Short communication

# Vanadate inhibits endoplasmic reticulum stress responses

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**ABSTRACT**

The disruption of endoplasmic reticulum function leads to an accumulation of unfolded proteins, which results in endoplasmic reticulum stress. In the present study, we investigated the effect of vanadate on such stress. Endoplasmic reticulum stress increased glucose-regulated protein 78 (GRP78) and CCAAT/enhancer-binding protein homologous protein (CHOP) expressions in glial cell cultures. We found that vanadate inhibited the endoplasmic reticulum stress-induced increase in GRP78 and CHOP expressions at both mRNA and protein levels. Thus, these results suggest that vanadate modulates endoplasmic reticulum stress responses and that novel vanadate-responsive protein(s) might be involved in these processes.

**Key Words:** endoplasmic reticulum stress; vanadate; glucose-regulated protein 78 (GRP78); CCAAT/enhancer-binding protein homologous protein (CHOP)

## 1. **Introduction**

Endoplasmic reticulum stress has been implicated in the pathogenesis of diseases such as neurodegenerative diseases, diabetes, and virus infection (Katayama et al., 1999; Imai et al., 2001; Kaufman et al., 2002). When unfolded proteins accumulate in the endoplasmic reticulum, cells activate the endoplasmic reticulum stress response pathways for cellular protection, such as translational attenuation, the induction of endoplasmic reticulum chaperones (i.e., induction of glucose-regulated protein 78 (GRP78)), and degradation of unfolded proteins (Mori et al., 2000). On the other hand, when endoplasmic reticulum functions are severely impaired, the apoptotic pathway is activated. This apoptosis is mediated by factors such as caspases (Nakagawa et al.,2000; Hitomi et al., 2004) or C/EBP homologues protein (CHOP) (Zinszner et al., 1998; Harding et al., 2000). We previously reported that phosphatidylinositide-3-OH kinase (PI3K) and heat shock protein 90 (HSP90) inhibitors induced CHOP expression through 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)-sensitive serine protease (Hyoda et al., 2006; Hosoi et al., 2007a; Hosoi et al., 2007b). However, the intracellular mechanisms of these endoplasmic reticulum-regulated integrated stress responses are not well-understood.

Vanadate is a potent inhibitor of protein-tyrosine phosphatases (Swarup et al., 1982a; Swarup et al., 1982b), $Na^+/K^+$ ATPase (Cantley et al., 1977), and alkaline phosphatases (Lopez et al., 1976; Seargeant et al., 1979). In contrast, vanadate has been reported to activate adenylate cyclase (Schwabe et al., 1979). Moreover, it has been reported to alter a number of cellular functions (Shechter et al., 1980; Seglen et al., 1981; Carpenter et al., 1981). However, the pharmacological actions of vanadate in mammalian cells have not been well-clarified. In the present study, we investigated the possible involvement of vanadate in the endoplasmic reticulum stress response.

We found that vanadate inhibited endoplasmic reticulum stress-induced GRP78 and CHOP expressions at both mRNA and protein levels. These unique observations suggest that novel vanadate-responsive protein(s) might be involved in endoplasmic reticulum stress, and the possible physiological significance of this finding was discussed.

## 2. **Materials and Methods**

### *2.1. Materials and reagents*

Tunicamycin and thapsigargin were obtained from Wako Pure Chemical Ltd. (Japan). Sodium orthovanadate was purchased from Nacalai Tesque (Japan).

### *2.2. Cell culture*

The mouse DBT astrocytoma cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Nacalai Tesque, JAPAN) at 37°C in humidified 5% $CO_2$, 95% air.

### *2.3. Preparation of primary cultured glial cells*

Glial cells were prepared from the whole brains of neonatal C57BL/6 mice, as described previously (Hosoi et al., 2000). The cells were allowed to grow to confluency (10 days) in DMEM medium with 10% FCS and antibiotics (100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Nacalai Tesque, JAPAN). All cultured cells were kept at 37°C in 5% $CO_2$/95% air. Subsequently, mixed glial cells were shaken at 120 rpm for 18 h, cultured again for 5 to 7 days in 35 mm dishes, and then used in the following experiments. At this point, the astrocyte cultures were routinely >95% positive for glial fibrillary acidic protein.

