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Report

Analysis of OSW-1-Induced Stress Responses in HT-29 Cells

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OSW-1, a promising compound that is toxic to diverse tumor cell lines, is a saponin from Ornithogalum saundersia. In this study, we analyzed the stress responses induced by OSW-1 using the human colon cancer cell line HT-29 cells and compared it with the commonly used ER and Golgi stress inducers brefeldin A (BFA), thapsigargin (Tg), and tunicamycin (Tm). OSW-1 induced few ER stress-related factors, but there was an increase in expression of TFE3 protein, one of the Golgi stress response factors. A shift in the molecular weight of TFE3 was also found, likely attributable to dephosphorylation. Conversely, the impact of OSW-1 on the expression of the TFEB protein, another member of the MiTF/TFE family, was minimal. Cleavage of CREB3, another Golgi stress sensor, was apparently induced only by BFA. LC3-II and p62, autophagy-related factors, were increased in all drug treatments. Unexpectedly, OSBP protein levels, one of the targets of OSW-1, were increased by not only three reagents but also OSW-1. Taken together, OSW-1 treatment of HT-29 cells induced atypical Golgi stress that strongly activated the TFE3 pathway and did not involve the CREB3 pathway or the ER stress response. Although OSW-1 was also found to affect the autophagy system, it was suggested that the effects of OSW-1 may not be mediated by OSBP depletion. These findings will contribute to the development of OSW-1-based cancer therapies and to our understanding of Golgi stress responses.

Key words CREB3, Golgi stress, OSW-1, TFE3, TFEB

INTRODUCTION

OSW-1 is a member of the steroidal saponins, first isolated in 1992 from the bulbs of *Ornithogalum saundersiae*.[1] This saponin has a unique structure in that it contains an acylated disaccharide moiety attached to the C16β-OH position of the D ring of the sapogenin aglycon and its structure is markedly different from many other saponins.[2,3] This OSW-1 is considered one of the most promising anti-tumor compounds because of its toxicity at very low concentrations against various types of tumor cells.[3,4] Although oxysterol binding protein (OSBP) and its homologues have been reported as one target,[3,5,6] the detailed mechanisms of OSW-1-induced cellular damage are not fully understood. The ORP family including OSBP has evolutionally conserved sterol-binding domains and controls the transport and sensing of lipid/cholesterol transport. OSBP-VAP complex at the membrane contact sites between the ER and Golgi apparatus is reported to be involved in the exchange and transport of cholesterol and phosphatidyl inositol 4-phosphate.[6] Recently, it was reported that this OSW-1 induces Golgi stress signaling resulting from Golgi damage or dysfunctions.[7] Abnormalities in the ER involved in vesicular transport to the Golgi apparatus are recognized by IRE1, ATF6, and PERK. These stress sensors on the ER membrane subsequently induce several downstream factors.[8,9] On the other hand, the Golgi stress response has been proposed to involve the MiTF/TFE family regulating Golgi structural proteins, N-glycosyltransferases, vesicular transport-related factors, and CREB3 and Hsp47.[10-13] CREB3 is a transcription factor that resides in the ER, similar to ATF6, while Hsp47 functions as a collagen chaperone. However, the details of the Golgi stress response are not fully understood.

Recently, we elucidated the OSW-1-induced stress responses on Neuro2a cells and found OSW-1 triggered atypical Golgi stress response that is activated by TFE3/TFEB but not by CREB3.[14] Based on the findings, we analyzed the OSW-1-in-

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duced stress responses including the expression of ER and Golgi stress-related factors using a different tumor cell line, a human colon cancer cell line HT-29. As observed in Neuro2a cells, OSW-1-treated HT-29 cells showed atypical Golgi stress response. Particularly, TFE3 protein was remarkably increased by OSW-1. Unexpectedly, OSW-1 induced both OSBP and LC3-II protein expression in HT-29 cells. This suggests that these stress responses and cytotoxicity by OSW-1 are OSBP-independent, which will be useful for future development of OSW-1-based drugs.

MATERIALS AND METHODS

Cell Culture and Treatment HT-29 cells were maintained in RPMI1640 medium containing 5% heat-inactivated fetal bovine serum (Invitrogen). HT-29 cells in 96- or 6-well plates were treated with or without thapsigargin (Tg, 10 nM), tunicamycin (Tm, 2 μg/ml), brefeldin A (BFA, 0.5 μg/ml) or OSW-1 at the indicated concentration.

