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단보

# Dimethyl sulfoxide에 의한 세포내 칼슘이온 농도 증가가 안정적으로 형질전환된 초파리 S2 세포에서 재조합 사람 cyclooxygenase 1의 발현에 미치는 영향

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# An Increased Intracellular Calcium Ion Concentration in Response to Dimethyl Sulfoxide Correlates with Enhanced Expression of Recombinant Human Cyclooxygenase 1 in Stably Transfected *Drosophila melanogaster* S2 Cells

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**Abstract:** Dimethyl sulfoxide (DMSO) increased the intracellular calcium ion concentration in stably transfected *Drosophila melanogaster* S2 cells expressing recombinant cyclooxygenase 1 (COX-1). DMSO did not increase the *Drosophila NOS* (*dNOS*) transcript level in calcium chelator-treated cells. Expression of recombinant COX-1 due to DMSO was diminished in cells treated with calcium chelators or channel blockers. Our results indicate that an increased intracellular calcium ion concentration due to DMSO is associated with up-regulation of the *dNOS* gene, leading to enhanced expression of COX-1.

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**Keywords:** recombinant cyclooxygenase 1, dimethyl sulfoxide, *Drosophila melanogaster* S2 cells, calcium ion

## 1. Introduction

*Drosophila melanogaster* Schneider 2 (S2) cells have been widely used for heterologous gene expression [1]. S2 cells provide a null background for studying heterologous gene product and allow stable insertion of up to several hundred gene copies into the chromosomal DNA. In addition, S2 cells grow readily in suspension to high densities. The most important advantage of the S2 cell expression system is an availability of both strong constitutive and tightly regulated inducible promoters. The *Drosophila* actin 5C and metallothionein (MT) promoters are well characterized constitutive and inducible promoter, respectively, to allow high level expression of interest gene in *Drosophila* S2 cells [2,3].

Metallothioneins (MTs) are ubiquitous low-molecular-weight cysteine-rich proteins that bind heavy metals (i.e., Cu, Zn, Cd) and seem to protect organisms from toxic metals [4,5]. MTs are involved in zinc ion homeostasis. In experimental

transformation of animals and cultures, the MT gene regulatory 5′ region has been shown to be an efficient promoter in chimeric gene constructs. The *Drosophila* MT promoter is known to allow high-level, inducible expression of a gene of interest in S2 cells [6,7]. When used to express heterologous proteins, the MT promoter is extremely efficient and tightly regulated [7]. The MT promoter is inducible by addition of copper sulfate, cadium chloride, or metal ions to the culture medium [8].

Dimethyl sulfoxide (DMSO) is an effective cell permeabilizing agent that penetrates cellular membranes and helps the release of intracellular products from cells. DMSO has been reported to show several biological effects and is used as a stabilizing agent for protein [9]. DMSO increases the efficiency of DNA transfer in *Escherichia coli* by the combination with divalent calcium ion [10]. In mammalian cells, DMSO treatment increases the transfection efficiency after electroporation [11]. DMSO has been reported to induce morphological changes in mouse erythroleukemic cells [12]. DMSO regulates differentiation in neuroblastoma cells [13]. DMSO is thought to have a broad impact on many aspects of gene expression in animal and insect cells [14-16]. DMSO enhanced the production of recombinant proteins such as rotavirus VP7, human cyclooxygenase 1 and 2 (COX-1 and 2) from stably transfected S2 cells [17-19]. Recently, we reported that DMSO increased synthesis of *Drosophila* NOS (dNOS) at both the RNA and protein levels, and that dNOS expression is closely related to synthesis of recombinant COX-1 mRNA in stably transfected S2 cells [19]. The activity of the *dNOS* gene in the *Drosophila* genome is known to be calcium/calmodulin dependent [20]. We postulated that DMSO affects the changes in the intracellular calcium ion concentration associated with up-regulation of NOS activity, leading to enhanced expression of recombinant COX-1 in stably transfected S2 cells *via* up-regulation of the MT promoter. Therefore, in this study, we first attempted to elucidate the relationship between the intracellular calcium ion concentration and expression of recombinant COX-1 in stably transfected S2 cells after DMSO treatment.

