Cellular accumulation of Cys326-OGG1 protein complexes under conditions of oxidative stress

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ABSTRACT

The common Ser326Cys polymorphism in the base excision repair protein 8-oxoguanine glycosylase 1 is associated with a reduced capacity to repair oxidative DNA damage particularly under conditions of intracellular oxidative stress and there is evidence that Cys326-OGG1 homozygous individuals have increased susceptibility to specific cancer types. Indirect biochemical studies have shown that reduced repair capacity is related to OGG1 redox modification and also possibly OGG1 dimer formation. In the current study we have used bimolecular fluorescence complementation to study for the first time a component of the base excision repair pathway and applied it to visualise accumulation of Cys326-OGG1 protein complexes in the native cellular environment. Fluorescence was observed both within and around the cell nucleus, was shown to be specific to cells expressing Cys326-OGG1 and only occurred in cells under conditions of cellular oxidative stress following depletion of intracellular glutathione levels by treatment with buthionine sulphoximine. Furthermore, OGG1 complex formation was inhibited by incubation of cells with the thiol reducing agents β-mercaptoethanol and dithiothreitol and the antioxidant dimethylsulfoxide indicating a causative role for oxidative stress in the formation of OGG1 cellular complexes.

In conclusion, this study has provided for the first time evidence of redox sensitive Cys326-OGG1 protein accumulation in cells under conditions of intracellular oxidative stress that may be related to the previously reported reduced repair capacity of Cys326-OGG1 specifically under conditions of oxidative stress.

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1. Introduction

Reactive oxygen species (ROS) are ubiquitous in the intracellular environment and are generated by numerous endogenous processes including, for example the mitochondrial electron transport chain, reviewed by Murphy [1]. In addition, exposure to both physical (e.g. ionising radiation) [2] and chemical agents (e.g. toxic metals) [3] can further increase ROS formation inside cells. DNA is susceptible to a range of oxidative modifications including oxidation of guanine to 7,8-dihydro-8-guanine (8-oxo G), which, if not repaired has the capacity to mis-pair during DNA replication resulting in the formation of GC to TA transversion mutations [4,5] and oxidative modifications to DNA are considered to contribute to the aetiology of a range of human pathological conditions including cancer [6–8]. The major pathway for the repair of 8-oxo G in genomic DNA is base excision repair (BER), which is initiated by the DNA glycosylase 8-oxoguanine glycosylase 1 (OGG1). The

human OGG1 (*hOGG1*) gene is located on chromosome 3p26.2 [9], a region subject to deletion and loss of heterozygosity in human cancers. Furthermore, OGG1 knockout mice have been shown to accumulate elevated levels of 8-oxo G as they age and are more susceptible to a range of chemical stressors [10–14]. hOGG1 is polymorphic in the human population with a relatively common single nucleotide polymorphism – allele frequency of 0.22–0.27 in Caucasian populations – occurring at position 1245 in exon 7 resulting in a serine to cysteine amino acid substitution at position 326 (Ser326Cys) [15–17]. There is also evidence that post-translational modification to hOGG1 including: nitrosylation [18], phosphorylation [19–22], acetylation [23], ubiquitination [24] and redox modifications [25,26] modulate both the cellular localisation of hOGG1 and its catalytic activity and mechanistic studies focused on the Ser326Cys polymorphism indicate that the Cys326 variant is repair deficient, that this deficiency is enhanced under conditions of cellular oxidative stress [27] and that reduced repair activity may be related to oxidative modification of the Cys326 amino acid which resides in a positively charged sequence of amino acids (AD-LRQ[ser326cys]RHAQ) rendering the thiol group labile to oxidation [28]. As part of these data, work by Hill and Evans [29] has

demonstrated that purified Cys326 hOGG1 exists predominantly as a homo-dimer dependent on the Cys326 amino acid. Despite this, there is little if any direct evidence of OGG1 complex formation in cells and specifically how this is related to OGG1-genotype and to the cellular redox environment. Here we report the novel application of the imaging technique bimolecular fluorescence complementation (BiFC) to study OGG1 complex formation for the first time in the native cellular environment and show that this only occurs with the Cys326 variant of the protein and is specific to conditions of cellular oxidative stress.