### *2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*

Total RNA was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). RT-PCR was performed as described previously (Hosoi et al., 2007a). Specifically,

cDNA was synthesized from total RNA by reverse transcription using 100 U of Superscript Reverse Transcriptase (Invitrogen) and Oligo $(dt)_{12-18}$ primer (Invitrogen) in a 20 μl reaction mixture containing Superscript buffer (Invitrogen), 1 mM dNTP mix, 10 mM dithiothreitol (DTT), and 40 U of RNase inhibitor. Total RNA and Oligo $(dt)_{12-18}$ primer were incubated at 70°C for 10 min prior to the reverse transcription. After incubation for 1 h at 42°C, the RT reaction was terminated by denaturing the Reverse Transcriptase enzyme for 15 min at 70°C. For PCR amplification, 1.2 μl of cDNA was added to 12 μl of a reaction mix containing 0.2 μM of each primer, 0.2 μM of dNTP mix, 0.6 U of Taq polymerase, and reaction buffer. PCR was performed in a DNA Thermal Cycler (GeneAmp® PCR System 9700). The following primers were used: GRP78 upstream, 5'-ctg ggt aca ttt gat ctg act gg-3'; GRP78 downstream, 5'-gca tcc tgg tgg ctt tcc agc cat tc-3'; CHOP upstream, 5'-ccc tgc ctt tca cct tgg-3'; CHOP downstream, 5'-ccg ctc gtt ctc ctg ctc-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) upstream, 5'-aaa ccc atc acc atc ttc cag -3'; and GAPDH downstream, 5'-agg ggc cat cca cag tct tct-3'. The PCR products (10 μl) were resolved by electrophoresis in an 8% polyacrylamide gel in TBE buffer. The gels were stained with ethidium bromide and then photographed under ultraviolet light. cDNA for GAPDH, GRP78, and CHOP were amplified and these PCR reactions were run separately.

*2.5. Western blotting analysis*

Western blotting was performed as described previously (Hosoi et al., 2007c). Cells were washed with ice-cold PBS and lysed in a buffer containing 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM $Na_3VO_4$, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1% NP-40 for 20 min. The lysates were centrifuged at 15,000 rpm for 20 min at 4°C, and the

supernatants were collected. The samples were boiled with laemmli's buffer (62.5 mM Tris, 2% sodium dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue, pH 6.8) for 3 min, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred at 4°C to nitrocellulose membranes. The membranes were incubated with anti-KDEL (for GRP78) (Stressgen; 1:1,000), anti-CHOP (Santa Cruz; 1:500) and anti-phospho Tyr (Upstate; 1:1,000) antibodies and then with anti-horseradish peroxidase-linked antibody. Peroxidase was detected by chemiluminescence using an ECL system (Amersham). The protein bands that cross-reacted with antibodies were detected on X-ray films.

*2.6. Statistics*

Results are expressed as means ± S.E.M. Statistical analysis was performed with Student's *t*-test or the paired *t*-test.