Measurement of Cell Viability and Morphological Observation For the measurement of cell viability using a Cell Counting Kit (a WST-8 reagent) (Dojindo),[14] HT-29 cells in a 96-well plate (5000 cells/well) were treated with the indicated reagents or vehicle at the indicated concentration and cultured for 16, 24 or 48 h. During the last two hours, WST-8 solution was added to each well and incubated at 37°C according to the manufacturing instructions. The difference between absorbance at 450 and 650 nm was measured as an indicator of cell proliferation. Each absorbance in the untreated cells was respectively defined as 1.0.

Reverse Transcription Polymerase Chain Reaction To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with TRI reagent (Molecular Research Center), and equal amounts of total RNA from each sample were converted to cDNA by reverse transcription using a random nine-mer primer with Revertra Ace reverse transcriptase (RT) (TOYOBO) according to the manufacturing instructions. Each cDNA was added to a PCR mixture for amplification (Taq PCR Kit, Takara).[14,15] The PCR primers used in this study are shown in Table 1. The typical reaction cycling conditions were as follows: 30 sec at 96°C, 30 sec at 58°C and 30 sec at 72°C. The results represent 21-30 cycles of amplification. The products were separated by electrophoresis on 2.0 or 2.5% agarose gels and visualized using ethidium bromide. The expression level of each mRNA was analysed using the ImageJ software (National Institutes of Health), and the relative amount of each mRNA was calculated based on the G3PDH value obtained from the identical cDNA.[14,15]

Table 1. PCR primer pairs used in this study.

ABCA1 sense primer	5'-TTCTGAGCTTTGTGGCCTAC-3'
ABCA1 antisense primer	5'-CATGTCACTCCAGCTTCTCA-3'
Arf4 sense primer	5'-GTCACCACCATTCCTACCAT-3'
Arf4 antisense primer	5'-TACGAAGAGACTGAAGCCCT-3'
c-Myc sense primer	5'-TGCTGGGAGGAGACATGGT-3'
c-Myc antisense primer	5'-TGTCGTTGAGAGGGTAGG-3'
CREB3 sense primer	5'-CTGAAACGTGTGCGGAGGAA-3'
CREB3 antisense primer	5'-GAGGAGGCAGAAGGAGACTA-3'
GADD153 sense primer	5'-CGGAAACAGAGTGGTCATTC-3'
GADD153 antisense primer	5'-TGCGTATGTGGGATTGAGGGTC-3'
GRP78 sense primer	5'-ATGAAAGAAACCGCTGAGGCTT-3'
GRP78 antisense primer	5'-ATGTTCTTCTCCCCCTCCCTCT-3'
G3PDH sense primer	5'-ACCACAGTCCATGCCATCAC-3'
G3PDH antisense primer	5'-TCCACCACCCTGTTGCTGTA-3'
TFE3 sense primer	5'-GAGATGCGCTGGAACAAGG-3'
TFE3 antisense primer	5'-CCCCCCCTACATGGAACGT-3'
TFEB sense primer	5'-CATCAATACCCCCGTCCACT-3'
TFEB antisense primer	5'-ACTTGTTCCCATAGGTCTCG-3'
XBP1 sense primer	5'-CGGCCTTGTGGTTGAGAA-3'
XBP1 antisense primer	5'-ACTTGTCCAGAATGCCCA-3'

Western Blotting Analysis The cells were lysed and the protein concentration in each cell lysate was determined as described previously.[14,15] Each cell lysate was dissolved in an equal amount of 2× sodium dodecyl sulfate (SDS)-Laemmli sample buffer (62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol and 12% 2-mercaptoethanol), and equal amounts of protein were separated on 10 or 12.5% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (GE Healthcare) and identified by enhanced chemiluminescence (GE Healthcare) using antibodies against ATF4 (Santa Cruz Biotechnology), c-Myc (Santa Cruz Biotechnology), CREB3 (Proteintech), G3PDH (Proteintech), LC3 (MBL), OSBP (Proteintech), p62 (MBL), TFE3 (Cell Signaling Technology) and TFEB (Cell Signaling Technology). The expression level of each protein was analyzed using ImageJ software (National Institutes of Health), and the relative amount of each protein was calculated based on the G3PDH value obtained from the same lysate.[14,15]

Statistical Analysis The results are expressed as the mean ± SEM. Statistical analyses were carried out using one-way ANOVA followed by the Tukey-Kramer test (GraphPad Prism, GraphPad Software, CA, USA).[14,15] $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Cell Morphology and Viability Changes Induced by OSW-1 in HT-29 Cells First, HT29 cells were treated with different concentrations of OSW-1, and changes in cell morphology and cell viability by WST-8 assay were measured. As shown in Fig. 1, OSW-1 at 1 nM had little effect on HT-29 cells. On the other hand, OSW-1 at 10 and 100 nM caused slight changes in cell morphology after 24 h. Forty-eight hours after OSW-1 treatment, many cells lost intercellular adhesion and became rounded. Cell viability by WST-8 assay showed negligible change after 24 h treatment, but OSW-1 concentration-dependent cytotoxicity was observed after 48 h treatment, reflecting changes in cell morphologies.