2. Materials and methods

2.1. Preparation of BiFC vectors

Plasmids (pBluescript II KS-) containing either the N- or C-terminus of yellow fluorescent protein (YFP-N and YFP-C) were a generous gift from Dr. Saverio Brogna (University of Birmingham, B15 2TT). Full length Ser326- and Cys326-hOGG1 cDNA was amplified from *hOGG1* containing pcDNA3© plasmids [30] by PCR (Forward primer: 5′-GAGAGGATCCATGCCTGCCCGCGCGCTTCTG-3′ Reverse primer: 5′-GGCAGGATCCTTACTAGCCTTCCGGCCCTTTG-3′). Following overnight digestion with *BamHI* hOGG1 cDNA fragments were ligated into the pBluescript II vector in frame with YFP-N or YFP-C. Next hOGG1-YFP cDNA constructs were sub-cloned into the pcDNA3.1/Hygro© (+) mammalian expression vector (Invitrogen, USA) to generate: YFP-N-Ser326-hOGG1, YFP-C-Ser326-hOGG1, YFP-N-Cys326-hOGG1 and YFP-C-Cys326-hOGG1 constructs. Vectors containing only the YFP fragments were prepared in the same manner. All vectors were confirmed by sequencing and plasmids

were propagated using standard protocols in super competent JM109 *Escherichia coli* (Promega, UK) with antibiotic selection (ampicillin 100 μg/ml). Plasmids were recovered from bacteria using a Qiagen plasmid mini-prep kit according to manufacturer's instructions.

2.2. Cell culture

A549 lung carcinoma cells (HPA catalogue number 86012804) were cultured at 37 °C in T_{75} flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% v/v foetal calf serum, glutamine (2 mM), penicillin (100 μg/ml) and streptomycin (100 μg/ml). Cells were grown to approximately 80% confluence and passaged using trypsin–EDTA either into new T_{75} flasks or well culture plates for experimentation as appropriate.

