The dental resin monomers HEMA and TEGDMA have inhibitory effects on osteoclast differentiation with low cytotoxicity

Hiroyuki Inamitsu[1, 2], Kuniaki Okamoto[1], Eiko Sakai[1], Kazuhisa Nishishita[1], Hiroshi Murata[2], and Takayuki Tsukuba[1§]

Departments of Dental Pharmacology[1], and Prosthetic Dentistry[2], Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan

[§]Corresponding author
Department of Dental Pharmacology, Nagasaki University Graduate School of Biomedical Sciences, Sakamoto 1-7-1, Nagasaki 852-8588, Japan
Tel.: +81-95-819-7652; Fax: +81-95-819-7655
E-mail address: tsuta@nagasaki-u.ac.jp

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Abbreviations: HEMA, 2-hydroxyethyl methacrylate; TEGDMA, triethyleneglycol dimethacrylate; Abs, antibodies; RANKL, receptor activator of nuclear factor kappa-B ligand; BMMs, bone marrow-derived macrophages; TRAP, tartrate-resistant acid phosphatase; NFATc1, nuclear factor of activated T cells cytoplasmic-1; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor kappa B; JNK, Jun N-terminal kinase; Erk, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; α-MEM, α-minimal essential medium; IκBα, inhibitor of nuclear factor kappa B alpha; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; COX, cyclooxygenase

Abstract

The dental resin monomers 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) are released from the resin matrix due to unpolymerized monomers; once released, they influence various biological functions and the viability of cells in the oral environment. Although HEMA and TEGDMA have various effects on cells, including inflammation, inhibition of cell proliferation or differentiation, and apoptosis, the effects of these monomers on osteoclasts remain unknown. In this study, we investigated the effects of HEMA and TEGDMA on osteoclast differentiation of bone marrow-derived macrophages (BMMs) or murine monocytic cell line RAW-D. Both HEMA and TEGDMA inhibited osteoclast formation and their bone-resorbing activity at non-cytotoxic concentrations. Moreover, HEMA and TEGDMA decreased the expression of nuclear factor of activated T cells cytoplasmic-1 (NFATc1), a master regulator of osteoclasts differentiation, and of osteoclast markers that are transcriptionally regulated by NFATc1, including Src and cathepsin K. Regarding their effects on signaling pathways involved in osteoclast differentiation, HEMA impaired the phosphorylation of extracellular signal-regulated kinase (Erk) and Jun N-terminal kinase (JNK), whereas TEGDMA attenuated the phosphorylation of Akt and JNK. Thus, HEMA and TEGDMA inhibit osteoclast differentiation through different signaling pathways. This is the first report on the effects of the monomers HEMA and TEGDMA on osteoclasts.

Short abstract

The dental resin monomers 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) influence various biological functions and the viability of cells in the oral environment. However, the effects of these monomers on osteoclasts remain unknown. Here we demonstrated that HEMA and TEGDMA inhibit osteoclast differentiation from bone marrow-derived macrophages (BMMs) or murine monocytic cell line RAW-D *in vitro*. Although HEMA and TEGDMA had different inhibitory effects on signaling pathways of the osteoclast differentiation, they had little cytotoxic effects on osteoclasts.

Keywords: HEMA, TEGDMA, osteoclasts; cytotoxicity

1. Introduction

Dental resin monomers that are released from the resin matrix due to unpolymerized monomers influence various biological functions and the viability of cells in the oral environment (Bakopoulou et al., 2009; Schweikl et al., 2006). Among these, 2-hydroxyethyl methacrylate (HEMA) and triethylene glycol dimethacrylate (TEGDMA) are two major monomers that are released in relatively high amounts from dental restorative materials (Van Landuyt et al., 2011). These monomers are thought to enhance the formation of reactive oxygen species (ROS), leading to increased oxidative stress and perturbation of intracellular redox homeostasis (Krifka et al., 2013). Consequently, it is likely that these resin monomers have various effects on cells, including inflammation, inhibition of cell proliferation or differentiation, and apoptosis (Gallorini et al., 2014). Concerning inflammatory responses, HEMA induces them in human gingival fibroblast, such as increased expression of tumor necrosis factor-α and cyclooxygenase (COX)-2 and the release of prostaglandin E_2 (Di Nisio et al., 2013). Similarly, TEGDMA has been shown to induce the production of inflammatory mediators such as COX-2, prostaglandin-E_2, and prostaglandin-$F_2\alpha$ in human dental pulp cells (Chang et al., 2012). Treatment with HEMA and TEGDMA provoke the expression of COX-2 in murine macrophage RAW 264.7 cells. (Lee et al., 2009). Moreover, induction of HEMA and TEGDMA is known to cause allergic contact dermatitis *in vivo* (Aalto-Korte et al., 2007; Bando et al., 2014).