## 3. **Results**

### *3.1. Vanadate inhibited endoplasmic reticulum stress responses*

Endoplasmic reticulum stress triggers the cellular unfolded protein responses, which result in an increase in the unfolded protein response-regulated genes such as GRP78, an endoplasmic reticulum-resident chaperone, or CHOP, an endoplasmic reticulum stress-induced apoptotic transcription factor. Endoplasmic reticulum stress was induced by tunicamycin, which inhibits protein glycosylation (Wang et al., 1998; Iwawaki et al., 2001). To investigate whether endoplasmic reticulum stress was induced in the present experimental conditions, we measured GRP78 and CHOP expression. As assessed by RT-PCR, endoplasmic reticulum stress (Tm: tunicamycin, 0.1 μg/ml, 6 h) markedly increased GRP78 and CHOP expression in the DBT cell line (Fig. 2A-D). Moreover, endoplasmic reticulum stress (Tm: tunicamycin, 0.1 μg/ml, 24 h) also increased GRP78 and CHOP expression at protein levels (Fig. 3). Vanadate is known to be a potent inhibitor of tyrosine phosphatases. As shown in Figure 1, we observed an increase in phosphorylated tyrosine levels in vanadate-treated cells. Thus, we next investigated whether vanadate affects these endoplasmic reticulum stress responses. Vanadate (100 μM, 7 h) alone did not affect the expression levels (mRNA or protein levels) of GRP78 or CHOP (Figs. 2 and 3). However, vanadate (100 μM) completely inhibited the endoplasmic reticulum stress-induced increase in GRP78 and CHOP expression both at mRNA and protein levels in the DBT astrocytoma cell line (Figs. 2CD and 3AB). Similar results were obtained using mouse primary cultured glial cells. Vanadate inhibited endoplasmic reticulum stress (tunicamycin- or thapsigargin-induced endoplasmic reticulum stress)-induced GRP78 or CHOP expression in the primary cultured glial cells (Fig. 3C). The inhibitory effect of vanadate on the endoplasmic reticulum stress-induced expression of GRP78 or CHOP

was dose dependent (10-1,000 μM, Fig. 2AB). Thus, these results suggest that vanadate modulates endoplasmic reticulum stress responses, and that novel vanadate-responsive protein(s) might be involved in these processes.

On the other hand, we observed that the long-term treatment (36 h~) of cells with Tm alone increased cell death. Interestingly, we also found that vanadate alone increased cell death and that this was inhibited by treatment with Tm (unpublished observation). The mechanisms underlying these observations are not understood, and so future studies are needed.

## 4. **Discussion**

Increasing evidence has suggested that endoplasmic reticulum stress is involved in neurodegenerative diseases, diabetes, and viral infection (Katayama et al., 1999; Y. Imai et al., 2001; Kaufman et al., 2002). However, detailed mechanisms of endoplasmic reticulum stress responses are poorly understood. In the present study, we found that vanadate inhibited endoplasmic reticulum stress responses (GRP78 and CHOP induction), suggesting that vanadate-responsive factor(s) are involved in these processes.

Endoplasmic reticulum stress-regulated signal transducing sensor kinase is characterized as an endoplasmic reticulum-resident transmembrane kinase such as inositol-requiring protein-1 (IRE1) or double-stranded-RNA-dependent protein kinase-like endoplasmic reticulum kinase (PERK) (Tirasophon et al., 1998; Wang et al., 1998; Harding et al., 1999). In response to endoplasmic reticulum stress, IRE1 activates cJUN $NH_2$-terminal kinases (JNK) through TNFR-associated factor 2 (TRAF2), an adaptor protein that couples plasma membrane receptors to JNK activation (Urano et al., 2000). PERK activation phosphorylates eukaryotic initiation factor 2α (eIF2α) at Ser51, which results in the inhibition of translation initiation (Harding et al., 2000a). Thus, IRE1 and PERK activation is regulated through its Ser/Thr protein kinase activity. Indeed, okadaic acid, a protein Ser/Thr phosphatase inhibitor, has been reported to enhance GRP78 expression in endoplasmic reticulum-stressed cells (Price et al., 1992; Cao et al., 1995). On the other hand, vanadate is a potent inhibitor of protein tyrosine, but not protein serine phosphatases (Swarup et al., 1982a; Swarup et al., 1982b). We observed an increase in phosphorylated tyrosine levels in a vanadate-treated DBT cell line. As vanadate inhibited endoplasmic reticulum stress-induced GRP78 or CHOP expression, it is possible that protein tyrosine

