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In vitro inhibition of topoisomerase IIα by reduced glutathione

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In most cells, the major intracellular redox buffer is glutathione (GSH) and its disulfide-oxidized (GSSG) form. The GSH/GSSG system maintains the intracellular redox balance and the essential thiol status of proteins by thiol disulfide exchange. Topoisomerases are thiol proteins and are a target of thiol-reactive substances. In this study, the inhibitory effect of physiological concentration of GSH and GSSG on topoisomerase IIα activity *in vitro* was investigated. GSH (0–10 mM) inhibited topoisomerase IIα in a concentration-dependent manner while GSSG (1–100 μM) had no significant effect. These findings suggest that the GSH/GSSG system could have a potential *in vivo* role in regulating topoisomerase IIα activity.

Keywords: DNA topoisomerases, glutathione, redox regulation

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INTRODUCTION

DNA topoisomerases (TOPO) are key enzymes implicated in nearly all events related to DNA metabolism and are the primary cellular target for many effective antineoplastic agents (Bates & Maxwell, 2007; Schoeffler & Berger, 2008). The TOPOs, especially the TOPO IIα isoform which is highly expressed in proliferating tissues (Isaacs *et al.*, 1998), are thiol proteins (Sng *et al.*, 1999). The human enzyme TOPO IIα and IIβ isoforms have 13 and 17 cysteine residues, respectively (Tsai-Pfugfelder *et al.*, 1988; Jenkins *et al.*, 1992). SH-reactive agents (e.g., *N*-ethylmaleimide (NEM), menadione and disulfiram) inhibit TOPOs (Tirumalai *et al.*, 1996; Frydman *et al.*, 1997; Neder *et al.*, 1998; Yakisich *et al.*, 2001). This indicates that sulfhydryl groups on cysteines are important for the enzymatic activity and, thus, potential targets for TOPO-inhibiting drugs. Since the cellular concentration of protein SH groups is 10 mM to 30 mM (Kaplowitz *et al.*, 1985), it is likely that, due to the high number of cysteine residues on TOPO IIα, the activity of this enzyme could be modulated *in vivo* by cellular thiol-reactive substances. To assess this hypothesis, it is first essential to determine the effect of natural occurring thiol-reactive substances on the activity of purified TOPO enzymes. The thiol-reduced form of glutathione (GSH), which is also the biologically active form, is the dominant non-protein thiol in mammalian cells and occurs in virtually all animal cells, often at a relatively high (0.1–12 mM) concentration (Meister, 1995; Pastore *et al.*, 2003; Lu, 2009). It has been reported that expression of Bcl-2 in HeLa cells produces redistribution of glutathione to the nucleus to give a concentration of 16 mM (74% of total cellular GSH within the nucleus compared to 32% when Bcl-2 expression is off) (Voehringer *et al.*, 1998). GSSG is present at much lower concentrations (20–40 μM) (Akerboom *et al.*, 1982; Gilbert, 1995). Available data also indicate that the GSH/GSSG redox potential is likely to be more reduced in nuclei than in the cytoplasm (Go & Jones, 2008). The GSH/GSSG system has important functions as an antioxidant, in detoxification of xenobiotics, maintenance of intracellular redox balance, storage and transport of cysteine (Meister, 1995; Lu, 1999; Dingren, 2000), and is essential for cell proliferation (Poot *et al.*, 1995; Lu, 1999; Dingren, 2000). Many enzymes and other endogenous compounds have been found to be modulated (activated or inhibited) by GSH and GSSG (Wang & Bellatori, 1998). To the best of our knowledge, except for plant mitochondrial TOPO I (Konstantinov *et al.*, 2001) and the use of high GSSG concentration (10 mM) for trapping Type1A TOPO/DNA complex (Li *et al.*, 2001), the redox regulation of TOPOs has not yet been evaluated.