Regarding the inhibitory effects on cell proliferation or differentiation, HEMA and TEGDMA have been reported to suppress the differentiation of human dental pulp cells into dentin (Kwon et al., 2015) and the osteogenic differentiation of human osteosarcoma MG63 cells under non-cytotoxic conditions (Kim et al., 2011). Moreover, HEMA induces apoptosis in RAW264.7 mouse macrophages via oxidative-DNA damage by an intrinsic mitochondrial pathway (Schweikl et al., 2014) or by increased ROS production (Krifka et al., 2012). However, to this date, there is no report regarding the effects of the monomers HEMA and TEGDMA on osteoclasts.

Osteoclasts are multinucleated giant cells that mainly participate in bone resorption (Chambers, 2000; Teitelbaum, 2000). Osteoclasts are differentiated from macrophages by two major cytokines: receptor activator of nuclear factor kappa-B ligand (RANKL) (Lacey et al., 1998; Yasuda et al., 1998) and macrophage colony-stimulating factor (M-CSF) (Elford et al., 1987). The interaction between RANKL-RANK activates osteoclast

differentiation through six major signaling pathways: nuclear factor of activated T cells cytoplasmic-1 (NFATc1), nuclear factor kappa B (NF-κB), phosphatidylinositol 3-kinase (PI3K)/Akt, Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (Erk), and p38 mitogen-activated protein kinase (MAPK) (Darnay et al., 1999; Matsumoto et al., 2000; Zhang et al., 2001., Boyle et al., 2003). Moreover, many osteoclast marker proteins have been identified. NFATc1 is a master regulator of osteoclast differentiation through Ca^{2+}/calmodulin-dependent calcineurin (Takayanagi et al., 2002), while c-Src is a non-receptor-type tyrosine kinase that regulates the formation of actin-rich podosomes in osteoclasts (Kim et al., 2010). Cathepsin K is a lysosomal cysteine proteinase specifically expressed in osteoclasts (Delaisse et al., 2003). RANK is a specific cell surface receptor for RANKL (Yasuda et al., 1998), whereas c-fms is an M-CSF receptor. Recent studies have reported that a transient increase in ROS production is essential for osteoclast differentiation (Lee et al., 2005). These findings prompted us to investigate the effects of the resin monomers HEMA and TEGDMA on osteoclasts. Given that osteoclasts are the most important cells for regulation of periodontitis, a chronic inflammatory disease of the gums with severe bone destruction, it is particularly important to determine whether these monomers could have either inflammatory or inhibitory effects on osteoclasts.

Thus, we investigated the effects of HEMA and TEGDMA on osteoclastogenesis, and examined some of the potential molecular mechanisms of these monomers, especially in cell signaling. We herein report the effects of HEMA and TEGDMA on osteoclast differentiation using *in vitro* cultured osteoclasts.

Materials and methods

Reagents

Both HEMA and TEGDMA were purchased from Tokyo Chemical Industry (Tokyo, Japan). α-Minimal essential medium (α-MEM) was from Wako Pure Chemicals, Japan. Recombinant RANKL was prepared as previously described (Hu et al., 2008). The following antibodies (Abs) were purchased: β-actin (Sigma-Aldrich, St. Louis, MO, USA), Src (Upstate, Lake Placid, NY, USA), c-fms, RANK, and NFATc1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), phospho-IκBα (Ser32), and phospho-Akt (Ser473; Cell Signaling Technology, Danvers, MA, USA). Cathepsin K

Ab was prepared as previously described (Kamiya et al., 1998). The Osteo Assay Stripwell Plate was purchased from Corning (Corning, NY, USA). All other reagents, including the protease inhibitor cocktail, were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Cell culture

Five-week-old male C57BL/6 mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained at 22 ± 2 °C with 50 ± 10 % humidity and a 12-h light/dark cycle under conventional conditions. The mice were fed *ad libitum* with a commercial diet (MF; Oriental Yeast Co., Ltd. Tokyo, Japan) and with sterilized water. The mice were euthanized within 2 weeks by carbon dioxide inhalation. All animals were handled in our facilities under the approved protocols of the Nagasaki University Animal Care Committee.