2.3. Assessment of oxidative stress and GSH levels

Cells were cultured in 6-well plates to confluence before treatment with buthionine sulphoximine (BSO, 1000 μM) for 24 h. Following treatment dichlorodihydrofluoroscein-diacetate (H_2DCF-DA, final concentration 10 μM) was added and cells incubated at 37 °C in the dark for 60 min. Cells were washed with PBS (2×2 ml), trypsin (1 ml) added and cells incubated for 10 min at 37 °C. Next PBS (1 ml) was added and cells transferred to centrifuge tube (15 ml). Cells were pelleted by centrifugation at $1500g$ (10 min), the supernatant removed and the cell pellet re-suspended in PBS (2 ml) and transferred to flow cytometry tubes for flow cytometry analysis (FACScalibur, BD Biosciences, USA). For each treatment the FITC fluorescence (517–527 nm) of
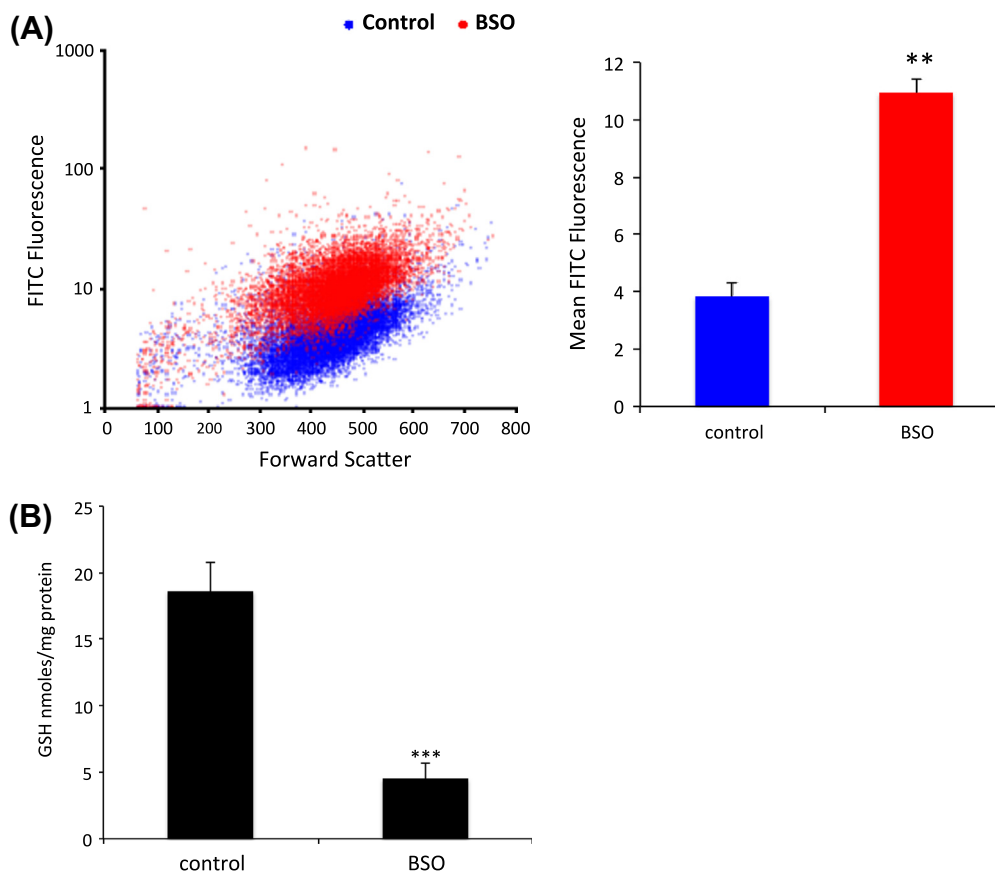
