibrosis is supported by a number of more recent studies where, for example, it has been demonstrated that over-expression of cytoglobin affords protection from chemically induced liver fibrosis (27) suggesting a role for cytoglobin in the cellular response to fibrosis-inducing agents. Very recently, a response element for the transcription factor c-ets1 [known to regulate the expression of other genes associated with fibrosis (28–30)] has been identified in the cytoglobin promoter (31).

Interestingly, cytoglobin is also expressed in a subpopulation of neurons in the central nervous system (26,32) where, in contrast to fibroblasts, expression appears to be at least partially localized to the nucleus. This and the fact that there is no evidence that collagen is produced in the brain suggest a distinct neuron-specific function of cytoglobin. There is evidence that cytoglobin expression can be up-regulated by hypoxia both in neuronal cells in vitro and in the brain in vivo (11,24) suggesting that like neuroglobin, cytoglobin has the potential to protect certain populations of neurons from the cellular effects of oxidative stress following ischaemic perfusion. Induction of 8-oxo deoxyguanine (8-oxodG) in DNA is a primary event in cells exposed to ROS and has the potential to cause cell death either via necrosis or initiation of apoptosis that may contribute to brain injury. In the current study, we used the photosensitizer Ro19-8022 [a synthetic source of reactive oxygen (33)] to investigate the hypothesis that over-expression of cytoglobin in vitro affords cytoprotection from Ro19-8022-induced oxidative DNA damage.

Materials and methods

Cell culture

The TE671 neuron-derived human medulloblastoma cell line (ECACC 89071904) was a generous gift from Dr R. Waring (School of Biosciences, The University of Birmingham, UK) and cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal bovine serum, 2 mM t-glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged twice weekly using a standard trypsin–EDTA protocol.

Vectors and transfection of cells

The pEGFP-N1 vector containing the full-length cytoglobin cDNA fused to GFP cDNA was a gift from Thorsten Burmester (The University of Mainz) and has been described previously (26); as a control the parent EGFP-N1 vector (Clontech Europe, 2 Avenue de President Kennedy, St Germain-en-Laye, France) was also used. Vectors were propagated by transformation into...
Escherichia coli (JM109, Promega, Delta House, Southampton Science Park, Southampton, UK) and selection with kanamycin antibiotic (30 μg/ml). Plasmid DNA was purified using a Qiagen plasmid miniprep kit. The day before transfection, TE671 cells were plated in 35-mm culture dishes (NUNC International, 75 Panorama Creek Drive, Rochester, NY, USA), so that the density on the day of transfection was approximately 70–80%. Cells were transfected using 1 μg DNA and 3 μl of GeneJuice transfection reagent according to the manufacturer’s instructions (Novagen, 441 Charnandy Drive, Madison, WI, USA). Cells were then cultured for 24 h prior to subsequent analysis. Following transfection, cells were examined by fluorescence microscopy (Zeiss Axiovert 135TV, ×20 objective lens) fitted with a 450–490-nm excitation filter and a barrier filter of 570 nm. A digital video camera (Kinetic Imaging TM-76) was used to record and capture the images in JPEG format (overall magnification was ×200). Images of approximately one hundred randomly selected cells were analysed per field. The observed transfection was highly efficient and we estimate that greater than 60% of cells expressed detectable levels of cytoglobin–GFP after 24 h of incubation.

**Treatment with Ro19-8022**

For treatment with Ro19-8022 (a gift from Dr Andrew Collins, now at The University of Oslo) confluent cells were incubated with media containing Ro19-8022 (0–50 μM) in the dark at 37°C for 1 h. Cultures were then placed in an ice-cold water bath (for cooling) and illuminated for 10 min using a 250 W halogen lamp at a distance of 19 cm. This illumination corresponds to 111 kJ/m² between 400 and 800 nm which is equivalent to 9.3 kJ/m² between 400 and 500 nm and results in photoactivation of Ro19-8022. We have previously demonstrated that this treatment results in oxidative DNA damage in a range of cells [e.g. (34)]. All treatments and subsequent analyses described below were carried out in six-well culture plates (Nunc) unless stated otherwise.

