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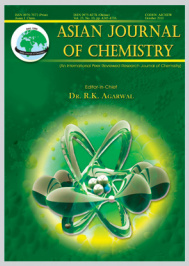

Expression of Metallothionein *MTT1* and *MTT2* from *Tetrahymena thermophila* Exposed to SO_2, NaCl and Tris-HCl

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The *m*RNA levels of two components of *Tetrahymena thermophila* metallothionein (MT) gene families, *MTT1* and *MTT2*, were evaluated using real-time quantitative-PCR when cells were exposed to SO_2 derivatives, high-salt and Tris-HCl. The *MTT1* expression level was higher by SO_2 derivatives and Tris-HCl stress than *MTT2*. The influence of high-salt for *MTT1* and *MTT2* *m*RNA levels were weak. The results provided evidence that *MTT1* protein act as an important role against the damage of SO_2 derivatives and beginning of starvation and *MTT1* and *MTT2* protein act a small role in anti-high-salt.

Key Words: Metallothionein, *Tetrahymena thermophila*, Gene expression, Quantitative real-time PCR.

INTRODUCTION

Sulfur dioxide (SO_2) is a ubiquitous air pollutant, present in low concentrations in the urban air and in higher concentrations in the working environment. Sulfur dioxide pollution is produced by combustion and processing of sulfur-containing fossil fuels[1]. As a ubiquitous air pollutant, gaseous SO_2 influences human health and also the global ecological system of animals and plants[2]. Sulfur dioxide can easily be hydrated in the environment to produce sulfurous acid, which subsequently dissociates to form bisulfite and sulfite derivatives (1:3 M/M in neutral fluid)[3]. These derivatives can be absorbed into the organism. Recently, Meng *et al.*[4,5] have reported that SO_2 and its derivatives are the systemic toxic agents, which can cause DNA damage and oxidative damage in lungs from mice. Many chemical reactions in the organisms also arise under the high salt and starvation.

The ciliated protozoan *Tetrahymena* are excellent eukaryotic unicellular model organisms for the study of toxic compounds in the environment[6,7]. Metallothioneins (MTs) were low-molecular-weight, cysteine-rich metal-binding proteins that act as a important role in resisting the external stress[8]. Metallothioneins may be induced by many stressors, *e.g.*, heavy metal, oxidants, hormones, pH, heat, starvation, etc, in order to reduce the damage of stressors[9]. In the single-celled organism *Tetrahymena*, usually multiple metallothionein isoforms are present in one organism. *Tetrahymena thermophila* having at least five isoforms, designated *MTT1* to *MTT5*[9,10]. *MTT1*, *MTT3* and *MTT5* belong to cadmium-inducible; *MTT2* and *MTT4* belong to copper-inducible. In *T. thermophila*, unique Cys-Cys-Cys clusters appear in *MTT1* and unique Cys-X-Cys clusters appear in *MTT2*, different structures correspond to different functions. *MTT1* and *MTT2* will provide good model protein for advance analyzing metallothionein mainly function. In this article, we made an evaluation for the expression level of *MTT1* and *MTT2* exposed to SO_2 derivatives, NaCl and starvation.

EXPERIMENTAL

Strains and culture conditions: *T. thermophila* strain CU428 was provided by Dr. Peter. J. Bruns (Cornell University). Cells were grown in 1 × SPP medium (1 % proteose peptone, 0.2 % glucose, 0.1 % yeast extract, 0.003 % EDTA ferric sodium salt) at 30 ºC.

SO_2 derivatives and NaCl impact on the proliferation of *T. thermophila*: *T. thermophil*a cells were inoculated into 5ml SPP at the initial concentration of 2×10^3 cells/ml. Then, the cells were treated with different concentrations of SO_2 derivatives (0, 0.01, 0.1, 0.5, 1, 2 and 4mM) and NaCl (0, 10, 40, 100, 154, 170 and 185mM), respectively.