Analysis of Stress Responses Induced by OSW-1 and Other Agents in HT-29 Cells Based on the reports that OSW-1 is known to target OSBPs involved in cholesterol transport from the ER to the Golgi apparatus and our previous study in Neuro2a cells,[3,5,14] we analyzed stress responses in HT-29 cells upon OSW-1. In addition, we compared these stress responses with those triggered by commonly utilised ER/Golgi stress inducers, BFA,[16] Tg[17] and Tm[18]. Treatment with BFA for 16 h caused the most significant morphological changes (Fig. 2A). On the other hand, treatment with OSW-1 for 16 h had marginal morphological effects. In parallel, cell viability was markedly decreased by BFA treatment (Fig. 2B). We then analyzed the changes in gene expression after 12 hours of treatment with each reagent. Three ER/Golgi stress inducers increased GADD153, GRP78 and sXBP1

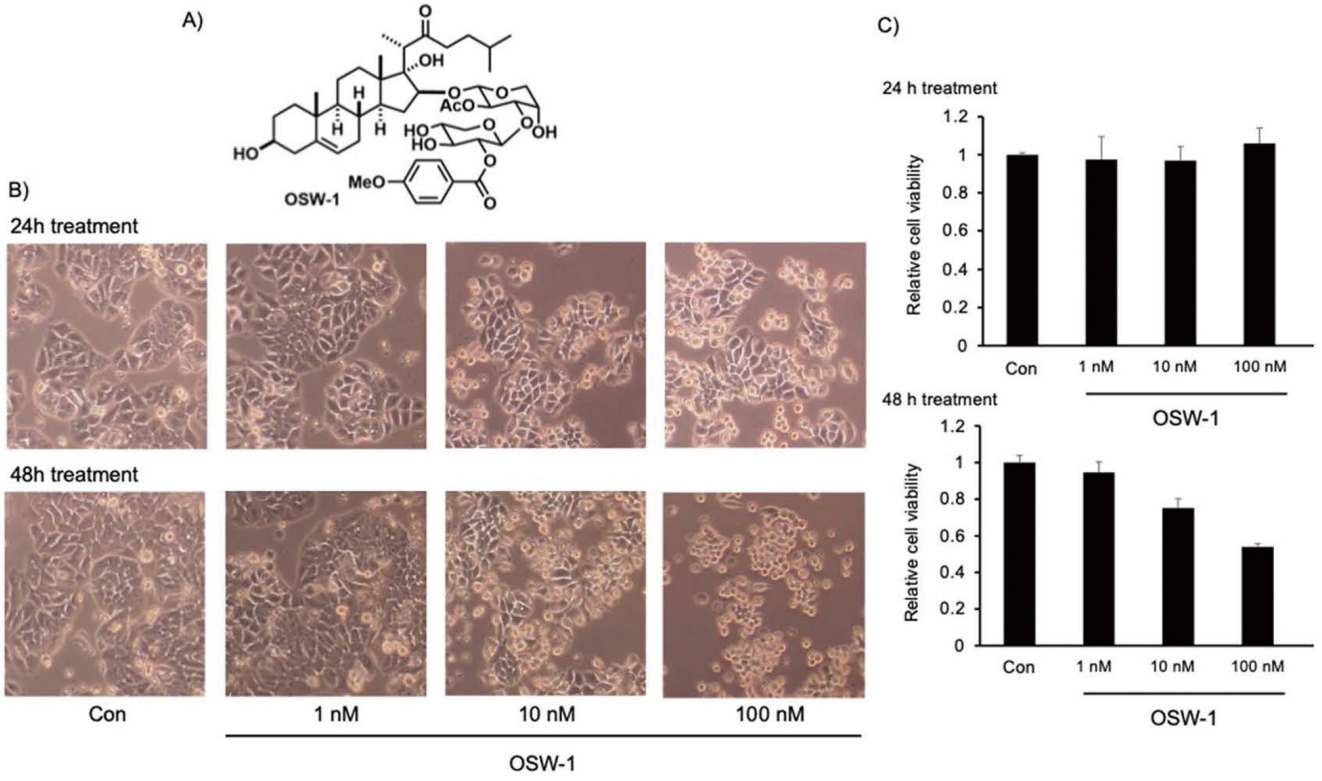

Fig. 1. OSW-1 Induced HT-29 Cell Damages in a Dose-Dependent Manner

A) Structure of OSW-1. B) HT-29 cells were treated with the indicated concentration of OSW-1 or vehicle for 24 or 48 h, and cell morphology was observed. C) HT-29 cells were treated with OSW-1 at the indicated concentration or vehicle for 24 or 48 h. Cell viability was measured as described in the materials and methods. The value obtained from the untreated cells was considered as "1.0". Each value represented the mean ± SEM from 6 independent cultures.