**Cytotoxicity (MTT and ATP) assays**

The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine mitochondrial function. Cells were grown to 90% confluency in a 96-well culture plate (Corning, 900 Celsnord Street, Lowell, MA, USA). Following treatment, cells were washed with phosphate-buffered saline (PBS), mock incubated with MTT (0.45 mg/ml) containing growth media and subsequently incubated at 37°C for 3 h. Media were aspirated before the addition of dimethyl sulfoxide (DMSO) to solubilize the blue formazan product. Culture plates were gently rocked for 1 h before the absorbance was determined at 530 ± 40 nm against a DMSO blank. Intracellular ATP concentration was measured using an assay kit according to the manufacturer’s instructions (Sigma Aldrich, Fancy Road, Poole, Dorset UK). Following treatment, the media were removed and cells washed with 3 ml PBS. Cells were scraped into 500 μl somatic cell ATP-releasing buffer and 150 μl added to a well of a 96-well plate. Distilled water (50 μl) was then added followed by 100 μl of the assay mix (1:25 dilution in buffer). The luminescence was then measured (Tecan, Theale Court, Cambridge House, Cambridge, UK) and normalized to protein content.

**Protein preparation and western blotting**

Cells were cultured to confluence in 60-mm plates and scraped into 100 μl RIPA buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na2EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS)] containing 10 μM mammalian protease inhibitor cocktail (Sigma). The suspension was incubated on ice for 30 min and mixed by vortexing every 10 min. Samples were then centrifuged at 13 000 r.p.m. for 10 min at 4°C and the supernatant retained for analysis.

Protein extracts (30 μg) were resolved on a 12.5% SDS–polyacrylamide gel, transferred to nitrocellulose and blocked overnight at 4°C with a blocking solution (3% bovine serum albumin, 10 mM DMSO and 1% Triton X-100). Following 3 × 10 min washes (3×10 min, TBS–0.05% Tween 20) the membranes were incubated with horseradish peroxidase-conjugated mouse and rabbit secondary antibodies (1:750 dilution, DAKO Ltd, Cambridge House, Cambridge, UK) in blocking buffer for 1 h at room temperature and washed (as above). Bands were detected using photographic film (Hyperfilm, ECL) and enhanced chemiluminescence detection (EZ-ECL, Geneflow Ltd, Bradly Business Centre, Bradley, Staffordshire, UK).

**Alkaline comet assay**

Following the appropriate treatment, cells were washed in cold PBS and gently scraped into fresh PBS (1 ml). Cells were centrifuged (200 × g, 5 min) and pellets re-suspended in PBS (150 μl). An aliquot of re-suspended cells (15 μl) was placed into a sterile tube containing low melting point agarose (300 μl) and this cell suspension transferred to two parallel glass microscope slide (150 μl per slide, MERCK, Hoddesdon, Hertfordshire, UK), pre-coated with 0.5% normal melting point agarose. Glass cover slips were added and slides placed on a metal tray over ice for 10 min. Cover slips were removed and slides incubated for 1 h at 4°C in lysis buffer (2.5 M NaCl, 0.1 M Na2EDTA, 10 mM Tris base, 1% sodium N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100). Following lysis, slides were placed in a moist three times in cold formamidopropylamine, DNA glycosylase (Fpg) enzyme buffer (40 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.1 M KCl, 0.5 mM Na2EDTA and 0.2 mg/ml bovine serum albumin). Fpg (1 U) in Fpg enzyme solution was subsequently added to the appropriate slides and glass cover slips added. In addition, a slide treated with buffer alone (50 μl) was prepared as a negative control. Slides were then placed in a moist three times in cold formamidopropylamine, DNA glycosylase (Fpg) enzyme buffer (40 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 0.1 M KCl, 0.5 mM Na2EDTA and 0.2 mg/ml bovine serum albumin). Fpg (1 U) in Fpg enzyme solution was subsequently added to the appropriate slides and glass cover slips added. In addition, a slide treated with buffer alone (50 μl) was prepared as a negative control. Slides were then washed (3×10 min) with 100 μl of lysis solution (0.4 M Tris, adjusted to pH 7.5). Slides were subsequently stained with 50 μl of 10× Sybr Gold (Invitrogen Ltd, 3 Fountain Drive, Paisley, UK). The slides were examined at ×320 magnification (×320/0.40 dry objective) using a fluorescence microscope (Zeiss Axiovit 10, Germany), fitted with a 515–560-nm excitation filter and a barrier filter of 590 nm. A USB digital camera (Merlin Allied Vision Technologies, Klein-Groeth Street, Ahrensburg, Germany) received the images, which were analysed using a personal computer-based image analysis system (Comet Assay IV, Perceptive Instruments). Images of one hundred randomly selected nuclei were analysed per slide.