RNA isolation and cDNA synthesis: Exponential phase cells were treated for 1 h with each of the following stressors: 185 mM NaCl and 0.5 mM SO_2 derivatives. When the concentration of *T. thermophila* cells reached 2-3 × 10^5, cells was washed twice with 10 mM Tris-HCl (pH 7.4). Total RNA of *T. thermophila* in each treatment was extracted using RNAiso

reagent (TaKaRa), digested by DNase (invitrogen). To produce first-strand cDNA, RNA was reverse transcribed at 37 ºC for 15 min using 1 µg of total RNA in 20 µL reaction mixture containing 0.5 µL of PrimeScript RT Enzyme Mix I, 0.5 µL Oligo dT Primer and Random 6 Mers.

Gene expression analysis by real-time quantitative PCR: Real time quantitative PCR was performed in a final volume of 20 µL using 1-10 ng cDNA, 1 × SYBR Green PCR Master mix (TaKaRa), 0.5 µM each *MTT1* and *MTT2* primers (*MTT1* and *MTT2* sense, *MTT1* and *MTT2* antisense) or 0.5 µM each ribosomal 17S primers (17S rRNA sense and 17S rRNA antisense) (Table-1). Thermal parameters in a Stepone Plus Real Time PCR System Cycler (Applied Biosystem) were as follow: 15 min at 95 ºC, followed by 40 cycles: 95 ºC for 15 s, 53 ºC for 30 s and 68 ºC for 35 s. A melting curve of PCR products (60-90 ºC) was also performed to ensure the absence of artefacts. The relative expression levels of *MTT1* and *MTT2* was normalized against *T. thermophila* 17S ribosomal RNA gene. The amplification efficiency and the linearity over a range of three orders of magnitude concentration were confirmed. For each analyzed target, the PCR efficiency value was averaged from three different experiments. The melting curve of PCR products (60-85 ºC) was also performed to ensure the absence of artifacts. To calculate the normalized relative gene expression levels (as *n*-fold induction), data were analyzed using real time PCR System Cycler own software in which the mathematical model used is based on mean threshold cycle (Ct) differences between the sample and the control group and the expression ratio results of the investigated transcripts are tested for significance by a pair-wise fixed reallocation randomization test[11].

TABLE-1
REAL-TIME QUANTITATIVE PCR PRIMER

Name	Sequence (5'-3')
17S rRNA R	AAATGTTTACTCCCTAAGTCGAACC
17S rRNA F	CCTGAGAAACGGCTACTACAACTAC
MTT1F	TGCTGCACAGACCCTAACAG
MTT1R	TGCATCCCTCTCCTGTACCAG
MTT2F	ATCCCTGCTCTTGTAATCCC
MTT2R	AGTTGGAAGTAGAACCGCA

RESULTS AND DISCUSSION

NaCl and SO_2 derivatives effect on *T. thermophila* growing rate: In SPP medium containing less than 154 mM NaCl, the proliferation of *T. thermophila* is slightly faster than that in NaCl-free medium. While NaCl concentration is greater than 185 mM, *T. thermophila* stopped growing and cells became very small owing to dehydration. *T. thermophila* can grow in less than 4 µM medium with SO_2 derivatives, but the proliferation of *Tetrahymena* cell decreased step by step with SO_2 derivatives concentration increasing (Fig. 1A-B).