To isolate bone marrow-derived macrophages (BMMs), marrow cells from mice femurs and tibias were cultured overnight in α-MEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin in the presence of M-CSF (50 ng/mL) at 37 °C in a 5% CO_2 atmosphere. After harvesting non-adherent cells, stroma-free bone marrow cells were cultured in the presence of 50 ng/mL M-CSF. After three days, non-adherent cells were washed out and adherent cells were used as BMMs. BMMs were replated, and then further cultured with fresh medium containing M-CSF (30 ng/mL) and RANKL (50 ng/mL) for the times indicated. The murine monocytic cell line RAW-D was kindly provided by Prof. Toshio Kukita (Kyushu University, Japan) and cultured in α-MEM containing 10% FBS with RANKL (50 ng/mL) (Watanabe et al., 2004). HEMA and TEGDMA were dissolved in PBS and dimethyl sulfoxide (DMSO), respectively. The final concentration of DMSO (vehicle) was 0.005% (v/v). To analyze the activity of tartrate-resistant acid phosphatase (TRAP), cells were treated with or without 0.1–1.0% HEMA or TEGDMA, and the cells were subsequently stimulated with RANKL (50 ng/mL) in α-MEM containing 10% FBS for 3 days. Next, the cells were fixed with 4% paraformaldehyde and stained for TRAP activity using a previously described method (Hotokezaka et al., 2002). TRAP-positive (red) cells with three or more nuclei were considered mature osteoclasts. The bone-resorbing activity of osteoclasts was assayed using the Osteo Assay Stripwell Plate after five days of culture. The resorption

area was determined using the Image J software (http://rsbweb.nih.gov/ij/).

Cell viability assay

Cells seeded in 96-well culture plates were rinsed twice with PBS, and incubated with the Cell Counting Kit-8 solution (Dojindo, Kumamoto, Japan) for 1 h. Absorbance at 450 nm was measured with a microplate reader (Bio-Rad iMark; Hercules, CA, USA).

Western blot analysis

BMMs were stimulated with or without RANKL in the presence of M-CSF for the indicated times. Cells were rinsed twice with ice-cold PBS, and lysed in a cell lysis buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl) containing 2 mM sodium orthovanadate, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor cocktail. The protein concentration of each sample was measured using the BCA Protein Assay Reagent (Thermo Pierce, Rockford, IL, USA). Five micrograms of lysate protein per lane was resolved by SDS-PAGE, and proteins were electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked with 3% skimmed milk in 20 mM Tris-HCl, pH 7.5 containing 150 mM NaCl and 0.1% Tween 20 for 1 h at room temperature, probed with various antibodies overnight at 4 °C, washed, incubated with HRP-conjugated secondary antibodies, and finally detected with ECL-Prime (GE Healthcare Life Sciences, Amersham Place, UK). The immunoreactive bands were analyzed by LAS4000 mini (Fuji Photo Film, Tokyo).

Statistical analysis

All values are expressed as means ± standard deviations. Data were analyzed by One-way ANOVA followed by the Tukey-Kramer post hoc test multiple comparison (*P < 0.05 or **P < 0.01).

Results

HEMA and TEGDMA inhibit osteoclastogenesis of RAW-D cells and native BMMs

To examine the effects of HEMA and TEGDMA on osteoclastogenesis, we first investigated osteoclast differentiation of the RANKL-stimulated murine monocytic cell line, RAW-D. Analysis by TRAP staining, which is the most reliable method to assess osteoclast formation, showed that treatment with HEMA markedly inhibited osteoclast formation from mononuclear into multinuclear cells (Fig 1A). The number of TRAP-positive multinucleated osteoclasts after HEMA treatment was significantly decreased with 0.2 mM HEMA, and abolished with 0.5 mM HEMA (Fig. 1B). The viability of osteoclasts after treatment with 0.1–0.5 mM HEMA was slightly increased, although it was decreased after treatment with high concentrations of 1.0-2.0 mM HEMA (Fig. 1C). Similarly, TRAP staining analysis revealed that TEGDMA abolished RANKL-induced osteoclastogenesis of RAW-D cells (Fig 1D). TEGDMA treatment markedly reduced the number of TRAP-positive multinucleated RAW-D cells even at the concentrations of 0.1 mM, and completely abolished it at 1 mM (Fig. 1E). The viability of osteoclasts after treatment with 0.2–0.5 mM TEGDMA was slightly increased, although it with 2.0 mM TEGDMA was still detectable (Fig. 1F).