Fig. 1. (A) Induction of oxidative stress and (B) depletion of reduced glutathione (GSH) in A549 cells following treatment with BSO (1000 μM, 24 h). The results represent the mean of four independent experiments (±SD, $n = 4$) carried out in duplicate, *** and ** significantly different from controls (Student *t*-test), $P < 0.001$ and 0,01 respectively.
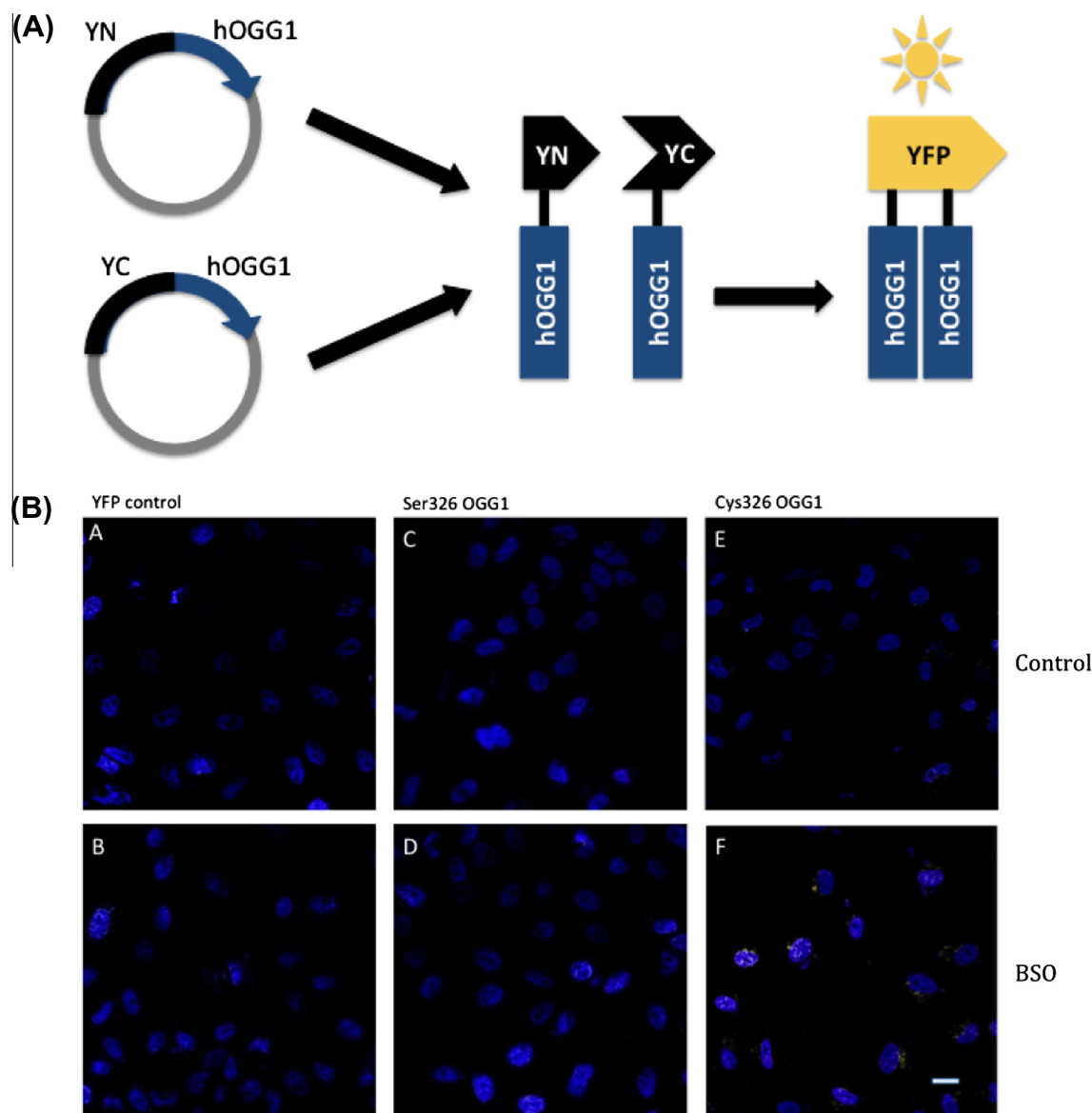
Fig. 2. (A) Principle of the BiFC assay: Cultured A549 cells were co-transfected with YC- and YN-YFP hOGG1 constructs. In the event of hOGG1 dimer formation the YC and YN fragments of YFP come into close physical proximity resulting in YFP fluorescence, which can be detected by confocal microscopy. (B) BiFC fluorescence is only observed in cells transfected with Cys326 N/C and only under conditions of oxidative cellular oxidative stress. (A) and (B) control vectors, (C) and (D) Ser326 N and C, (D) and (E) Cys326 N and C. (A), (C) and (E) untreated, (B), (D) and (E) treated with BSO (1000 μM, 24 h). Scale bar is 10 μm. In all images, nuclear DNA is counterstained with Hoechst 33258.