Measurement of per cent tail DNA was chosen to assess the extent of DNA damage in this assay because it has been shown to suffer much less from inter-run variation than other comet parameters because it is independent of electrophoresis voltage and run time (35). Median values of three separate experiments were analysed using analysis of variance and post hoc Student’s t-test, as recommended by Duez et al. (36).

**Assessment of lipid peroxidation**

Lipid peroxidation was assessed by measuring loss of cis-parinaric acid (PNA) fluorescence as described previously (37). Briefly, cells were incubated with 10 μM PNA (Molecular Probes, Invitrogen Ltd) at 37°C for 30 min in the dark. The media were then removed and cells washed three times with 3 ml warm PBS to remove unincorporated dye. Cells were then treated with Ro19-8022 as described above prior to analysis. After treatment, cells were scraped into 2 ml PBS using a rubber policeman. The suspension was added to a fluorescence cuvette and the emission fluorescence of wavelength 455 nm (slit width 5 nm) measured using an excitation wavelength of 312 nm (slit width 5 nm). A blank (unlabelled cells) was measured and subtracted from all readings.

**Assessment of reactive oxygen**

Intracellular ROS were measured by measuring intracellular oxidation of dichlorofluorescein described by Carini et al. (37). Briefly, cells were incubated with 10 μM dichlorofluorescein diacetate (Molecular Probes) at 37°C for 30 min in the dark. The media were then removed and cells washed three times with 3 ml warm PBS to remove unincorporated dye. Cells were then treated as appropriate prior to analysis. After treatment, cells were scraped into 2 ml PBS using a rubber policeman. The suspension was added to a fluorescence cuvette and the emission fluorescence of wavelength 520 nm (slit width 5 nm) measured using excitation wavelength of 495 nm (slit width 5 nm) measured using excitation wavelength of 495 nm (slit width 5 nm). A blank (unlabelled cells) was measured and subtracted from all readings.

**Results**

**Assessment of parameters related to oxidative stress**

Levels of dichlorofluorescein fluorescence in untransfected and transfected control cells were 57.3 ± 1.5 and 28.6 ± 8.8 fluorescence units per milligram of total protein, respectively. Treatment with Ro19-8022 (0–50 μM) resulted in a concentration-dependent increase in levels of ROS as assessed by dichlorofluorescein fluorescence (Figure 1A). Interestingly, the level of ROS was statistically reduced in cytoglobin-transfected cells compared to untransfected cells at all the Ro19-8022 concentrations investigated (Figure 1A). Parallel treatments showed no evidence for lipid peroxidation in either control or transfected cells following treatment with Ro19-8022.
Fig. 1. Effect of expression of cytoglobin–GFP on levels of ROS (A) as assessed by dichlorodihydrofluorescein fluorescence and lipid peroxidation (B) as assessed by loss of PNA fluorescence in Te671 cells treated with Ro19-8022 (0–50 μM) + light. The results represent the mean of three experiments carried out in duplicate ± standard deviation (n = 3). ## and # significantly different from untransfected cells (P < 0.05 and P < 0.01, respectively). Open bars represent untransfected controls and closed bars cytoglobin–GFP-transfected cells.