mRNA, but little increase was observed upon OSW-1 stimulation (Fig. 2C and D). There were also marked differences between OSW-1 and the other three stimuli with respect to the expression of CREB3, TFE3, and TFEB mRNA, which are presumed to be Golgi stress sensors.[10] OSW-1 stimulation hardly fluctuated expression of either mRNA in HT-29 cells, but the other three stimuli significantly induced CREB3 mRNA and decreased TFEB mRNA. Arf4, which is reported to be regulated by CREB3 was significantly increased by BFA.[19] Tg and Tm slightly but not significantly increased Arf4 mRNA and OSW-1 did not. ABCA1 mRNA, which was increased only in OSW-1-treated Neuro2a cells,[14] was similar in HT-29 cells. This increase may reflect the fact that OSW-1, which has a cholesterol skeleton, affects cholesterol transport systems such as OSBP-VAP.[20] Although c-Myc is well known as an important factor in the development and progression of various cancers,[21] none of the stimuli had any effect on c-Myc mRNA expression.

Analysis of TFE3, TFEB, and CREB3 Protein Expression in Response to Treatments Next, changes in expression of TFE3, TFEB and CREB3 proteins involved in Golgi stress signaling were analyzed after 16 h treatment with each reagent. It is known that TFE3 and TFEB are in a hyperphosphorylated state when inactivated and are activated by dephosphorylation.[11,22-24] As shown in Fig. 3A, treatment with OSW-1 significantly increased the two TFE3 bands, and the lower band seems to be slightly shifted downward by OSW-1 and BFA. As observed in GADD153, GRP78, and sXBP1 mRNA induction, the signaling pathway triggered by ER stress inducers (Tg and Tm) appears to be different from that of OSW-1. However, they showed similar effects on TFE3 protein expression. In contrast, TFEB protein expression after OSW-1 treatment was different from other stimuli. OSW-1 marginally elevated high molecular weight TFEB, but other stimuli resulted in a reduction in high molecular weight TFEB. Interestingly, Tg treatment significantly increased low molecular weight TFEB. In this study, the changes in TFE3/TFEB mRNA expression induced by OSW-1 and other stimuli did not coincide with changes in each protein expression. It has been reported that phosphorylation of TFE3/TFEB by mTOR is involved in ubiquitination and its subsequent degradation at proteasomes;[23,24] however, the precise mechanisms in HT-29 cells are unclear. Since mTOR is closely related to the regulation of authophagy as described below, it may be an important target for elucidating the mechanism of OSW-1-induced cellular damage. In addition, it is reported that TFE3/TFEB is involved in integrated stress response, these fluctuations of TFE3/TFEB in HT-29 cells may be involved in diverse stress responses besides Golgi stress.[25]

Expression of CREB3 protein was not altered by OSW-1 stimulation, similar to the previous results in Neuro2a cells.[14] Interestingly, there was a marked increase in full-length in parallel to an increase in CREB3 mRNA in BFA-stimulated HT-29 cells. Tg stimulation did not induce truncated CREB3, but did induce the full-length form. In addition, the amount of cleaved CREB3 protein by BFA treatment is markedly increased compared to the full-length form when unstimulated. This implies that the regulation of cleaved CREB3 pro-