phosphatases are involved in endoplasmic reticulum stress. Indeed, protein-tyrosine phosphatase 1B (PTP-1B) has been reported to potentiate IRE1 signaling during endoplasmic reticulum stress (Gu et al., 2004). Thus, it is possible that protein-tyrosine phosphatases may positively regulate endoplasmic reticulum stress responses. On the other hand, in agreement with previous reports (Price et al., 1992; Cao et al., 1995), we observed that genistein, a tyrosine kinase inhibitor, inhibited the endoplasmic reticulum stress-induced expression of GRP78 and CHOP (unpublished observation). These results raise the possibility that the inhibitory effect of vanadate on endoplasmic reticulum stress would be mediated independently through inhibiting protein tyrosine phosphatases. Vanadate has other pharmacological actions in addition to inhibiting protein tyrosine phosphatases. Thus, it is possible that the inhibitory effect of vanadate on endoplasmic reticulum stress would be mediated through these other vanadate-responsive factor(s). It is possible that the effect of vanadate on the endoplasmic reticulum stress signal would be mediated through $Na^+/K^+$ ATPase and/or alkaline phosphatases, as vanadate has been reported to inhibit these factors (Cantley et al., 1977; Seargeant et al., 1979). Na+/K+ ATPase has been reported to be inhibited up to 50% by 40 nM vanadate under optimal conditions (28 mM $Mg^{2+}$) (Cantley et al., 1977). In addition, vanadate was shown to be a potent competitive inhibitor ($K_i$ less than 1 μM) of purified alkaline phosphatase from the human liver, intestine, or kidney (Seargeant et al., 1979). On the other hand, vanadate has been reported to activate adenylate cyclase in membranes isolated from rat fat cells (Schwabe et al., 1979). The stimulatory effect of vanadate was concentration (10-1,000 μM)-dependent, which led to significant activation at 10 μM (Schwabe et al., 1979). Moreover, vanadate dose-dependently (10-80 μM) activated both NF-κB and c-Jun N-terminal kinase (JNK) in macrophages (Chen et al., 1999). Furthermore, vanadate has been shown to induce

cyclooxygenase (COX) isoenzymes, key enzymes in prostaglandin synthesis (Hirai et al., 1997). In the present study, we found that vanadate dose-dependently (10-1,000 μM) inhibited endoplasmic reticulum stress responses. The present dose range of the pharmacological effects of vanadate would correspond with these reported concentrations. Thus, it is possible that these vanadate-sensitive proteins would be involved in endoplasmic reticulum stress signal transduction. However, as the experimental conditions of the previous reports are different from the present ones, detailed analysis is needed in future experiments. At present, it is unknown whether and what endoplasmic reticulum-residing proteins are responsible for vanadate-sensitive endoplasmic reticulum stress signals, but the results provide novel information to aid in the understanding of endoplasmic reticulum stress-related diseases.