SO_2 derivatives impact on the expression of *MTT1* and *MTT2*: Expression of the *MTT1* and *MTT2* transcripts was assessed after *in vivo* exposure (1 h) to SO_2 derivatives (0.5 mM). Concentrations used were approximately the semi-inhibition value of *T. thermophila* growth. To produce scarce effects on the organism physiology, data were normalized for rRNA 17S expression. SO_2 derivatives produced a stimulation

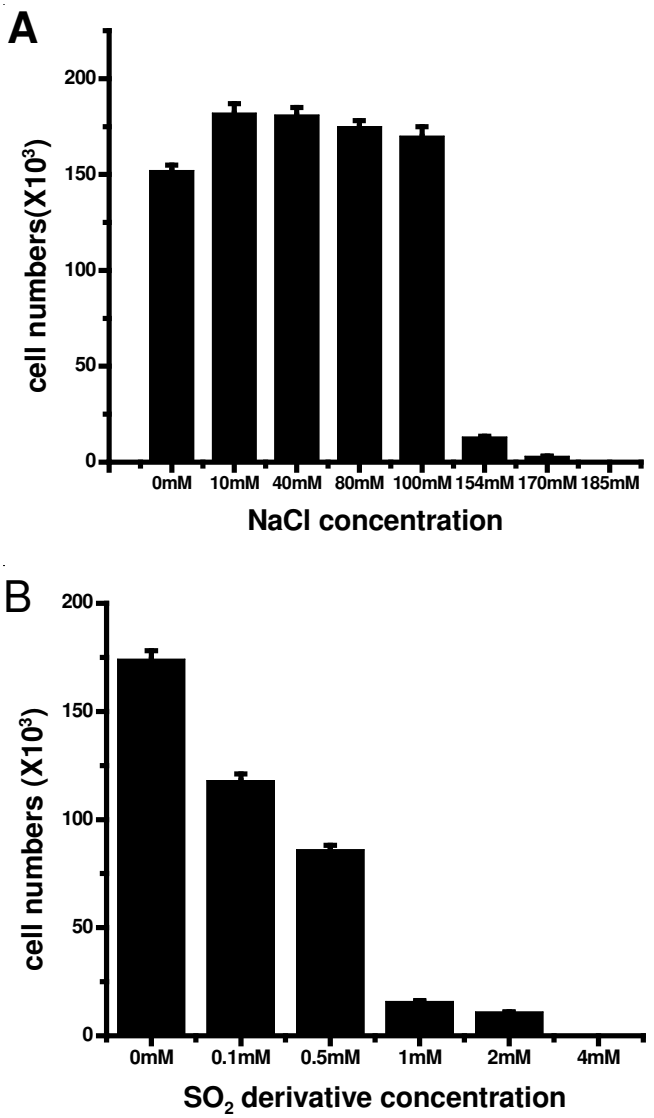

Fig. 1. NaCl and SO_2 derivatives effect on *T. thermophila* proliferation. The cell vaccination number of each sample was the same at begin and at the time stress elements (NaCl and SO_2 derivatives) was added. When the number of control cell reached 10^5 mL, all sample numbers were counted. Experiment was repeated 3 times. (A): NaCl stress; (B) SO_2 derivatives stress

of the *MTT1* gene whose relative expression levels with respect to the control were comprised within one order of magnitude, showing the higher effect (13-fold) (Fig. 2A). By contrast, the effect of SO_2 derivatives to *MTT2* was lower since the expression was 4-fold with respect to the control (Fig. 2B).

NaCl and Tris-HCl impact on the expression of *MTT1* and *MTT2*: All inducers produced a stimulation for *MTT1* gene expression (Fig. 3). Exposure to 10 mM Tris-HCl (pH 7.4) showed the more effective induction of mRNA level (54-fold) and 185 mM NaCl was the lower (3-fold) (Fig. 3A). By contrast, Tris-HCl (2-fold) and NaCl (4.5-fold) exposure elicited much lower accumulation of the *MTT2* transcript (Fig. 3B).