10,000 cells were quantified with unlabelled cells used as a blank to control for background fluorescence. Weasel software (Walter and Eliza Hall Institute of Medical Research, Australia) was used for the analysis of results and to calculate the mean fluorescence of each population of cells. Total reduced GSH was measured in cells using the method of Hissin and Hilf [31], as described previously [32].

2.4. Transfection of cells with GFP-OGG1

A549 cells were cultured on sterile round 13 mm glass coverslips (PAA Laboratories Ltd.) until approximately 65% confluent before being transfected with OGG-GFP constructs [33] using Turbofect (Thermo-Fisher Scientific Inc.) according to the manufactures instructions. 48 h later cells were fixed and analysed by confocal microscopy and flow cytometry to confirm location of OGG1-GFP and transfection efficiency respectively.

2.5. Detection of BiFC fluorescence

Cells were cultured on sterile round 13 mm glass coverslips as above. At approximately 65% confluence cells were transfected with pairs of BiFC constructs (4 μg each) described above using Turbofect according to the manufacturers instructions (Thermo-Fisher Scientific Inc.), 24 h after transfection cells were treated with BSO (1000 μM, 24 h). In some experiments the disulphide reducing agents β-mercaptoethanol (1 mM) or dithiothreitol (1 mM) were added for the final four hours of incubation. In other experiments cells were co-incubated with BSO (1000 μM) and the antioxidant DMSO (1% v/v). Following treatment the media was removed, cells washed with PBS (2 ml) and fixed at room temperature (15 min) with neutral buffered formalin (10%, pH 7.4, 2 ml). Following further washing with PBS (2 × 1 ml) cells were counterstaining with 1 ml of PBS containing Hoechst 33258(0.6 μM, Invitrogen, UK) for 5 min, washed again with PBS (2 × 1 ml) before been
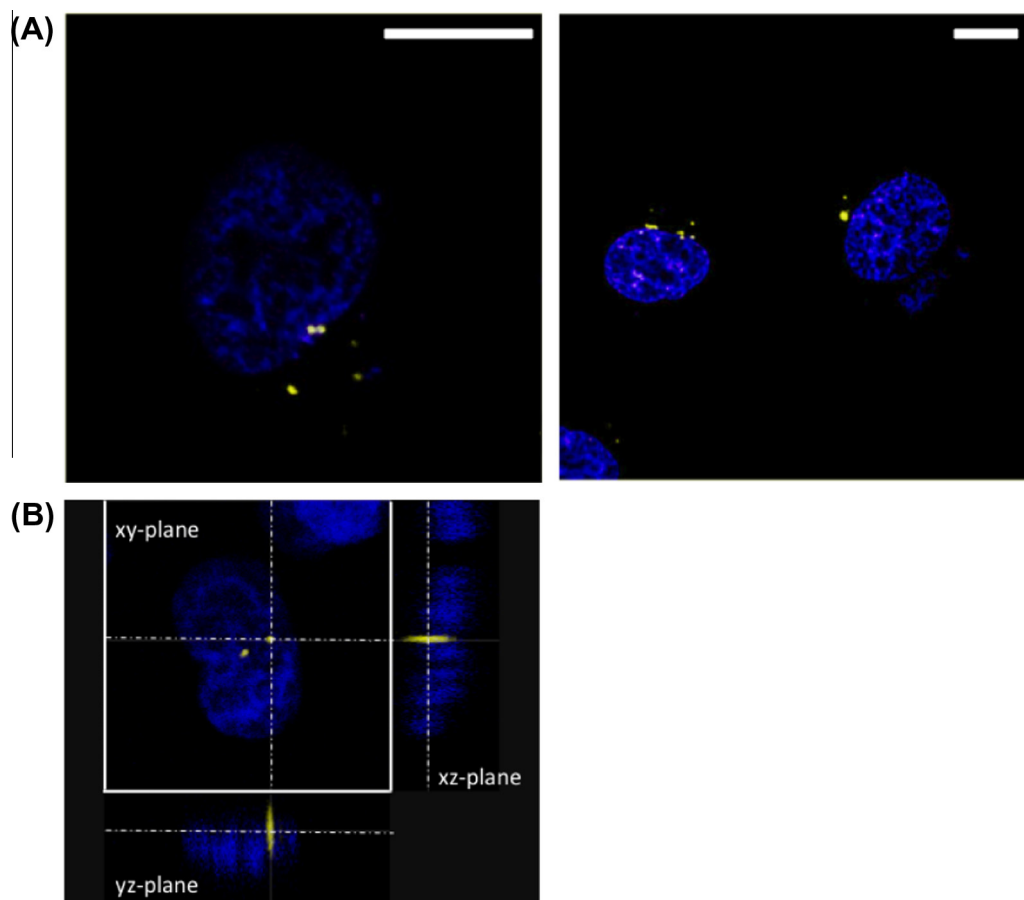
Fig. 3. (A) Higher magnification representative images of BiFC fluorescence in Cys326 N/C transfected cells following treatment with BSO (1000 μM, 24 h). Scale bar is 10 μm, nuclear DNA is counterstained with Hoechst 33258. (B) Three-dimensional distribution of yellow BiFC fluorescence in the nucleus of a cell transfected with Cys326-OGG1 vectors following treatment with BSO (1000 μM, 24 h). Nuclei are counterstained with Hoechst 33258.

mounted onto a glass microscope slide with Hydromount Vecta Mountant (approximately 50 μl, National Diagnostics, UK). Excess mountant was carefully removed by dabbing with a paper towel and slides were stored at 4 °C in the dark for a maximum period of 48 h prior to analysis by confocal microscopy. Image acquisition was performed using a Leica TCS SP2 confocal microscope (Leica Microsystems) using an oil immersion objective (63x). Fluorophores were excited using a 488 nm laser for YFP and 405 nm laser for Hoechst 33258. Images were analysed using Leicalite software and post-processing was carried out using Adobe Photoshop CS5 extended.