We further analyzed the effects of HEMA and TEGDMA on RANKL-induced osteoclast differentiation of native BMMs. TRAP staining confirmed that HEMA inhibited osteoclast differentiation of BMMs (Fig 2A). The number of TRAP-positive BMM-derived osteoclasts was significantly decreased by treatment with HEMA at concentrations greater than 0.1 mM (Fig. 2B). Interestingly, a small increase in viability was observed in osteoclasts treated with 0.1-1.0 mM HEMA (Fig. 2C).

Similar results were observed after TEGDMA treatment. TRAP staining showed that TEGDMA abolished BMM-derived osteoclast differentiation (Fig. 2D and E). The cell viability of cells treated with 0.1–1.5 mM TEGDMA was significantly increased, although the viability of cells treated with TEGDMA was indistinguishable from untreated cells at 2 mM (Fig. 2F). These results indicate that HEMA and TEGDMA inhibit osteoclast differentiation of native BMMs with low cytotoxicity.

HEMA and TEGDMA impair the bone-resorbing activity of osteoclasts

To test whether HEMA or TEGDMA inhibits the bone-resorbing activity of

osteoclasts, we performed a pit formation assay with BMM-derived osteoclasts after induction with M-CSF and RANKL. Treatment of BMM-derived osteoclasts with HEMA or TEGDMA inhibited their bone-resorbing activities (Fig. 3A and B). The calculated resorption area of osteoclasts treated with 0.2 or 0.5 mM HEMA was markedly lower than that of untreated osteoclasts (Fig. 3B). Similarly, the calculated resorption area of osteoclasts treated with TEGDMA was gradually decreased in a dose-dependent manner (Fig. 3B). These results indicate that HEMA and TEGDMA inhibit the physiological bone resorbing activity of osteoclasts.

HEMA and TEGDMA interfere with the expression of osteoclast marker proteins

To further investigate the effects of HEMA and TEGDMA on osteoclast differentiation, we analyzed the expression of osteoclast marker proteins by western blotting. As shown in Fig. 4, the expression levels of NFATc1 in BMM-derived osteoclasts was markedly decreased after treatment with either HEMA or TEGDMA. It should be noted that the expression of NFATc1 after treatment with 0.2 mM HEMA almost disappeared, whereas with 0.2 mM TEGDMA it was slightly detectable (Fig. 4). Upon treatment with HEMA, the expression of c-Src, which is transcriptionally regulated by NFATc1, was strikingly decreased even at 0.2 or 0.5 mM (Fig. 4). However, upon TEGDMA treatment, the expression of c-Src was also decreased, but it was still detectable at concentrations of 0.2 or 0.5 mM (Fig. 4). The expression of cathepsin K, which is also transcriptionally regulated by NFATc1, was drastically decreased after treatment with either HEMA or TEGDMA (Fig. 4). The expression levels of RANK were virtually unchanged, whereas those of c-fms were gradually decreased (Fig. 4). These results indicate that HEMA and TEGDMA inhibit the expression of NFATc1 and NFATc1-regulated proteins such as cathepsin K and c-Src.

Both HEMA and TEGDMA attenuate osteoclast differentiation, but in distinct signaling pathways

Finally, we analyzed the effects of HEMA and TEGDMA on RANKL-induced intracellular signaling pathways during osteoclast differentiation of BMMs. Because the effects of HEMA and TEGDMA on NFATc1 were described above, we investigated the

effects of HEMA and TEGDMA on the remaining five signaling pathways; specifically on the phosphorylation of Erk, p38 MAPK, Akt, JNK, and IκBα, using western blotting. BMMs were preincubated with or without 1.0 mM HEMA or 1.0 mM TEGDMA for 12 h and subsequently stimulated with RANKL.

As shown in Fig. 5A, HEMA treatment markedly suppressed phosphorylation of Erk and JNK, and slightly inhibited phosphorylation of Akt and p38 MAPK. IκBα was phosphorylated 5 min after RANKL stimulation in untreated cells, but this was decreased and delayed 10 min after HEMA treatment (Fig. 5A). In contrast, addition of TEGDMA remarkably repressed phosphorylation of Akt and JNK, whereas it slightly inhibited phosphorylation of p38 MAPK (Fig. 5B). However, upon TEGDMA treatment, phosphorylation of Erk and IκBα was almost intact (Fig. 5B). These results indicate that HEMA and TEGDMA attenuate intracellular signaling pathways in osteoclasts in different ways.