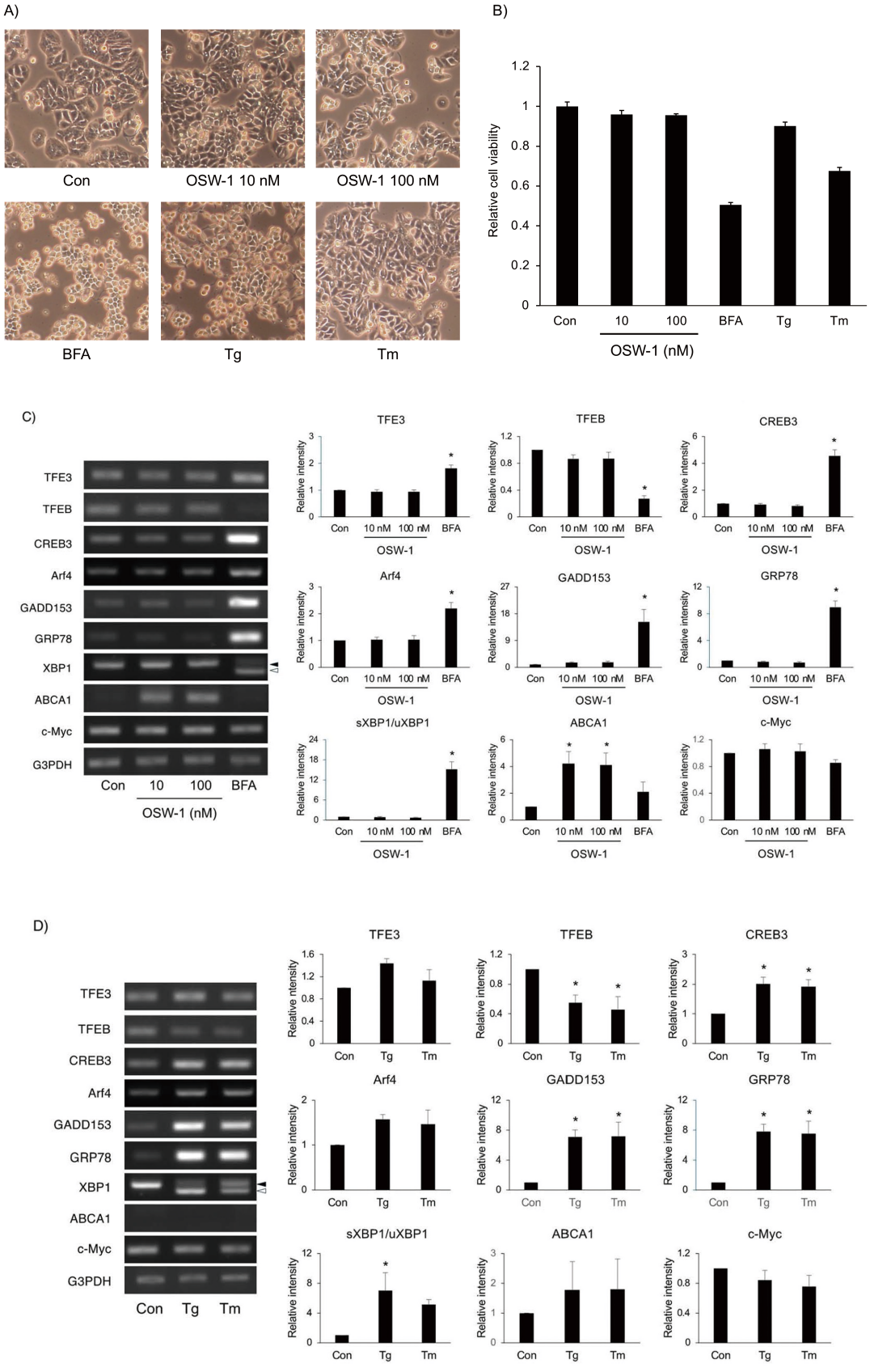

Fig. 2. Analysis of Gene Expression in OSW-1 Treated HT-29 Cells

HT-29 cells were treated with the indicated concentration of OSW-1, BFA (0.5 μg/ml), Tg (10 nM), Tm (2 μg/ml) or vehicle for 16 h, and cell morphology (A) and cell viability (B) was evaluated. Cell viability was measured as described in the materials and methods. The value obtained from the untreated cells was considered as "1.0". Each value represented the mean ± SEM from 4 independent cultures. C, D) HT-29 cells were treated with the OSW-1 (10 or 100 nM), BFA (0.5 μg/ml), Tg (10 nM), Tm (2 μg/ml) or vehicle for 12 h and the indicated gene expression was evaluated by RT-PCR as described in the materials and methods. The amount of each mRNA in the untreated control cells (con) was considered "1.0". Each value represents the mean ± SEM from 4-5 independent cultures. The filled and open arrowheads indicate the unspliced and spliced XBP1, respectively. Values marked with asterisks are significantly different from the value of untreated cells (* $p < 0.05$).