Fig. 2. Effect of expression of cytoglobin-GFP on levels of frank strand breaks (A) and FPG-dependent strand breaks (B) in Te671 cells treated with Ro19-8022 (0–50 μM) + light as assessed by the comet assay. The results represent the mean of three experiments carried out in duplicate ± standard deviation (n = 3). * and ** significantly different from untreated controls (P < 0.05 and 0.01, respectively). ## significantly different from untransfected cells (P < 0.01). Open bars represent untransfected controls and closed bars cytoglobin–GFP-transfected cells.

Cytoglobin affords protection from oxidant-induced DNA strand breaks

Treatment of cells with Ro19-8022 (0–50 μM) resulted in a concentration-dependent increase in frank (single-stranded DNA breaks and alkali-labile sites) but there was no statistically significant difference between transfected and untransfected cells at any of the concentrations investigated (Figure 2A). Treatment with Ro19-8022 also resulted in a statistically significant increase in levels of DNA strand breaks as assessed by the Fpg-modified comet assay (Figure 2B). However, interestingly levels of DNA damage in transfected cells were significantly reduced (P < 0.01) with a 2.6- and 2.2-fold reduction in strand breaks compared to untransfected cells following treatment with 25 and 50 μM Ro19-8022, respectively (Figure 2B). In contrast, levels of DNA strand breaks in cells transfected with GFP alone were not significantly different from untransfected cells (data not shown). We wanted to confirm that the changes in DNA damage observed in the median values following treatment represented a shift in the distribution of the entire population of cells rather than the result of the existence of a small subpopulation of highly sensitive cells. A representative population plot (100 cells per treatment) is shown in Figure 3 and confirms an overall shift in the median damage value following Ro19-8022 treatment as assessed by the Fpg-modified comet assay. In addition, this population plot also clearly demonstrates that the cytoprotection afforded by cytoglobin is also a generalized population effect rather than the existence of a subpopulation of protected cells.

Partial nuclear localization of cytoglobin

Levels of endogenous cytoglobin expression were below the levels of detection as assessed by reverse transcription–polymerase chain reaction and western blotting (data not shown).
Interestingly, transfection of cells with the cytoglobin–GFP fusion protein (Figure 4A) resulted in fluorescence with an apparent partial nuclear localization in a subpopulation of cells (15%, see Figure 4B). In comparison, transfection of cells with GFP vector alone resulted in a cytoplasmic distribution of green fluorescence (Figure 4B). Similar results were obtained in another human neuronal cell line (SKNSH, data not shown). In contrast, we observed no evidence of nuclear localization of the cytoglobin–GFP fusion protein when it was expressed in a range of non-neuronal cell lines derived from liver (HepG2, MCP13), lung (A549), cervix (HeLa) and mouse embryonic fibroblasts (data not shown).

Discussion

Since its initial discovery by Kawada et al. (23) in a proteomic screen of rodent liver, a definitive physiological role of cytoglobin has remained difficult to identify and is the focus of active debate. Although it now seems most likely that the role of cytoglobin in cells of a fibroblast lineage is related to fibrosis and extracellular matrix (e.g. collagen) deposition, the function of cytoglobin expressed in cells of the central nervous system remains underdetermined.

Various physiological functions for neuroglobin and cytoglobin have been hypothesized including the following: intracellular oxygen transportation, detoxification of ROS and nitrogen species, oxygen sensing and interaction with cell-signalling pathways. For example, yeast two-hybrid studies have identified several potential protein partners for neuroglobin including flotillin-1 (38), the Na\(^+/K^+\) ATPase β2 subunit (39) and cystatin c (40). In addition, Wakasugi et al. (41) have suggested that oxidized neuroglobin may function as an inhibitor of G\(\alpha\) protein guanine nucleotide dissociation inhibition factor inhibitor. In our laboratory, we have failed to identify protein interactors with cytoglobin using either yeast two-hybrid or immunoprecipitation/mass spectrometry studies (Hodges et al., unpublished data). Therefore, it seems unlikely that cytoglobin is involved in interacting with cell-signalling pathways.