3. Results

3.1. Confirmation of oxidative stress

As expected and consistent with previous studies, treatment of A549 cells with BSO (1000 μM) for 24 h resulted in a statistically significant ($P < 0.01$) induction of reactive oxygen species (Fig. 1A) as assessed by the oxidation of DCF as well as depletion of glutathione (Fig. 1B) in the absence of cytotoxicity as assessed by the MTT assay (data not shown). To confirm nuclear localisation of OGG protein in this cell line, cells were transfected with GFP-tagged proteins and imaged by confocal microscopy. OGG-GFP was found to be nuclear located (Fig. S1) and consistent with previous studies in MEF cells [34], the nuclear sub-localisation of OGG1 was not influenced by OGG1 genotype and was not affected by treatment with BSO (1000 μM) for 24 h.

3.2. Oxidative stress induces BiFC fluorescence only in cells expressing Cys326-OGG1

BiFC is a powerful technique for the visualisation of direct protein–protein interactions (Fig. 2A). To investigate possible OGG1 protein complex formation, A459 cells were transfected with either Ser326-OGG1 half YFP vectors, Cys326 half YFP vectors or YFP only half vectors as a control prior to treatment with 1000 μM BSO (24 h) to induce oxidative stress and deplete intracellular GSH before BiFC fluorescence was assessed by confocal microscopy. Little to no BiFC fluorescence was observed in YFP control or Ser326-OGG1 transfected cells either before or after treatment with BSO (1000 μM, Fig. 2B panels A, B and C, D). In contrast, although there was no evidence of BiFC fluorescence in cells transfected with Cys326-OGG1 in the absence of treatment with BSO (Fig. 2B panel E) there was clear BiFC fluorescence following treatment with BSO (1000 μM, 24 h, Fig. 2B panel F). BiFC fluorescence appeared as discrete foci that were mainly located to the nucleus as mapped by counterstaining with the nuclear stain Hoescht 33258. YFP fluorescence was also apparent in peri-nuclear regions; this is shown at higher magnification in Fig. 3A. A similar pattern of fluorescence was observed when cells were treated with the pro-oxidant hydrogen peroxide (data not shown). The presence of BiFC fluorescence inside of the nucleus was confirmed by a z-scan that clearly shows yellow BiFC fluorescence staining in the same plane as the Hoechst 33258 counterstained nucleus (Fig. 3B). Co-transfection experiments with YFP-N-Cys326-hOGG1 and YFP-C-Ser326-hOGG1 or YFP-N-Ser326-hOGG1 and YFP-C-Cys326-hOGG1 constructs did

(A)