Discussion

In this study, we demonstrated that HEMA and TEGDMA inhibited osteoclast differentiation from BMMs or RAW-D cells *in vitro*; furthermore, both HEMA and TEGDMA suppressed the bone-resorbing activity of osteoclasts. As for the inhibitory mechanism of signaling, HEMA and TEGDMA down regulated the expression of NFATc1 and its target proteins Src and cathepsin K. Moreover, HEMA markedly suppressed the phosphorylation of Erk and JNK, and slightly inhibited the phosphorylation of Akt and p38 MAPK. TEGDMA remarkably suppressed the phosphorylation of Akt and JNK, whereas it slightly inhibited the phosphorylation of p38 MAPK. Thus, to our knowledge, this is the first study to report that HEMA and TEGDMA have a powerful inhibitory effect on osteoclastogenesis.

The monomers HEMA and TEGDMA have inhibitory effects on osteoclastogenesis in both BMMs and RAW-D cells. In the case of native BMM-derived osteoclasts, 0.1-0.2 mM HEMA and TEGDMA have inhibitory effects on osteoclast differentiation. Similarly, in RAW-D-derived osteoclasts, 0.2-0.5 mM HEMA and TEGDMA have inhibitory effects on osteoclastogenesis. Because of the lower concentrations, the inhibitory effects of the monomers are not probably due to cytotoxic effects. Instead, the inhibitory effects of the monomers on RANKL-stimulated osteoclasts are reminiscent of those of LPS-stimulated

macrophages. In LPS-stimulated macrophages, HEMA and TEGDMA reduce pro-inflammatory cytokine production from THP-1 monocytes (Noda et al., 2003) and mouse macrophages RAW264.7 cells (Bolling et al., 2013; Krifka et al., 2010). Given that osteoclast differentiation is mediated by RANKL-stimulation, it is speculated that HEMA and TEGDMA may inhibit the function and differentiation of "stimulated" cells. This notion is supported by findings with osteoblasts and dental pulp cells. Upon treatment with differentiation medium, HEMA and TEGDMA suppress the differentiation of "stimulated" osteoblasts (Imazato et al., 2009) or "stimulated" dental pulp cells (Kwon et al., 2015). In contrast, under unstimulated conditions, it is likely that HEMA and TEGDMA induce inflammatory responses. Indeed, in unstimulated-RAW 264.7 cells, HEMA and TEGDMA induce COX-2 expression (Lee et al., 2009). In normal human gingival fibroblasts, HEMA induces an increased expression of tumor necrosis factor-α and COX-2, and the release of prostaglandin E_2 (Di Nisio et al., 2013). In unstimulated human dental pulp cells, TEGDMA has been shown to induce inflammatory eicosanoid production (Chang et al., 2012). Therefore, it would be of interest to determine differences between the inhibitory effects of these monomers in "stimulated" vs. unstimulated cells.

Concerning cell viability, HEMA and TEGDMA had a different cytotoxic profile for osteoclasts derived from BMMs and those derived from RAW-D cells. Namely, both HEMA and TEGDMA had little cytotoxic effects on RANKL-stimulated BMMs (Fig. 2), whereas they had a moderate toxic effect on RANKL-stimulated RAW-D cells at concentrations of 1-2 mM (Fig. 1). Although the detailed molecular mechanisms behind the different degrees of cytotoxicity between cell types remain unclear, one possible explanation for these differences is the cytotoxicity between the two cell types. Specifically, it is speculated that HEMA and TEGDMA may have little cytotoxicity in native cells; however, they have a powerful inhibitory effect on the proliferation of certain types of cancer cells. For this reason, almost all of the experiments, with the exception of TRAP staining and cell viability tests, were performed with native osteoclasts derived from BMMs.

Significant differences were observed between the effects of HEMA and TEGDMA on intracellular signaling pathways during BMM-derived osteoclast differentiation. The inhibitory effects of HEMA on osteoclastogenesis occurred through a broad range of signaling pathways, whereas those of TEGDMA occurred mainly through Akt- and JNK-dependent pathways. The major signaling pathways for RANKL-induced osteoclast

differentiation are known to include NFATc1, NF-κB, PI3K/Akt, JNK, Erk, and p38 MAPK–dependent pathways (Darnay et al., 1999; Matsumoto et al., 2000; Zhang et al., 2001). Both HEMA and TEGDMA inhibited the expression of NFATc1. HEMA strongly blocked the phosphorylation of Erk and JNK, and sufficiently reduced the phosphorylation of Akt, IκBα, and p38 MAPK. However, TEGDMA predominantly attenuated the phosphorylation of Akt and JNK. These differences may be due to different cytotoxic effects on macrophages, as reported by a previous study. Lee *et al.* (Lee et al., 2009) showed that the cytotoxic effects of HEMA and TEGDMA are likely to be significantly different in murine macrophage RAW 264.7 cells. Namely, the cytotoxicity in RAW 264.7 cells of TEGDMA is about 4.4-fold higher than that of HEMA, and the toxic concentration (TC_{50}) of HEMA is about 1.7 mM whereas that of TEGDMA is about 7.5 mM (Lee et al., 2009).