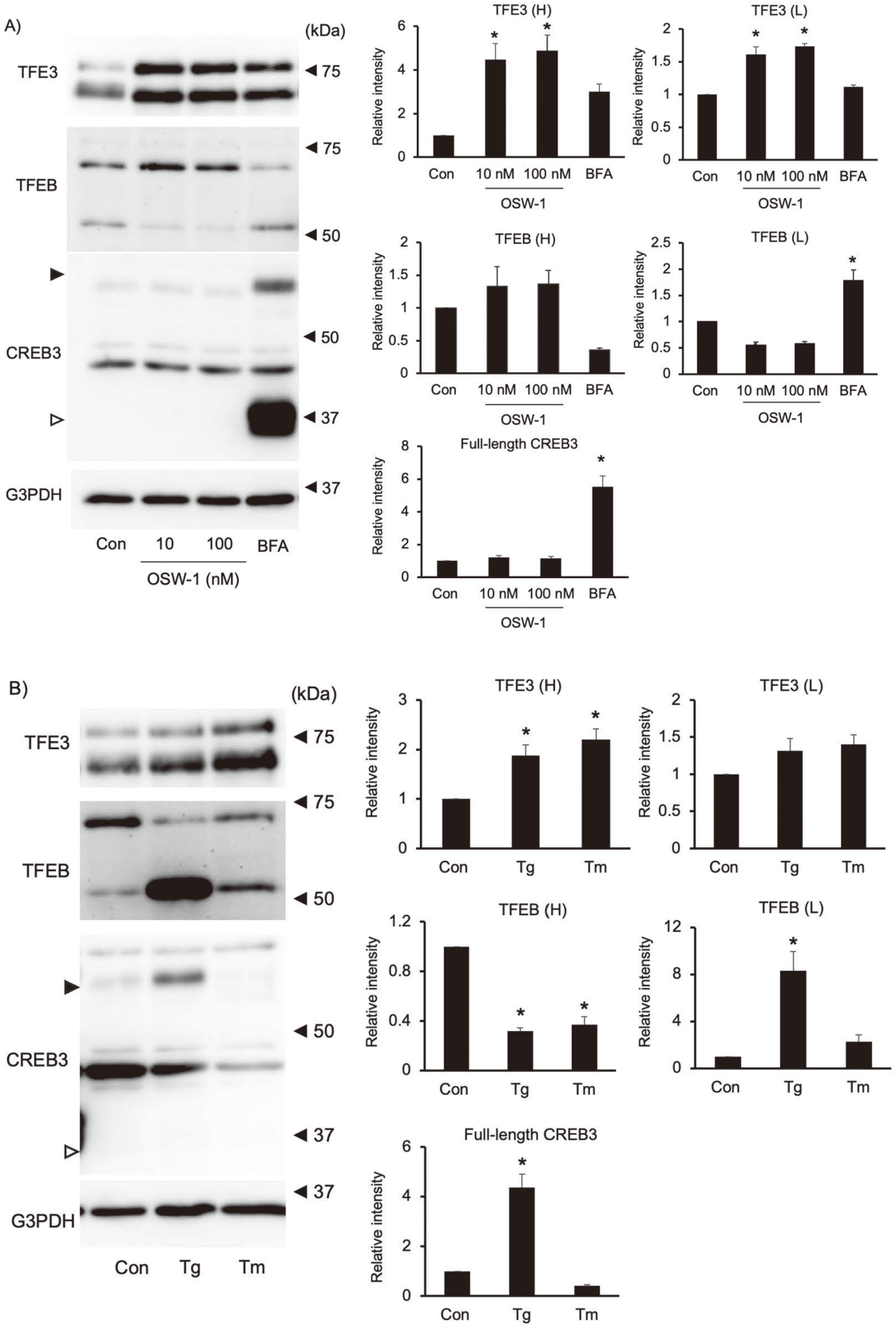

Fig. 3. OSW-1 Induced Atypical Golgi Stress in HT-29 Cells

HT-29 cells were treated with the indicated concentration of OSW-1 (10 or 100 nM), BFA (0.5 μg/ml) or vehicle (A) or Tg (10 nM), Tm (2 μg/l) or vehicle (B) for 16 h and the indicated protein expression was evaluated by western blotting analysis. The higher and lower bands of TFE3 and TFEB are shown as TFE3(H)/TFEB(H) and TFF3(L)/TFEB(L), respectively. The filled and open arrowheads indicate the full-length and cleaved CREB3, respectively. The relative amount of each protein was calculated as described in the materials and methods. The values obtained from the untreated control cells were considered as "1.0". Each value represented the mean ± SEM from 4-5 independent cultures. Values marked with asterisks are significantly different from the value of untreated cells (* $p < 0.05$).