BSO

BSO + β-ME

BSO + DTT

BSO + DMSO

(B)
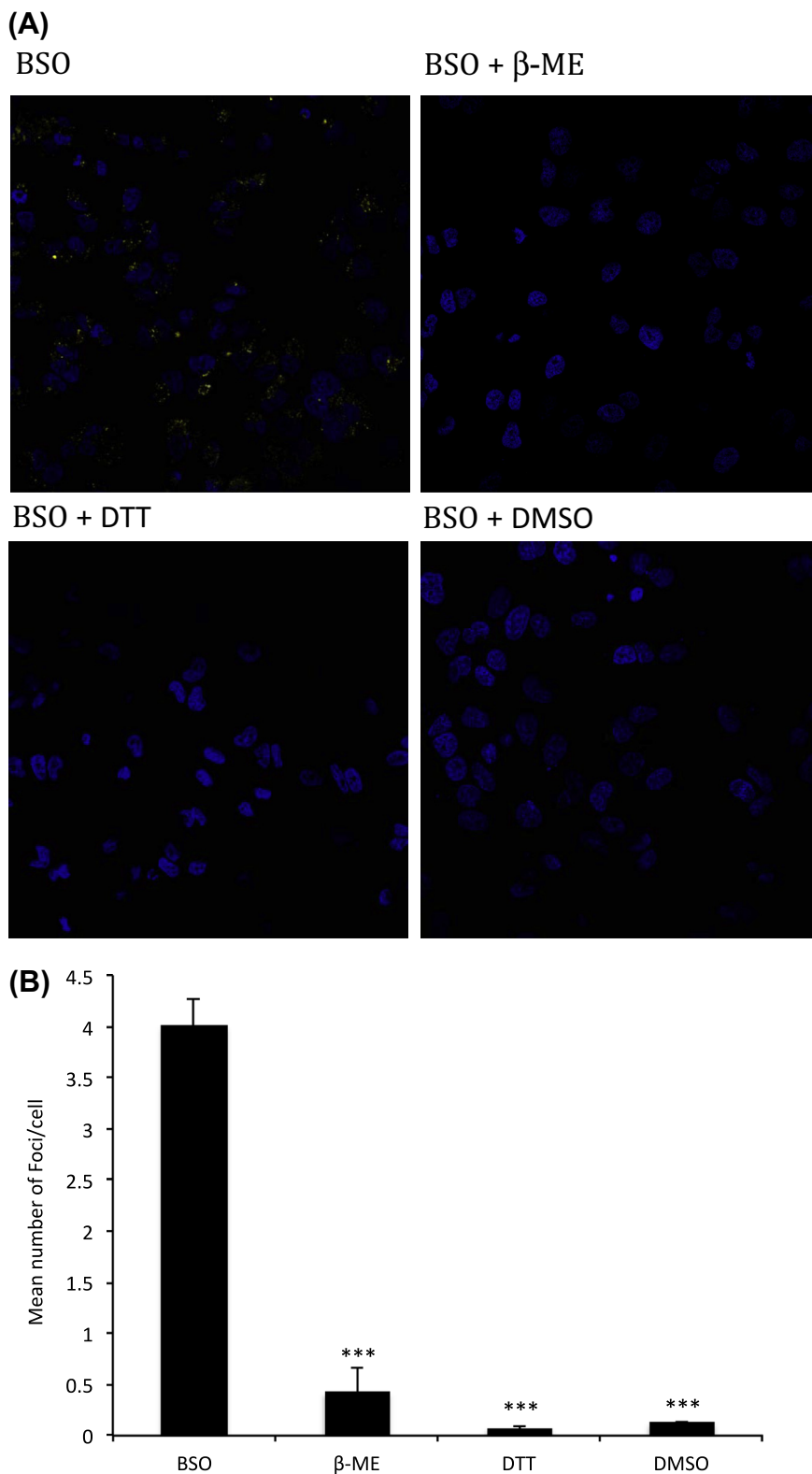
Fig. 4. (A) Inhibition of BiFC fluorescence in Cys326 OGG1 transfected cells treated with BSO (1000 μM), 24 h after co-incubation with the antioxidant DMSO (1% v/v) or addition of the disulphide reducing agents β-mercaptoethanol (1 mM) of dithiothreitol (1 mM) for the final four hours of incubation. (B) The bar graph represents the average number of BiFC fluorescent foci per cell ± SD for three independent fields of view; the average number of cells per field was 57.3 ± 13.33. *** Significantly different from BSO treated cells alone ($P < 0.001$, _t_-test).

not result in any BiFC fluorescence (data not shown) suggesting that BiFC fluorescence was entirely dependent on Cys326-OGG1 protein and did not involve an interaction between Ser326-OGG1 and Cys326-OGG1.

3.3. Antioxidants inhibit BiFC fluorescence

To further investigate the role of disulphide bond formation in the observed BiFC fluorescence in Cys326-OGG1 expressing cells following oxidative stress cells, the disulphide reducing agents β-mercaptoethanol and dithiothreitol were added for the final four hours of culture. Under these experimental conditions the number of BSO-induced fluorescence foci per cell was significantly reduced (Fig. 4A and B), strongly supporting our hypothesis that OGG1 complex formation within cells is dependent on the formation of a disulphide bond. Furthermore, co-incubation of cells with BSO and the antioxidant DMSO for 24 h also inhibited BiFC fluorescence suggesting that ROS generation is also important in the generation of BiFC fluorescence (Fig. 4A and B).

4. Discussion

Regulation of nuclear localisation of OGG1 has been putatively linked to phosphorylation of Ser326, with the Cys326 variant of OGG1 apparently excluded from the nucleolus during S-phase of the cell cycle [19,21]. Campalans et al. [35] have shown that following UVA irradiation, OGG1 is relocated from the nuclear matrix to form complexes that co-localise in "nuclear speckles" and it has been suggested that they represent foci of OGG1 and other BER components at sites of active repair of oxidative DNA damage. Although similar to the fluorescence observed in the current study, localisation of OGG1 to nuclear speckles described by Campalans et al. [35] were observed in cells expressing wild type OGG1-GFP protein. In contrast, we report BiFC fluorescence specifically in cells expressing Cys326 OGG1-YFP only under conditions of oxidative stress. Interestingly, we observed BiFC fluorescence in the form of discreet foci within the nucleus suggesting that the BiFC fluorescence observed represents the accumulation of Cys326-OGG1 complexes at sites of active DNA repair but further work is required to confirm this. Regardless of the exact nature of Cys326-OGG1 at sites of nuclear BiFC fluorescence, in regions of active repair it seems likely that the concentration of OGG1 protein would be relatively high facilitating the formation of BiFC fluorescent complexes of OGG1. Alternatively as discussed below, BiFC fluorescence may represent sites where oxidatively damaged Cys326-OGG1 has accumulated in the cell.