**Figure legends**

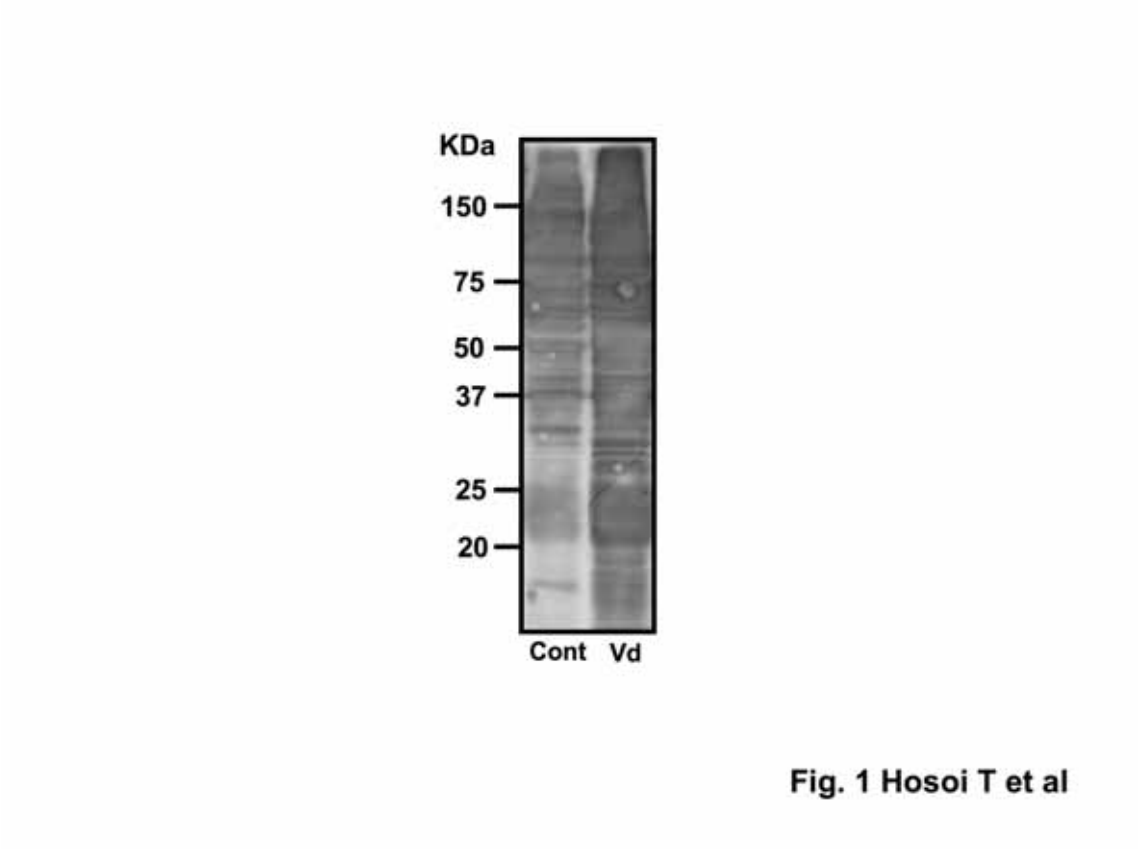


**Fig.1 Effect of vanadate on phosphorylated tyrosine levels in DBT cell line.**

DBT cells were treated with vanadate (Vd: 100 μM) for 6 h, and Western blotting was performed using phospho-tyrosine antibody. Vanadate markedly increased phospho-Tyr levels in the DBT cell line.

