CELLULAR & MOLECULAR BIOLOGY LETTERS
Volume 9, (2004) pp 423 – 427
http://www.cmbl.org.pl

Received 10 February 2004
Accepted 29 April 2004

Short Communication

DNA REPLICATION REACTION IN *XENOPUS* CELL-FREE SYSTEM IS SUPPRESSED BY HIGH PRESSURE

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Abstract: Previously, we demonstrated that when mouse erythroleukemia cells are exposed to a pressure of 80 MPa, the cell-cycle progression of S-phase cells is retarded. To examine the effects of high pressure on DNA replication, we used a *Xenopus* cell-free system. From cell-cycle progression of sperm nuclei, it was found that sperm nuclei are stable to a pressure of 80 MPa, whereas egg extracts are susceptible to high pressure. Similarly, biotin-16-dUTP was incorporated into 80 MPa-treated sperm nuclei in pressure-untreated extracts, but not into naïve sperm nuclei in 80 MPa-treated extracts. These results indicate that DNA replication in *Xenopus* cell-free system is suppressed by the susceptibility of the extracts to a pressure of 80 MPa.

Key Words: Cell Cycle, DNA Replication, High Pressure, *Xenopus*

INTRODUCTION

When the cells are exposed to physicochemical stresses such as UV irradiation [1], heating [2], and high pressure [3, 4], specific damages in cellular components occur. For instance, UV irradiation damages cellular DNA such as thymine dimers and nucleotide delition. Protein denaturations are observed in heated cells. Heat shock proteins are induced by heating the cells [2]. High pressure induces the dissociation of oligomeric (or subunit) proteins [5]. Multisubunit proteins such as ribosomal proteins, DNA polymerases, and ATP synthase play an important role in cellular events such as protein synthesis, DNA replication, and ATP production, respectively. Thus, it seems likely that these cellular events are affected by high pressure.

Abbreviations used: MCM - minichromosome maintenance; ORC - origin recognition complex; PI - propidium iodide; UV - ultraviolet.

When mouse erythroleukemia cells are exposed to a pressure of 80 MPa and then cultured at atmospheric pressure, the proliferation of the cells is significantly suppressed [4]. On the basis of cell-cycle analysis, it was found that the cell-cycle progression of S-phase cells is retarded and these cells arrest at G2-phase [4]. To understand the sensitivity of S-phase cells to high pressure, we have attempted to examine the high-pressure effect on cell-cycle progression in a *Xenopus* cell-free system. In the present work, we demonstrate that the DNA replication in the *Xenopus* cell-free system is suppressed by a pressure of 80 MPa.

MATERIALS AND METHODS

Preparation of egg extracts and sperm nuclei

The preparation of S-phase egg extracts and sperm nuclei was performed by the methods described by Murray [6].

Pressure treatment

Pressure treatment of samples was carried out as follows. Samples (50-100 μl)of cycling extracts or freezing extracts were inserted into a glass tube of 5 mm diameter. Sperm nuclei were suspended in 10 volumes of EB buffer (100 mM KCl, 2.5 mM $MgCl_2$, 50 mM Hepes-KOH, pH 7.5). The glass tubes were filled with EB buffer and setted in the glass syringe containing EB buffer. The samples were exposed to pressures of 40-80 MPa for 30 min at 23 °C. After decompression, samples for sperm nuclei were added to NIB (50 mM KCl, 5 mM $MgCl_2$, 0.5 mM spermidine, 0.15 mM spermine, 1 mg/ml leupeptin, 1 mg/ml pepstatin, 50 mM Hepes-KOH, pH 7.6) containing 15% sucrose and centrifuged for 5 min at 6,200 g and 4°C .

Nuclear morphology

To examine the nuclear morphology, the mixture of cycling extracts and sperms (1 x 10^6 sperms/ml) was incubated for 150 min at 23°C and atmospheric pressure (0.1 MPa). Aliquots (5 µl) at indicated times were mixed with 5 µl of fixing buffer (3% formaldehyde, 80 mM KCl, 15 mM NaCl, 50% glycerol, 15 mM Pipes, pH 7.2) containing Hoechst 33258 (5 µg/ml). Sperm nuclei were observed by a fluorescence microscope.

DNA replication

To measure DNA replication, freezing extracts (50 µl) containing biotin-16-dUTP (8 µM) were incubated with sperm nuclei (10^6 nuclei/ml) for 60 min at 23°C. Aliquots (15 µl) were added to S buffer (250 mM sucrose, 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 50 mM Hepes-KOH, pH 7.5) containing 25% glycerol [6] and centrifuged at 1,200 g for 15 min at 4°C. Sperm nuclei were collected onto poly-L-lysine-coated cover glass. The sperm nuclei were fixed, washed with S buffer, and incubated with avidin-FITC for 30 min at room temperature. After incubation, the sperm nuclei were washed with EB

buffer, stained with PI (100 µg/ml) for 5 min, and washed with EB buffer. The intensity of FITC and PI of about 30 nuclei was measured by image processor (ARGUS-20). The value of FITC was normalized by intensity of PI.

RESULTS AND DISCUSSION

When sperm nuclei were incubated in cycling extracts, the morphology of sperm nuclei changed depending on the cell-cycle progression, as shown in Fig. 1A. The condensation of sperm chromatin in S phase was observed at 30 and 75 min. On the other hand, the breakdown of the nuclear envelope corresponding to M phase appeared at 60 and 135 min. To examine the sensitivity of cycling extracts to high pressure, the extracts were subjected to pressures of 40, 60, and 80 MPa. In the case of 40 MPa, the cell cycle of sperm nuclei progressed as with pressure-untreated ones. At 60 MPa, morphological changes of sperm nuclei were observed or not. In the case of 80 MPa, no progression of the cell cycle were observed in 80 MPa-treated cycling extracts (Fig. 1A).