Important cellular end points of oxidative stress following ischaemic reperfusion are lipid peroxidation and oxidative damage to DNA [e.g. 8-oxodG, reviewed by Warner et al. (42)] which can result in cellular necrosis and/or induction of apoptosis contributing to brain injury. Singlet molecular oxygen (\(^1\)O\(_2\)) is a direct derivative of molecular oxygen that is a by-product of membrane lipid peroxidation and is also formed as a product of the Haber–Weiss reaction between hydrogen peroxide and the superoxide radical (43). Singlet molecular oxygen is highly reactive; can oxidize many organic cellular molecules, e.g. lipids, proteins, DNA, carbohydrates and thiols (43) and has been implicated in ischaemic reperfusion injury in both the heart and the brain (44–50). In relation to this, another possible physiological role for cytoglobin is protection of neuronal cells from ischaemic reperfusion injury. Interestingly, a recent study (51) reported that cytoglobin expression (and to a lesser extent neuroglobin) in adult mouse brain is induced by chronic hypoxia. Furthermore, expression is in regions of the brain known to be responsive to hypoxia (e.g. hippocampus, thalamus and hypothalamus). In the current study, we have shown that a cytoglobin–GFP fusion protein affords statistically significant protection from Ro19-8022 (singlet oxygen generator)-induced oxidative DNA damage as assessed by the Fpg-modified comet assay. Although the transfection efficiency of this cell line was high (>60%), we acknowledge that some of the cells assessed in the comet assay will not be expressing cytoglobin–GFP. However, this will result in a conservative estimate of the degree of protection afforded because a proportion of cells scored will not be expressing cytoglobin–GFP and therefore will not be protected from Ro19-8022-induced oxidative...
damage. Overall, these data are consistent with a possible role for cytoglobin in cellular protection from pro-oxidant-induced DNA damage.

Although the detailed mechanism remains to be determined, cytoglobin has been demonstrated to possess peroxidase activity (23) and it may also have the ability to detoxify other ROS including singlet oxygen. For this mechanism to function effectively, a cytoglobin ‘reductase’ would be required to reduce ferric cytoglobin. Although nothing is known regarding possible cytoglobin reductases, recent evidence indicates that neuroglobin has only a low affinity for cytochrome b5 (a candidate reductase) (52) and it appears unlikely that cytochrome b5 is a physiologically relevant ferric neuroglobin reductase in vivo. However, it is possible that other unidentified proteins may be able to carry out this role. Interestingly, in the same study (52), Fago et al. report that neuroglobin has a high affinity (1 μM) for cytochrome c and suggest that a possible physiological role for this interaction could be sequestration of cytochrome c released from damaged mitochondria in metabolically active cells. In contrast, initiation of apoptosis by appropriate signals would result in release of high levels of cytochrome c that would overwhelm this protective mechanism. These observations provide another potential mechanism by which neuroglobin (and potentially cytoglobin) could offer cytoprotection during oxidative stress.

Interestingly, in the current study, we also observed that in a significant subpopulation of transfected cells (~15%), there was evidence for a nuclear localization of the cytoglobin–GFP transgene product. This was specific to cell lines derived from the central nervous system and not observed in a range of other cell lines investigated. Despite early reports to the contrary (53), a nuclear localization of cytoglobin appears to be specific to cells of a neuronal origin (26,32). Nuclear localization of cytoglobin is in concordance with the hypothesis that cytoglobin plays a role in the protection of genomic DNA from oxidant-induced oxidative DNA damage during ischaemic reperfusion injury. However, cytoglobin contains no known nuclear localization signals and the mechanism of nuclear transport remains to be determined. Our observation of nuclear localization in a subset of transfected cells suggests that nuclear localization is a response to an environmental signal and that nuclear localization represents a homeostatic response. Although further studies are required to determine the nature of this signal, we hypothesize that the signal is intracellular oxidative stress and that nuclear translocation of cytoglobin represents an adaptive response to this physiological condition.

In summary, we provide experimental evidence for a role of cytoglobin in protection against pro-oxidant-induced oxidative DNA damage; this mechanism may afford cellular protection of neuronal cells during ischaemic reperfusion injury.

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N. J. Hodges et al.


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