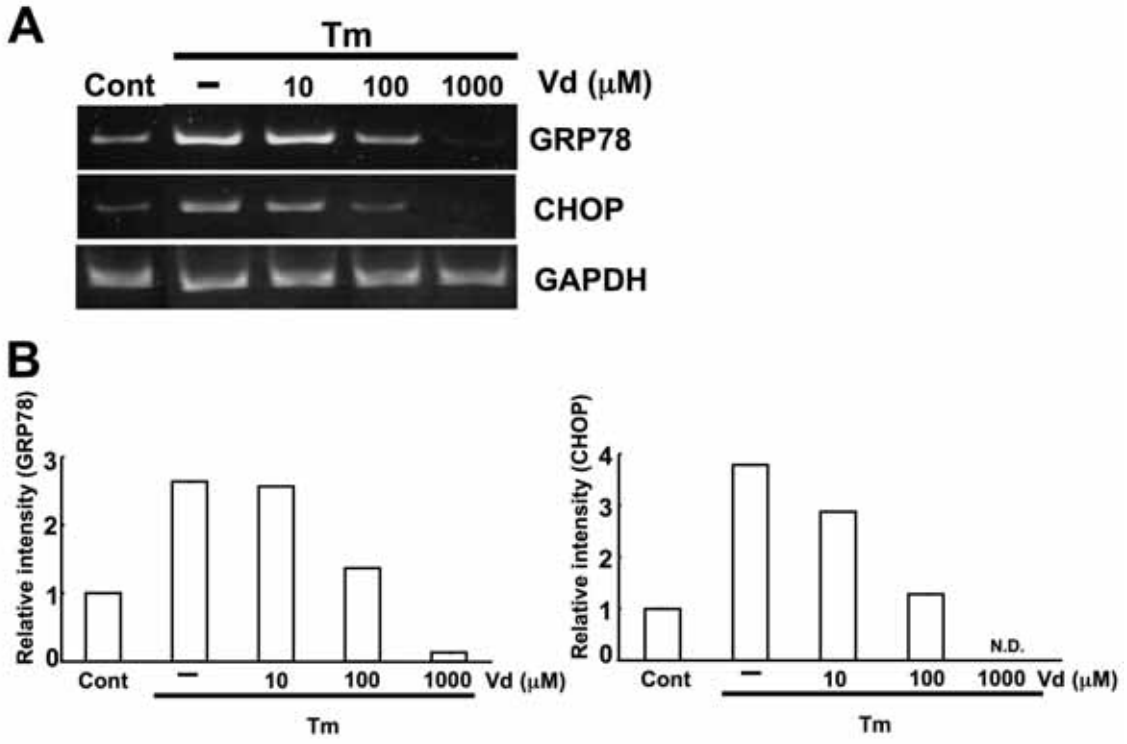


Fig.2 (AB) Hosoi T et al

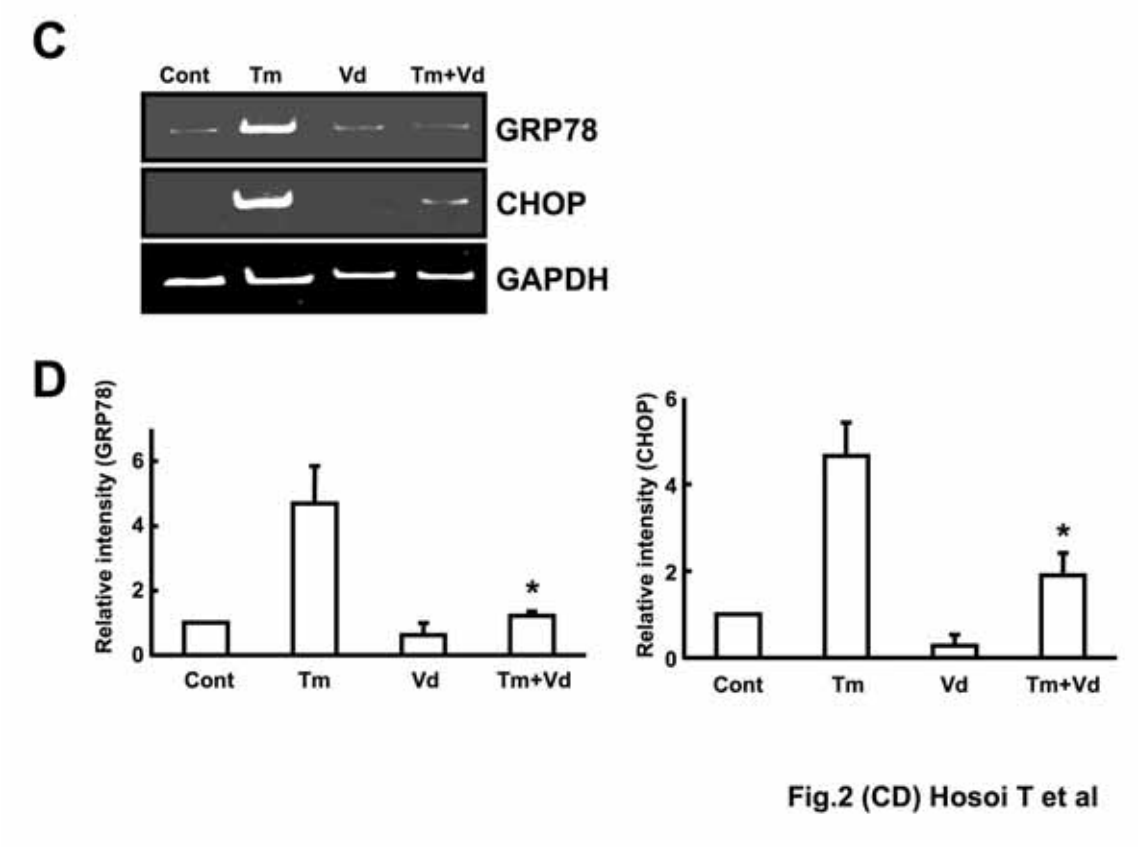


**Fig. 2 Effect of vanadate on endoplasmic reticulum stress-induced GRP78 and CHOP mRNA expression.**

(A) DBT cells were pre-treated with vanadate (Vd: 10-1000 μM) for 1 h and then with tunicamycin (Tm: 0.1 μg/ml) for 6 h. RT-PCR analysis was performed using specific primers for each mRNA. (B) Quantification of the RT-PCR analysis was performed by densitometric analysis using image-analyzing software. (C) DBT cells were pre-treated with vanadate (Vd: 100 μM) for 1 h and then with tunicamycin (Tm: 0.1 μg/ml) for 6 h. RT-PCR analysis was performed using specific primers for each mRNA. Data are from an experiment representative of three independent experiments. (D) The quantification of RT-PCR analysis was performed by densitometric analysis using image-analyzing software. Values are presented as means ± S.E.M. (n=3 per group). GRP78 and CHOP: $*P < 0.05$ (Student's *t*-test: significant difference between

Tm and Tm+Vd)