MATERIAL AND METHODS

Dimethylsulfoxide (DMSO), reduced and oxidized glutathione (GSH and GSSG, respectively), etoposide, camptothecin were purchased from Sigma (Sweden), TOPO I, TOPO IIα and pBR322 plasmid DNA were purchased from Inspiralis (UK). All other reagents were of analytical grade or the highest grade available. Etoposide and camptothecin were prepared as stock solutions (25 mM) in DMSO. GSH and GSSG were prepared as stock solutions (200 and 300 mM, respectively) in sterile distilled water. Dithiothreitol (DTT) was purchased as 1 M stock solution. Except DTT (stored at 4 °C) all stock solutions were stored at –20 °C and diluted accordingly before use. Topoisomerase activity was measured as previously described (Yakisich *et al.*, 2001). Briefly, TOPO I and TOPO IIα activity was measured by the relaxation activity of superhelical plasmid pBR322 (400 ng/reaction) using appropriate DTT-free solutions and protocols adapted to the supplier recommendations. For TOPO I, the reactions were started by the addition of the enzyme (1 U) and allowed to proceed at 37 °C for 30 min. For TOPO IIα, the reactions were started by the addition of the enzyme (5 U) and allowed to proceed at 30 °C

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Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; TOPO, topoisomerase; DMSO, dimethylsulfoxide; DTT, dithiothreitol

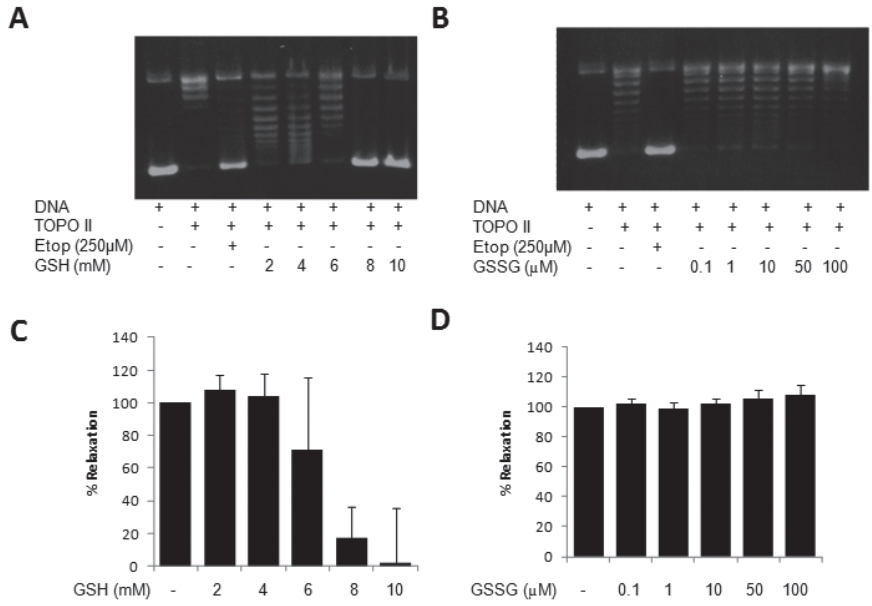

Figure. 1. Effect of GSH and GSSG on relaxation activity of TOPO IIα
Supercoiled (S) pBR322 plasmid DNA was incubated with different concentrations of GSH (A) or GSSG (B). Equivalent concentrations of vehicle alone (H_2O and DMSO) were used in control reactions containing only plasmid and TOPO IIα (second lanes). Etoposide (Etop) was used as positive control (third lanes). Representative gels are shown. Quantitative data for GSH and GSSG are shown in panels C and D, respectively. Data are the mean ± S.D. of three independent experiments.