Interestingly, BiFC fluorescent foci were also present outside of the nucleus of cells. There are several possible explanations that could account for this observation – it may represent redox sensitive Cys326-OGG1 complex formation at sites of protein synthesis in the cell. Alternatively it could represent accumulation of mis-folded or inactive Cys326-OGG1 protein that has been targeted for degradation. In support of this second hypothesis there is emerging evidence that base excision repair proteins are regulated and targeted for proteasome-mediated degradation by the activity of ubiquitin ligases [36–38] and it is possible that similar pathways may act to regulate OGG1 protein. Increased turnover and degradation of damaged Cys326-OGG1 protein would account for the reduced repair activity of this form of OGG1 observed under conditions of oxidative stress and we have observed previously that treatment of cells with the pro-oxidant sodium dichromate results in reduced levels of OGG1 protein expression and activity [39].

At the molecular level there is emerging evidence that reduced activity of Cys326-OGG1 under oxidative conditions may be related to redox modification of the Cys326 amino acid. Hill and Evans [29] have shown that purified Cys326-OGG1 exists predominantly as a homo-dimer whose formation is dependent on the short c-terminal loop containing the Cys326 residue. Studies in cellular systems indicate that DNA repair rates of Cys326-OGG1 are reduced 2–4-fold compared to wild type protein [25,29,33,40,41]

and the existence of a Cys326-OGG1 dimer might be expected to reduce catalytic activity by approximately 2-fold because only one protein of each dimer would have access to the substrate at a time. A recent study [42] has utilised mass spectrometry to identify the formation of redox sensitive disulphide bond formation in Cys326-OGG1 involving Cys28 and Cys326 amino acids but conclude that this is only possible under conditions where folding of the protein is altered because the bond distance between Cys326 and Cys28 residues is too large to form a intra-molecular disulphide bridge when OGG1 is in its native folded state. It is possible that under conditions of oxidative stress Cys326-OGG1 becomes at least partially unfolded and an intra-molecular Cys28-Ser326 disulphide bond is formed. This would explain the occurrence of discreet foci of fluorescence, which may represent accumulation of mis-folded Cys326-OGG1 under conditions of oxidative stress and would also explain the reduced repair capacity of Cys326-OGG1 specifically under conditions of oxidative stress observed previously [27]. It is also possible that an inter-molecular disulphide bridge between two Cys326 residues may be formed in cellular environments and that the BiFC fluorescence observed represents Cys326-dependent OGG1 homo-dimer formation. Consistent with a role for redox dependent Cys326-OGG1 complex formation, BiFC fluorescence was specifically observed in cells expressing Cys326-OGG1 and only under conditions of oxidative stress and GSH depletion. The observation that incubating cells with disulphide reducing agents inhibits BSO-induced BiFC fluorescence also strongly supports the hypothesis that Cys326-OGG1 complex formation in cells is dependent on the formation of a redox sensitive disulphide bond possibly between two Cys326 amino acids and is in agreement with the previous studies of Hill and Evans in purified OGG1 protein [29]. Furthermore, the observation that BSO-induced BiFC was also inhibited by co-incubation with the antioxidant DMSO appears to confirm that BSO-mediated generation of ROS is also important for Cys326-OGG1 BiFC fluorescence. In conclusion, this study has demonstrated for the first time the application of BiFC to study a component of the BER pathway and provided further insight into the molecular mechanisms of the reduced repair capacity Cys326-OGG1. The novel finding of redox dependent Cys326-OGG1 complex accumulation and possible dimer formation within the native cellular environment provides further support to an increasing body of data implicating redox modification and disulphide dimer formation in the mechanism of the reduced repair capacity of Cys326-OGG1 observed.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.044.

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