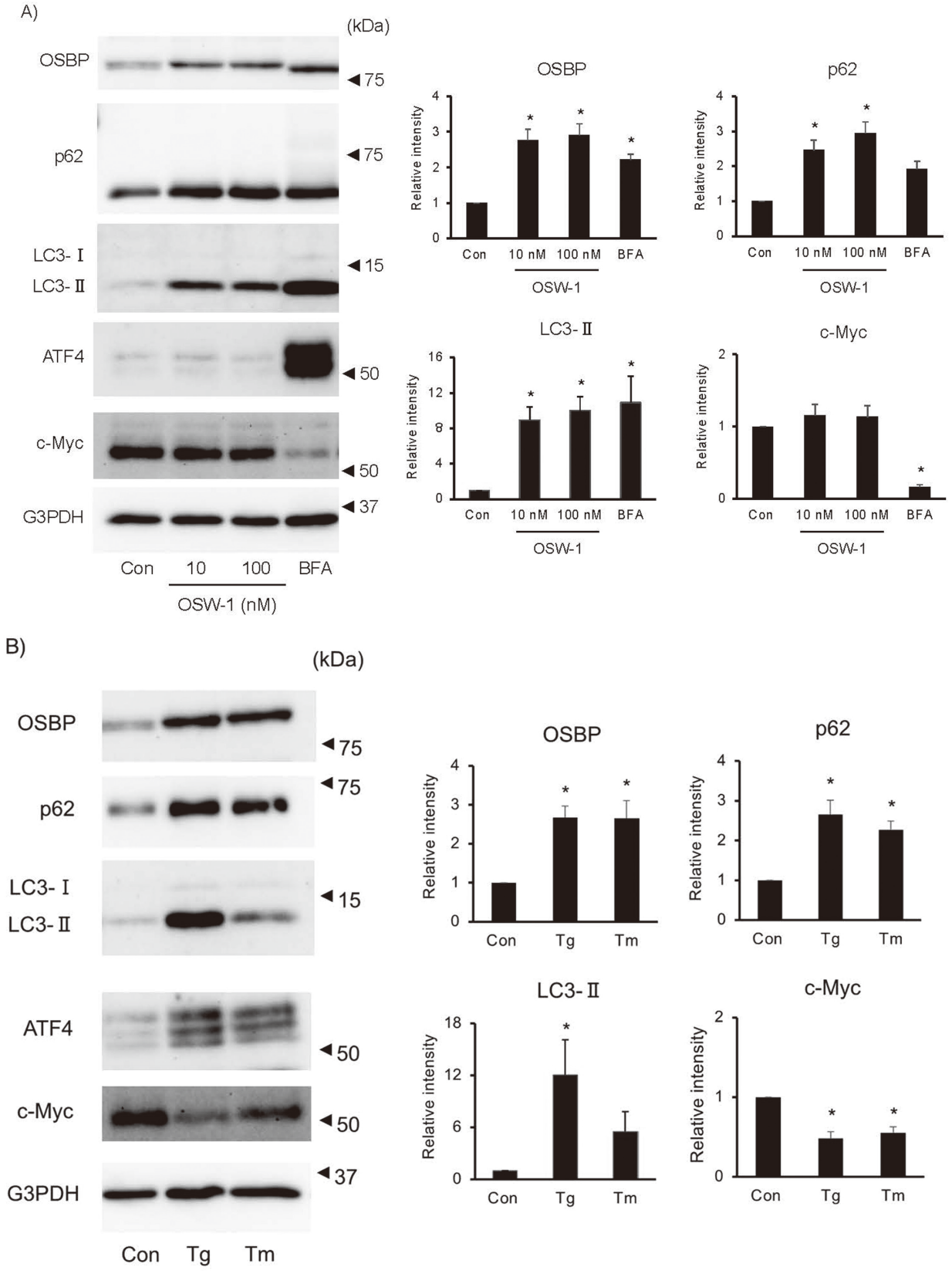

Fig. 4. OSW-1-Stimulated Protein Changes in HT-29 Cells Were Not Accompanied by an Increase in ATF4 Expression

HT-29 cells were treated with the indicated concentration of OSW-1 (10 or 100 nM), BFA (0.5 μg/ml) or vehicle (A) or Tg (10 nM), Tm (2 μg/ml) or vehicle (B) for 16 h and the indicated protein expression was evaluated by western blotting analysis. The relative amount of each protein was calculated as described in the materials and methods. The values obtained from the untreated control cells were considered as "1.0". Each value represented the mean ± SEM from 4-5 independent cultures. Values marked with asterisks are significantly different from the value of untreated cells (* $p < 0.05$).