## 2. Materials and Methods

### 2.1. Cell culture

Stably transfected S2 cells expressing recombinant COX-1 were generated *via* co-transfection with pMT/BiP-His-COX-1 and pCoHygro [19]. Stably transfected S2 cells were grown at 27˚C in T-25 flasks in 3 mL of Shields and Sang M3 insect medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% insect medium supplementary (IMS, Sigma-Aldrich) and 300 μg/mL hygromycin B. Expression of recombinant COX-1 from stably transfected S2 cells was induced by 0.5 mM $CuSO_4$ after the start of the run. To determine the effect of DMSO on gene expression, DMSO was added at 6% (v/v) to the cultures, 5 days post-innoculation.

### 2.2. Measurement of intracellular calcium ion concentration

Intracellular calcium ion concentrations were determined by calcium measurements using Wallac VICTOR$^2$ plate reader (PerkinElmer Life Sciences, Inc., Boston, MA, USA), according to the manufacturer's instruction. Briefly, stably transfected S2 cells ($5 \times 10^5$ cells) were incubated for indicated times in the presence or absence of 6% DMSO. Cells were washed with Hank's basal saline solution (HBSS; 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2.5 mM probenecid, pH 7.0). Cells were resuspended in 100 μL of HBSS containing 4.5 μM Fluo-AM (Molecular Probes, Eugene, OR, USA) and 0.01% pluronic acid, followed by an incubation in darkness for 40 min at 27˚C. After washing twice with HBSS, cells were resuspended in 50 μL of HBSS and transferred to 96-well plates. The fluorescence was measured with Wallac VICTOR$^2$ plate reader. The excitation and emission wavelengths were 488 nm and 530 nm, respectively.

### 2.3. SDS-PAGE and Western blot analysis

Stably transfected S2 cell cultures were centrifuged at 3,000 rpm for 5 min to separate the cells. The supernatant was used to identify extracellular recombinant proteins. The cell fraction was rocked for 1 h in a lysis buffer (50 mM Tris-Cl at pH 8.0, 150 mM NaCl, 0.02% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1% Triton X-100) and subjected to three freeze-thaw cycles of 10 min in a -70˚C freezer and 5 min in a 37˚C water bath. After centrifuging the cell extracts at 14,000 rpm for 15 min to remove cell debris, the supernatant was used to identify intracellular recombinant proteins. Protein samples were separated by electrophoresis on 8% polyacrylamide-sodium dodecyl sulfate (SDS) gel and transferred to a nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ, USA). The membranes were blocked with 3% BSA (bovine serum albumin) and incubated in 1% BSA with mouse anti-human COX-1 monoclonal antibody (1 : 1,000 dilution; Cayman Chemical, Ann Arbor, MI, USA). After probing with alkaline phosphatase-conjugated goat anti-mouse-IgG antibody (1 : 1,000 dilution; Sigma-Aldrich). The membranes were washed and a BM purple AP substrate solution (Boehringer Mannheim, Germany) was applied to detect protein bands. The reaction was quenched with distilled water.

### 2.4. Northern blot analysis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen,

Valencia, CA, USA) from stably transfected S2 cells treated with DMSO and BAPTA-AM [1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); Sigma-Aldrich]. RNAs were separated by electrophoresis in 1% agarose gel under denaturing conditions containing formaldehyde, transferred to a Hybond-$N^+$ membrane (Amersham Pharmacia) using the capillary transfer method, and fixed by exposure to ultraviolet (UV) light (CL-1000 Ultraviolet Crosslinker; UVP, Upland, CA, USA). The *dNOS* gene fragment was labeled using the Prime-A-Gene Labelling System (Promega, Madison, WI, USA) with [α-$^{32}$P]dATP, then used as a probe. The membrane was hybridized overnight at 65˚C in a hybridization mixture (6x SSC, 0.2% Denhardt's solution, 0.5% SDS, 100 µg/mL of salmon sperm DNA) containing the [α-$^{32}$P]dATP-labelled probe. The membrane was then washed three times with a washing solution (0.1x SSC, 0.1% SDS) at 65˚C for 10 min, followed by autoradiography at -70˚C using X-ray film (Kodak, Rochester, NY, USA).