Clinically, such high concentrations of HEMA and TEGDMA, 0.1–0.2 mM, are not detectable in the oral environment. However, our results are important for periodontitis research because they help determine whether these resin monomers activate or inhibit osteoclasts. The inhibitory effects of the monomers on osteoclasts indicate their usefulness in preventing periodontitis.

In conclusion, both HEMA and TEGDMA inhibit osteoclast differentiation of BMMs and RAW-D cells. Furthermore, HEMA and TEGDMA have little cytotoxic effects on BMM-derived osteoclasts. Regarding the signaling pathways associated in osteoclast differentiation, HEMA interferes with osteoclastogenesis through NFATc1, Erk, and JNK-mediated pathways, whereas TEGDMA interferes mainly through NFATc1, Akt, and JNK-mediated pathways.

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Figure Legends

Fig. 1. Effects of HEMA and TEGDMA on osteoclast differentiation of RAW-D cells
(A, D) RAW-D cells were cultured for 72 h with the indicated concentrations of HEMA (A) or TEGDMA (D) in the presence of RANKL (50 ng/mL). TRAP staining was performed after fixation. The data are representative of three independent experiments. Scale bars represent 100 μm. (B, E) The number of TRAP-positive multinucleated osteoclasts after treatment with the indicated concentrations of HEMA (B) or TEGDMA (E) at 72 h of culture was counted. (significance compared with mock-treated cells $^{**}P < 0.01$). (C, F) Cell viability of RAW-D-derived-osteoclasts after treatment with the indicated concentrations of HEMA (C) or TEGDMA (F) at 72 h of culture was analyzed using the Cell Counting Kit. The data are shown as means ± standard deviations of four wells. (significance compared with mock-treated cells $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 2. Effects of HEMA and TEGDMA on osteoclast differentiation of BMMs
(A, D) BMMs were cultured for 72 h with the indicated concentrations of HEMA (A) or TEGDMA (D) in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL). TRAP staining was performed after fixation. The data are representative of three independent experiments. Scale bars represent 100 μm. (B, E) The number of TRAP-positive multinucleated osteoclasts after treatment with the indicated concentrations of HEMA (B) or TEGDMA (E) at 72 h of culture was counted. (significance compared with mock-treated cells $^{**}P < 0.01$). (C, F) Cell viability of BMM-derived-OCLs after treatment with the indicated concentrations of HEMA (C) or (F) at 72 h of culture was analyzed using the Cell Counting Kit. The data are shown as means ± standard deviations of four wells. (significance compared with mock-treated cells $^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3. Effects of HEMA and TEGDMA on the bone-resorbing activity of osteoclasts
BMMs were cultured for 4 days with the indicated concentrations of HEMA or TEGDMA in the presence of M-CSF (30 ng/mL) and RANKL (50 ng/mL). (A) Representative photograph of the bone-resorbing activity of osteoclasts. (B) The resorption area was determined using Image J software. The data are shown as means ± standard deviations of three wells. (significance compared with PBS or DMSO-treated cells, $^{**}P < 0.01$). The data are representative of three independent experiments.

Fig. 4. Effects of HEMA or TEGDMA on the expression of osteoclast marker proteins
BMMs were cultured with M-CSF (30 ng/mL) and RANKL (50 ng/mL) for 72 h in the presence of HEMA or TEGDMA at the indicated concentrations (0, 0.2, 0.5, and 1 mM). Cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by western blotting with Abs specific to NFATc1, c-Src, cathepsin K, RANK, and c-fms. β-Actin was used as a loading control. The data are representative of three independent experiments.