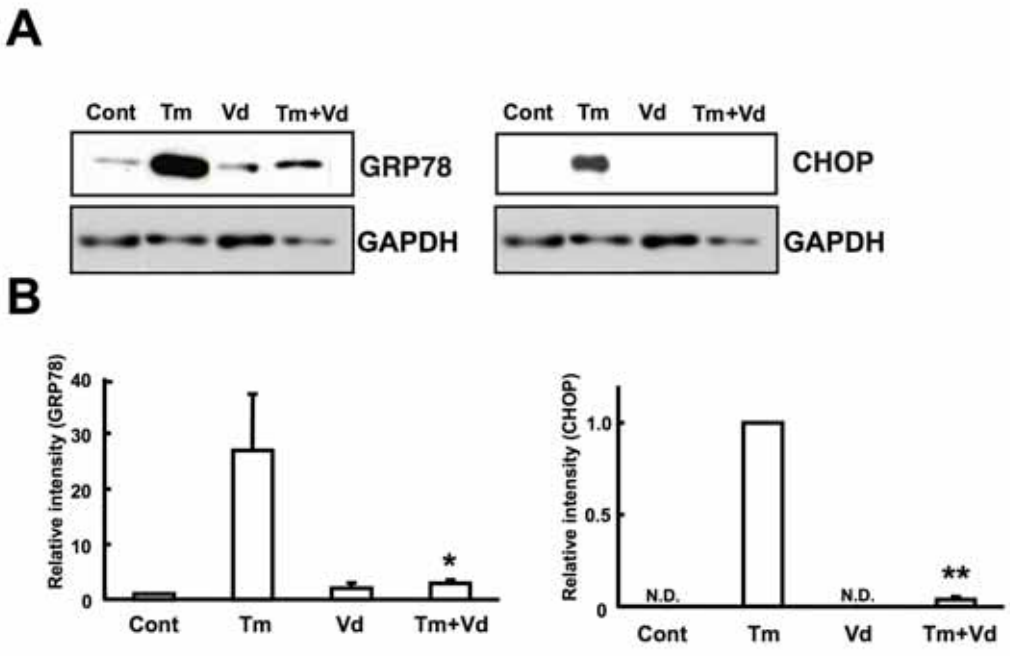


**Fig.3 (AB) Hosoi T et al**

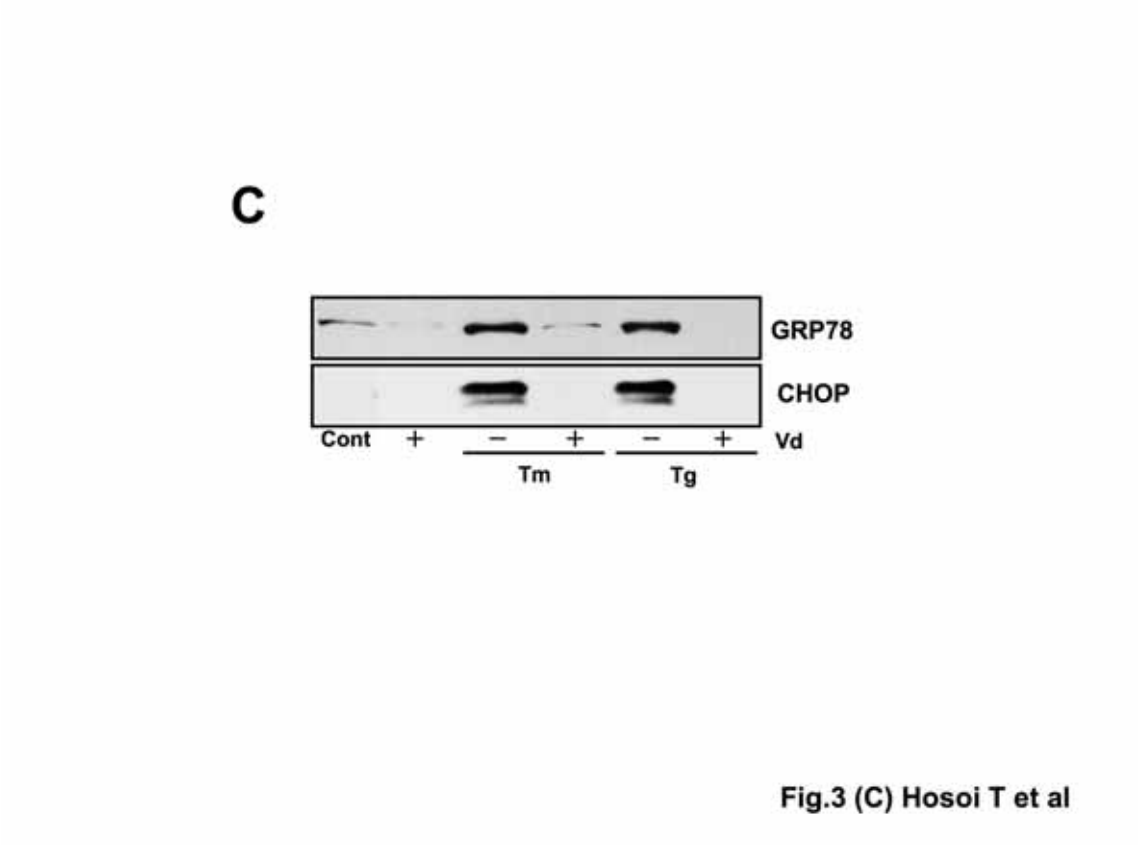


**Fig. 3 Effect of vanadate on endoplasmic reticulum stress-induced GRP78 and CHOP protein expression.**

(A) DBT cells were pre-treated with vanadate (Vd: 100 μM) for 1 h and then with tunicamycin (Tm: 0.1 μg/ml) for 24 h. Samples were separated by SDS gel electrophoresis followed by immunoblot analysis using anti-KDEL (GRP78) or anti-CHOP antibodies. CHOP expression was not detected (N.D.) in control (Cont) and vanadate (Vd)-treated cells. Data are from an experiment representative of three independent experiments. (B) The quantification of Western blot analysis was performed by densitometric analysis using image-analyzing software. Values are presented as means ± S.E.M. (n=3 per group). GRP78: $*P < 0.05$ (Student's *t*-test: significant difference between Tm and Tm+Vd), CHOP: $**P < 0.01$ (Paired *t*-test: significant difference between Tm and Tm+Vd). (C) Mouse primary cultured glial

cells were treated with vanadate (Vd: 500 μM) for 1 h and then stimulated with tunicamycin ( Tm: 0.1μg/ml) or thapsigargin (Tg: 0.1 μM) for 24 h. Samples were separated by SDS gel electrophoresis followed by immunoblot analysis using anti-KDEL (GRP78) or anti-CHOP antibodies.

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