## 3. Results and Discussion

### 3.1. Effect of DMSO on expression of recombinant COX-1 from stably transfected S2 cells

In our previous work, we generated stably transfected *Drosophila* S2 cells expressing recombinant COX-1 [19]. Recombinant COX-1 was expressed in the cellular fraction of stably transfected S2 cells with molecular weights of 68 kDa and 74 kDa, due to glycosylation. A 72 kDa COX-1 was observed in the medium fraction of stably transfected S2 cells [19]. To determine the effects of DMSO on expression of recombinant COX-1, DMSO was added to the cultures at 5 day post-inoculation in the presence or absence of $CuSO_4$. Supplementation of cultures with 6% DMSO increased recombinant COX-1 production by 180% compared to $CuSO_4$-treated cells [19]. DMSO induces expression of recombinant COX-1 without $CuSO_4$ supplementation. Enhancement of recombinant COX-1 production due to DMSO was only observed in S2 cells transfected with a construct of the MT promoter, but not the actin 5C promoter. This suggests that DMSO increases production of recombinant COX-1 though up-regulation of the MT promoter.

### 3.2. Effect of DMSO on intracellular calcium ion concentration

DMSO has been reported to transiently increase the calcium ion concentration in various cell types including primary cultures of chicken ovarian granulose cells, undifferentiated P19 embryonal carcinoma cells, 3T3-L1 fibroblasts, Friend murine erythroleukemia (MEL) cells, and hepatocytes [21,22]. To determine the effect of DMSO on the cellular calcium ion concentration in S2 cells, stably transfected S2 cells were incubated for indicated times in the presence and absence of 6% DMSO and the intracellular calcium ion concentrations were determined. The intracellular calcium ion concentration in stably transfected S2 cells cultured in the presence of 6% DMSO dramatically increased up to 9 h, after then gradually decreased (Fig. 1). In RPMI-8402 human T lymphoblasotoid cells, DMSO increased the intracellular calcium ion concentration with a maximum concentration at 24 h after DMSO treatment [23]. This indicates that DMSO increases the intracellular calcium ion concentration in S2 cells and also suggests that the modification of intracellular calcium ion concentration due to DMSO might affect the induction of MT promoter.

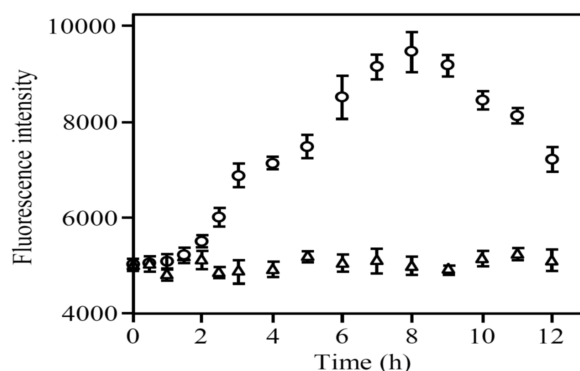


**Fig. 1.** The effect of DMSO on the cytoplasmic calcium ion concentration in stably transfected S2 cells. Open circle and up-triangle indicate relative fluorescence units of stably transfected S2 cells incubated in the presence and absence of 6% DMSO, respectively. Data represent the mean ± standard deviation from three independent experiments.