Fig. 5. Effects of HEMA and TEGDMA on essential signaling pathways of osteoclast differentiation
BMMs were cultured with M-CSF (30 ng/mL) for 12 h in the presence or absence of 0.5 mM HEMA (A) or 0.5 mM TEGDMA (B). After 2 h of cell culture in serum-free media, cells were subsequently stimulated with RANKL (50 ng/mL) for the indicated times (0, 5, 10, 15, and 30 min). Cell lysates with equal amounts of protein were subjected to SDS-PAGE, followed by western blotting with Abs specific to p-Akt, p-Erk, p-JNK, p-IκBα, and p-p38 MAPK. β-Actin was used as a loading control. The results are representative of three independent experiments.

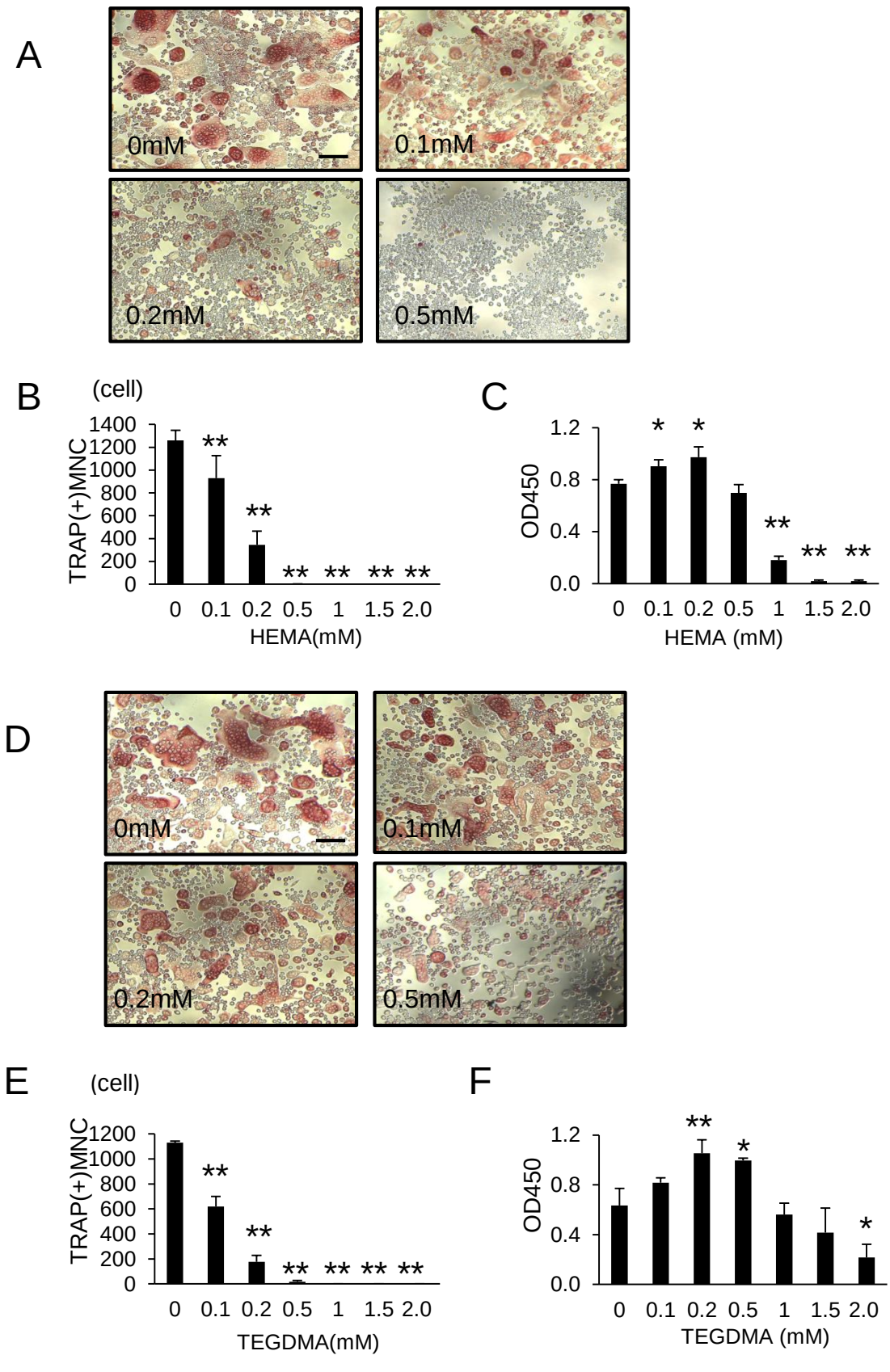

A
0mM
0.1mM
0.2mM
0.5mM
B
(cell)
TRAP(+)MNC
1400
1200
1000
800
600
400
200
0
0 0.1 0.2 0.5 1 1.5 2.0
HEMA(mM)
C
OD450
1.2
0.8
0.4
0.0
0 0.1 0.2 0.5 1 1.5 2.0
HEMA (mM)
D
0mM
0.1mM
0.2mM
0.5mM
E
(cell)
TRAP(+)MNC
1200
1000
800
600
400
200
0
0 0.1 0.2 0.5 1 1.5 2.0
TEGDMA(mM)
F
OD450
1.2
0.8
0.4
0.0
0 0.1 0.2 0.5 1 1.5 2.0
TEGDMA (mM)