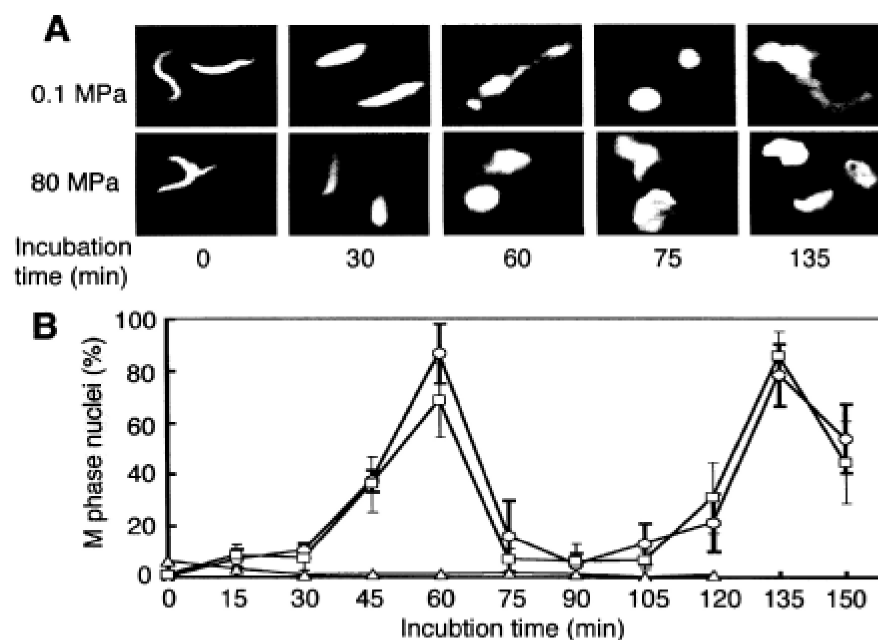

Fig. 1. Pressure effects on cell-cycle progression of sperm nuclei. (A) Sperm nuclei were incubated in pressure-untreated (0.1 MPa) or 80 MPa -treated cycling extracts at 23°C and atmospheric pressure. (B) Pressure effects on appearance of M-phase nuclei. Sperm nuclei were incubated at 23°C in pressure-untreated cycling extracts (○) and 80MPa-treated ones (Δ). On the other hand, 80 MPa-treated sperm nuclei (□) were incubated in pressure-untreated cycling extracts. Values are means ± SD for three independent experiments.

Appearance of M-phase nuclei was examined as a function of incubation time (0-150 min) using a light microscope (Fig. 1B). Most of sperm nuclei entered into the M-phase at 60 and 135 min in the cycling extract, but M-phase nuclei did not appear during the incubation time in 80 MPa-treated cycling extracts. On the other hand, sperm nuclei were also exposed to a pressure of 80 MPa and then incubated with cycling extracts. In this case, morphological changes of sperm nuclei occurred as usual. These results suggest that egg extracts are susceptible to a pressure of 80 MPa, whereas sperm nuclei are stable to its pressure.
To clarify the causes of the inhibition of the cell-cycle progression, we examined the incorporation of biotin-16-dUTP into sperm nuclei (Fig. 2).

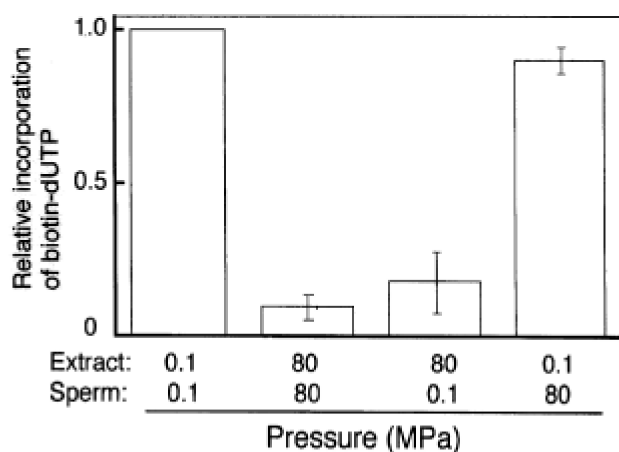

Fig. 2. Pressure effects on incorporation of biotin-16-dUTP into sperm nuclei. Relative amount of incorporated biotin-16-dUTP was estimated from fluorescence intensities of avidin-FITC and PI. Values are means ± SD for three independent experiments.

The DNA replication was suppressed upon exposure to 80 MPa of the freezing extracts, whereas 80 MPa-treated sperm nuclei replicated DNA in pressure-untreated freezing extracts. Thus, the suppression of DNA replication induces no progression to M-phase of sperm nuclei. The mechanism of DNA replication has been investigated by using the *Xenopus* cell-free system. Egg extracts contain all the cell cycle activities. So, exogenously added sperm nuclei are able to replicate their DNA without protein synthesis [7, 8]. In fact, sperm nuclei replicated DNA in the freezing extracts containing cycloheximide (10 μg/ml) (data not shown). These data suggest that the suppression of DNA replication by high pressure is not associated with protein synthesis. The licensing of DNA is an important event for DNA replication. Licensing factors such as MCMs bind on DNA preloaded with ORC and Cdc 6 [9]. After the licensing is complete, replication factors containing DNA polymerases are recruited on DNA. Thus, multiprotein complexes are formed on DNA. Further studies are necessary to understand how these protein complexes are affected by pressure. Our findings that a pressure

of 80 MPa gives severe damages to S-phase cells by suppressing DNA replication suggest a possibility of application of high pressure to proliferating cells such as cancer ones.

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