### 3.3. Effect of the intracellular calcium ion concentration on up-regulation of the dNOS transcript by DMSO

Calcium is known to act both in the cytoplasm and the nucleus to activate signaling pathways that stimulate gene expression through different DNA regulatory elements [24]. In our previous work, DMSO increased synthesis of *Drosophila* NOS (dNOS) at both the RNA and protein levels. dNOS expression was closely related to synthesis of recombinant COX-1 mRNA in stably transfected S2 cells [19]. Nitric oxide synthases (NOSs) synthesize nitric oxide (NO), a short-lived molecule that reacts with $O_2$, transition-metal ions, and thiols, during conversion of L-arginine to L-citrulline in many cell types [25,26]. The *dNOS* located on the second chromosome at cytological position 32B is the only gene for NOS in the *Drosophila* genome. Its activity is known to be calcium/ calmodulin dependent [20]. To determine the effect of the intracellular calcium ion concentration on up-regulation of the *dNOS* transcript by DMSO, stably transfected S2 cells were treated with 6% DMSO in the presence of BAPTA-AM, an intracellular calcium ion chelator. Total RNA was isolated from stably transfected S2 cells and Northern blot analysis

was performed to determine the level of the *dNOS* transcript. In stably transfected S2 cells, the *dNOS* transcript level was increased for up to 9 h, remained relatively constant for 12-18 h, then decreased slightly at 24 h [19]. However, the *dNOS* transcript level was not affected in stably transfected S2 cells treated with DMSO in the presence of BAPTA-AM (Fig. 2). This indicates that the increase of *dNOS* transcript level due to DMSO is mediated by enhancement of the intracellular calcium ion concentration.

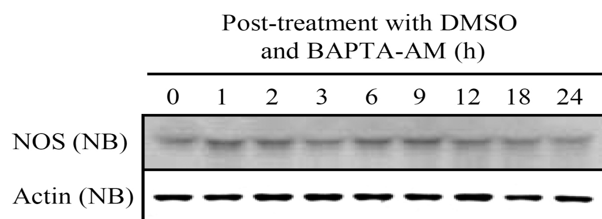


**Fig. 2.** The effect of DMSO on the *dNOS* transcript level in BAPTA-AM treated stably transfected S2 cells. Any noticeable effects on cell viability were not observed at a concentration of 250 μM BAPTA-AM. NB means Northern blot analysis.

### 3.4. Effect of calcium chelators on expression of recombinant COX-1 in DMSO-treated stably transfected S2 cells

The effect of BAPTA-AM on expression of recombinant COX-1 in DMSO-treated stably transfected S2 cells was further determined (Fig. 3(a)). DMSO and BAPTA-AM were added to cultures at 5 days post-inoculation. After 2 days of incubation, expression of recombinant COX-1 in both the cellular and medium fractions was determined using Western blot analysis with the anti-human COX-1 monoclonal antibody. The presence of BAPTA-AM decreased expression of recombinant COX-1 in response to DMSO. Expression of recombinant COX-1 due to DMSO in both the intracellular and medium fractions was diminished to the basal level at a concentration of 250 μM. Expression of recombinant COX-1 due to DMSO was also determined in calcium ion chelator, ethylene glycol tetraacetic acid (EGTA)-treated stably transfected S2 cells (Fig. 3(b)). EGTA treatment reduced expression of recombinant COX-1 in both the intracellular and medium fractions. In 10 mM EGTA-treated S2 cells, expression of recombinant COX-1 due to DMSO was reduced to the basal level. These results indicate that chelating the calcium ion reduces expression of recombinant COX-1 due to DMSO.

### 3.5. Effect of calcium channel blockers on expression of recombinant COX-1 in DMSO-treated stably transfected S2 cells

DMSO has been suggested to increase the calcium ion concentration *via* two mechanisms of 1) release of the ion from intracellular pools and, 2) stimulation of an influx across the plasma membrane [27]. Several classes of calcium entry channel have been documented in insects, including L-type calcium channel subunits. L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster* [28]. To evaluate whether the increase in the intracellular calcium ion level due to DMSO is caused by stimulation of an influx across the plasma membrane, expression of recombinant COX-1 due to DMSO was determined in calcium channel blocker-treated stably transfected S2 cells. DMSO and calcium channel blockers were added to the culture at 5 days post-inoculation. After 2 days of incubation, expression of recombinant COX-1 in both the cellular and medium fractions was determined