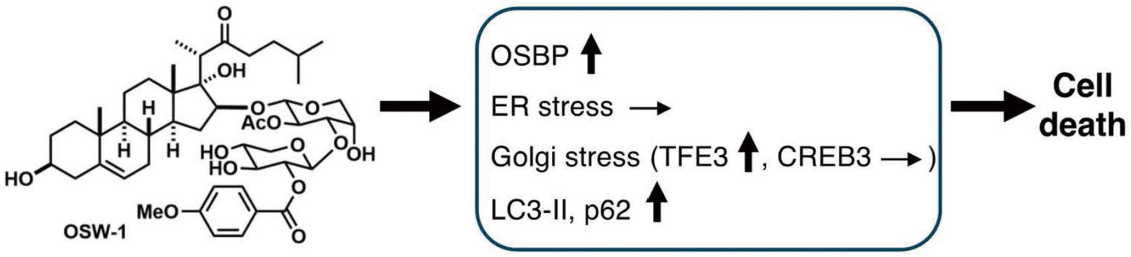

Fig. 5. Signaling Molecules Fluctuated by Treatment with OSW-1 in HT-29 Cells.

OSW-1 treatment induced atypical Golgi stress that strongly activated the TFE3 pathway and did not involve the CREB3 pathway or the ER stress response. OSW-1 also affected the autophagy system without OSBP downregulation.

tein in the cytoplasm and nucleus may be different from that of ER-localized CREB3 protein. Like full-length CREB3, cleaved CREB3 is also a proteasome substrate;[15,26] however, it is unclear what kinds of Ub ligase/deubiquitinase are responsible and whether it is altered by stresses such as BFA. To date, CREB3 has been implicated in the development and progression of multiple cancers.[27] Therefore, these findings on CREB3 in HT-29 cells would provide new insights into understanding CREB3 signaling pathway under pathophysiological conditions.

Analysis of Autophagy and Cell Stress Responses Induced by OSW-1 in HT-29 Cells OSW-1 induced cell damages in HT-29 cells with atypical Golgi stress induction that does not induce CREB3 activation, but its detailed target factors and signaling pathways are unknown. Finally, we elucidated several factors associated with cell stress responses and cell proliferation in HT-29 cells in response to each stimulus (Fig. 4). In consistent with no increase in ER stress-inducible mRNA expression in OSW-1 treated cells (Fig. 2C and D), OSW-1 did not induce ATF4 protein downstream of eIF2α kinase including PERK.[28] On the other hand, OSW-1 significantly increased LC3-II, one of the autophagy markers, as previously examined in Neuro2a cells.[14] The expression of p62, another autophagy factor,[29] in HT-29 cells was almost proportional to the amount of LC3-II. However, at present, the effects of OSW-1 on autophagy and its role in cellular damage are inconclusive.[14,30-32] Recently, Zang *et al*. reported that OSW-1 promotes early autophagic process but impairs autophagy flux.[30] On the other hand, c-Myc protein, a proteasome substrate, was not increased by OSW-1.[21] Therefore, OSW-1 is likely to affect the lysosome system, which may also be involved in TFE3 activation,[31] but further analysis is needed to determine whether it is essential for cellular damage.

One of the surprising points was that OSBP, a putative target of OSW-1, in HT-29 cells was increased not only in OSW-1 stimulation but also in BFA, Tg, and Tm. The regulation of OSBP in HT-29 cells may be exceptional, as OSBP has been reported to be downregulated by OSW-1 not only in our previous study with Neuro2a cells but also in another cancer cell line.[3,5,14] The fact that OSBP changes differ among cell types upon OSW-1 stimulation may mean that OSBP is not as important for OSW-1-stimulated cell death. This is consistent with our report that OSBP-deficient Neuro2a cells still induced OSW-1-induced cell death to the same extent as wild-type cells.[14] There are multiple homologs of OSBP with similar domains, and it was recently reported that ORP4 is a target of OSW-1.[5,6] Therefore, the search for OSW-1 binding factors including ORPs and their analysis will be useful in the development of anti-tumor compounds.

This study has several implications (Fig. 5). The study provides significant insights into the cellular mechanisms and pathways affected by OSW-1 in HT-29 cells, revealing its potential as a targeted cancer therapy. The selective activation of TFE3 and the specific increase in autophagy markers suggest that OSW-1 can modulate cellular stress responses in a unique manner, distinct from traditional ER stress inducers. This specificity could lead to more effective and precise cancer treatments with fewer side effects. Additionally, the involvement of the lysosome system and the atypical Golgi stress response highlight novel therapeutic targets and pathways for future drug development.

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Conflict of interest The authors declare no conflict of interest.

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