Many stressors can induce the expression of metallothionein whether it is in multicellular organisms or in the single cell organisms. Sulfur dioxide, a common air pollutant, is a gas that exists in aqueous solution at neutral pH as an equilibrium between bisulfite and sulfite ions (1:3, M/M)[3]. Bisulfite can produce free radicals in reactions of sulfite with inorganic

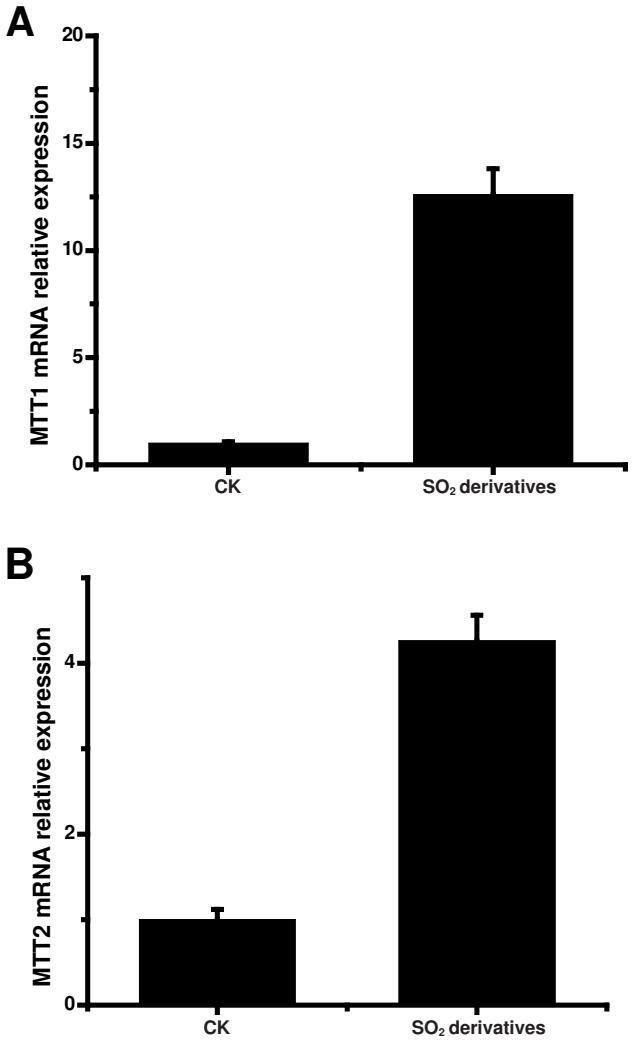

Fig. 2. Relative expression levels of *T. thermophila MTT1* and *MTT2* genes obtained by quantitative RT-PCR. Panels A and B: fold-induction for *MTT1* and *MTT2* after treatment by SO_2 derivatives. Gene expression levels are shown relative to an untreated control. Normalization of expression was achieved against the amplification of an endogenous gene (17S). Each bar of the histogram corresponds to an average value ± SD three independent experiments. Stress conditions are as reported in the experimental section