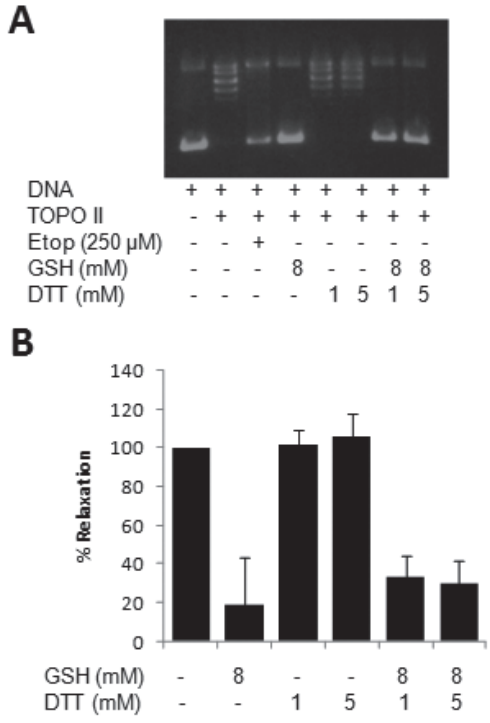

Figure 2. Effects of DTT on inhibitory activity of GSH
Supercoiled (S) pBR322 plasmid DNA was incubated with indicated concentrations of GSH alone or GSH+DTT and the relaxation activity of TOPO IIα was measured as described in Materials and Methods. Equivalent concentrations of vehicle alone (H_2O and DMSO) were used in control reaction containing plasmid and TOPO IIα (second lane). Representative gel is shown (A). Etoposide (Etop) was used as positive control (third lane). Quantitative data are shown in panel B. Data are the mean ± S.D. of three independent experiments.

for 15 min. The reactions of both assays were stopped by the addition of 5 μl of loading buffer. Aliquots (15 μl) were run in 1% agarose minigels at 2 V/cm for 16–20 h and stained with ethidium bromide for visualization with ultraviolet light. Quantitative determination of the bands was done using ImageJ (http://rsbweb.nih.gov/ij/).

RESULTS AND DISCUSSION

In the present work, we showed that physiologically relevant concentrations of GSH inhibited human TOPO IIα activity *in vitro*. Because TOPO I was only inhibited by high GSH concentration (we used camptothecin as positive control) of no physiological relevance (not shown) we did not further investigate this enzyme. TOPO IIα activity decreased with increasing GSH concentration (≥6 mM), reaching a maximum of inhibition at 10 mM GSH (Fig. 1A). GSH concentrations below 4 mM showed no inhibitory effect (Fig. 1 and data not shown). The lack of inhibitory effect at low GSH concentrations is in agreement with a recent article showing that 0.5 mM GSH had no effect on the decatenation activity of topoisomerase II (Wu *et al.*, 2011). Figure 1B shows that TOPO IIα activity was not inhibited by GSSG (1–100 μM). As described before, the cellular concentration of GSH varies from 0.2 to 12 mM (and GSSG from 10–40 μM). This means that GSH might affect *in vivo* the activity of TOPO IIα. The inhibitory effect of GSH was not affected by addition of 1 or 5 mM DTT (Fig. 2). However, it is possible that the DTT/GSH ratio (5 mM/8 mM = 0.625) used in our study may not be enough to prevent the inhibitory effect of GSH on TOPO IIα. For instance, the inhibitory effect of disulfiram on TOPO IIα was partially prevented by the addition of DTT to a high DTT/disulfiram (≥40) molar ratio (Yakisich *et al.*, 2001). A similar DTT/GSH molar ratio for the lowest GSH concentration (8 mM) that, in our study, showed a significant inhibitory effect on TOPO IIα (Fig. 1) was not possible to achieve due to the limited solubility of DTT (0.1 M at 20 °C). Thus, a thiol-disulphide exchange may still be important for the inhibitory effect of GSH as described for other SH-reactive agents such as disulfiram (Yakisich *et al.*, 2001). TOPO II enzymes contain 13–17 cysteine residues (Jenkins *et al.*, 1992; Tsai-Pfugfelder *et al.*, 1988; Wyckoff *et al.*, 1989). Interestingly, TOPO I has only eight cysteine residues (D'Arpa *et al.*, 1988). This suggests that the number of cysteine residues might be important for the modulatory effect of thiol-reactive substances that act directly on the cysteine residues of the enzymes and supports the (selectively) high effect of GSH on TOPO IIα activity (compared to TOPO I). The knowledge of the endogenous regulation of TOPOs by naturally occurring thiol substances, such as the GSH/GSSG system, might

be of importance for designing new therapeutic strategies for cancer treatment, protection of normal cells and for chemoprevention.

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