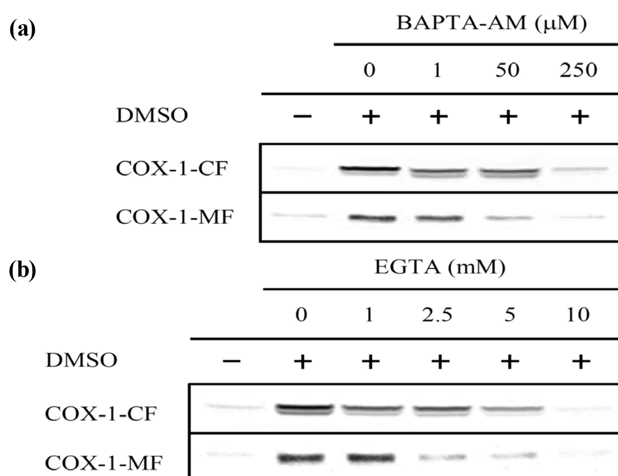


**Fig. 3.** The effect of the calcium chelators BAPTA-AM (a) and EGTA (b) on expression of recombinant COX-1 due to DMSO. Any noticeable effects on cell viability were not observed at a concentration of either 250 μM BAPTA-AM or 10 mM EGTA. CF and MF indicate cellular fraction and medium fraction, respectively.

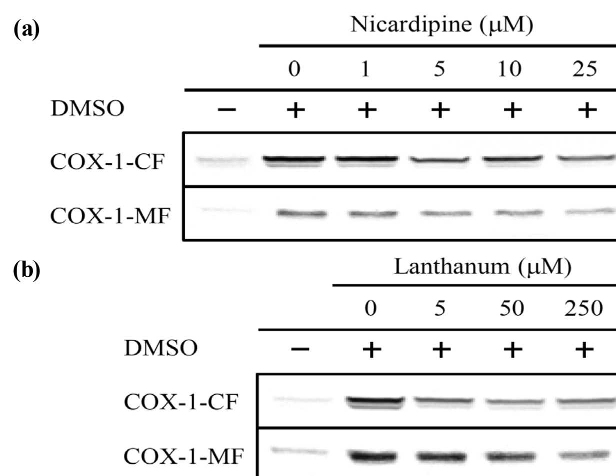


**Fig. 4.** The effect of the calcium channel blockers nicardipine (a) and lanthanum (b) on expression of recombinant COX-1 due to DMSO. Any noticeable effects on cell viability were not observed at a concentration of either 25 μM nicardipine or 250 μM lanthanum. CF and MF indicate cellular fraction and medium fraction, respectively.

by Western blot analysis. As shown in Fig. 4(a) and (b), expression of recombinant COX-1 due to DMSO was decreased by treatment with the calcium channel blockers nicardipine, an L-type voltage-dependent calcium ion blocker, and lanthanum, a calcium ion channel-entry blocker. Treatment with 25 μM nicardipine and 250 μM lanthanum reduced expression of recombinant COX-1 by 40% and 45%, respectively. In addition, exogenous supplementation with calcium chloride up to 28 mM did not improve expression of recombinant COX-1 in the absence of DMSO (data not shown). These indicates that an increase in the intracellular calcium ion concentration due to DMSO is probably caused by stimulation of a calcium ion influx across the plasma membrane.

## 4. Conclusions

DMSO increased intracellular calcium ion concentrations in stably transfected *Drosophila* S2 cells expressing recombinant COX-1. In calcium chelator-treated cells, DMSO did not increased the expression of *dNOS* transcript, which is closely related to synthesis of recombinant COX-1 mRNA in stably transfected S2 cells [19]. Expression of recombinant COX-1 due to DMSO was diminished in cells treated with calcium chelators or channel blockers. Our results indicate that an increased intracellular calcium ion concentration due to DMSO is associated with up-regulation of the *dNOS* gene, leading to enhanced expression of recombinant COX-1 in stably transfected S2 cells *via* induction of the MT promoter.

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