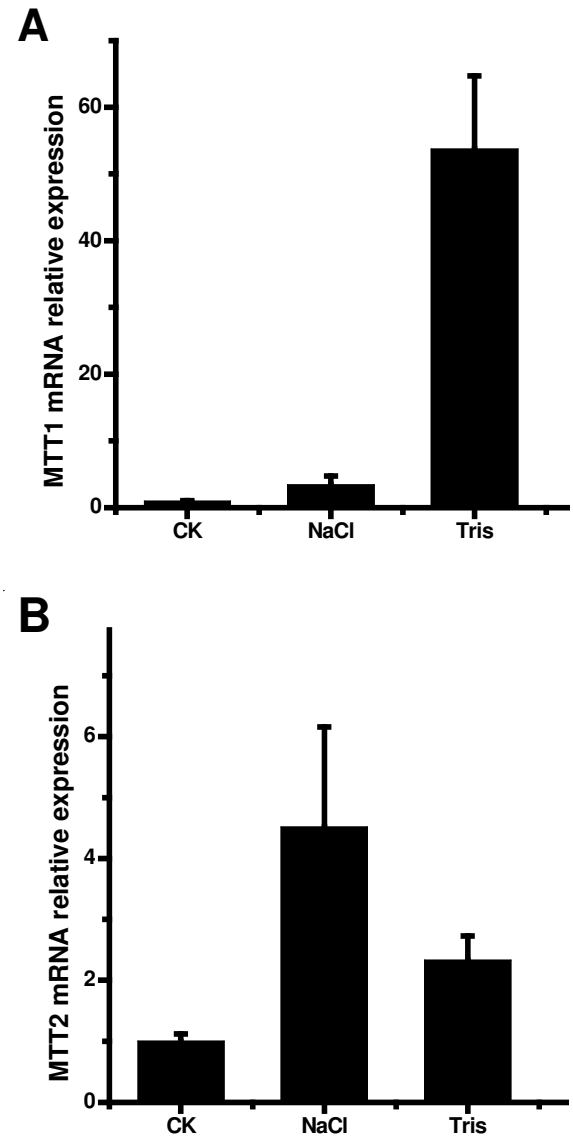

Fig. 3. Relative expression levels of *T. thermophila MTT1* and *MTT2* genes obtained by quantitative RT-PCR. Panels A and B: fold-induction for each gene (*MTT1* and *MTT2*, respectively) after treatment by different stress. Gene expression levels are shown relative to an untreated control. Normalization of expression was achieved against the amplification of an endogenous gene (*17S*). Each bar of the histogram corresponds to an average value ± SD three independent experiments. Stress conditions are as reported in experimental section

environmental pollutants and sulfur trioxide anion radicals can induce DNA damage[12,13]. After exposed to SO_2 derivatives, the response of *MTT1* gene for free radicals was stronger than *MTT2* gene, it was summarized that *MTT1* is prior to *MTT2* in antioxidant function. Based on the above results, we made a hypothesis that *MTT1* is stronger than *MTT2* in response to external oxidants.

MTT1 not only can be induced by heavy metal ions but also be induced by non-metallic, such as: arsenic, temperature (4 and 42 ºC, 2 h), pH (5 and 9, 24 h), paraquat (24 h) and starvation (24 h and 4d) which induced a weak expression of *MTT1* gene[9]. Here, we made 1 h high-salt stress for *T. thermophila* CU428. Expression level of *MTT1* and *MTT2* did not showed significantly enhancement, 3 fold and 4.5 fold, respectively. The results showed that *MTT1* and *MTT2* protein play a small role in anti-high-salt stress. Exponential phase cells were washed with 10 mM Tris-HCl (pH 7.4) and SPP medium was deprived, cells were transferred from nutritional condition to starvation condition (starvation treatment began). Starvation stress, mainly beginning exposure, effectively induces *MTT1* genes expression in *T. thermophila*. But *MTT2* gene expression was weekly influenced by beginning exposure to Tris-HCl. Exposed to starvation for 24 h, *MTT1* and *MTT3* gene from *T. thermophila* have lower expression[9]. However, *MTT5* from *T. thermophila* and Tros*MTT1* from *T. rostrata* are more sensitive to starvation stress, because these metallothionein genes are considerably induced by 24 h starvation[9,14]. Starvation can be considered as one of the most habitual types of stress that living cells can suffer from and metallothioneins are also expressed under this environmental stressor. In fact, *Saccharomyces cerevisiae* CUP1 metallothionein gene and mammalian metallothionein gene can also be induced by food deprivation[15,16].

From the above experiments results, we made a hypothesis for metallothioneins gene expression induced by starvation. First, it is known that almost half of all enzymes must associate with a particular metal to function[17] and exponential phase cells metabolism is stronger because they have adequate nutrition. But after food was deprived, metabolism must be decreased. Decreased metabolism may be occured by metallothioneins owing to the capacity of metallothioneins binding metal ions. So metallothioneins can be effectively induced when food was just deprived. In addition, starvation can increase hormones level of *Tetrahymena* and hormones can also induced the expression of metallothioneins[18,19], so it is still a mystery whether they function simultaneously or hormones only induce metallothioneins expression.

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