Figure 1

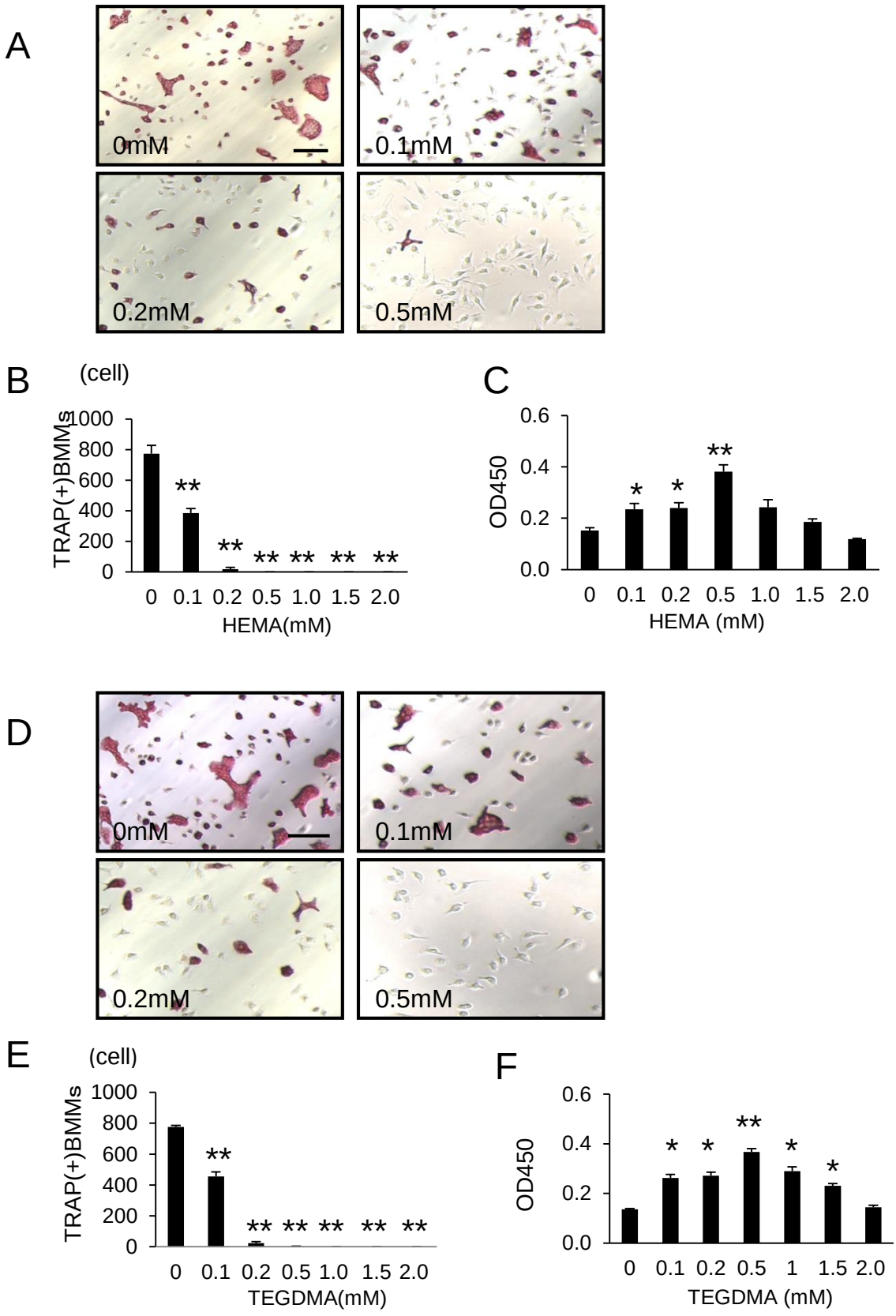
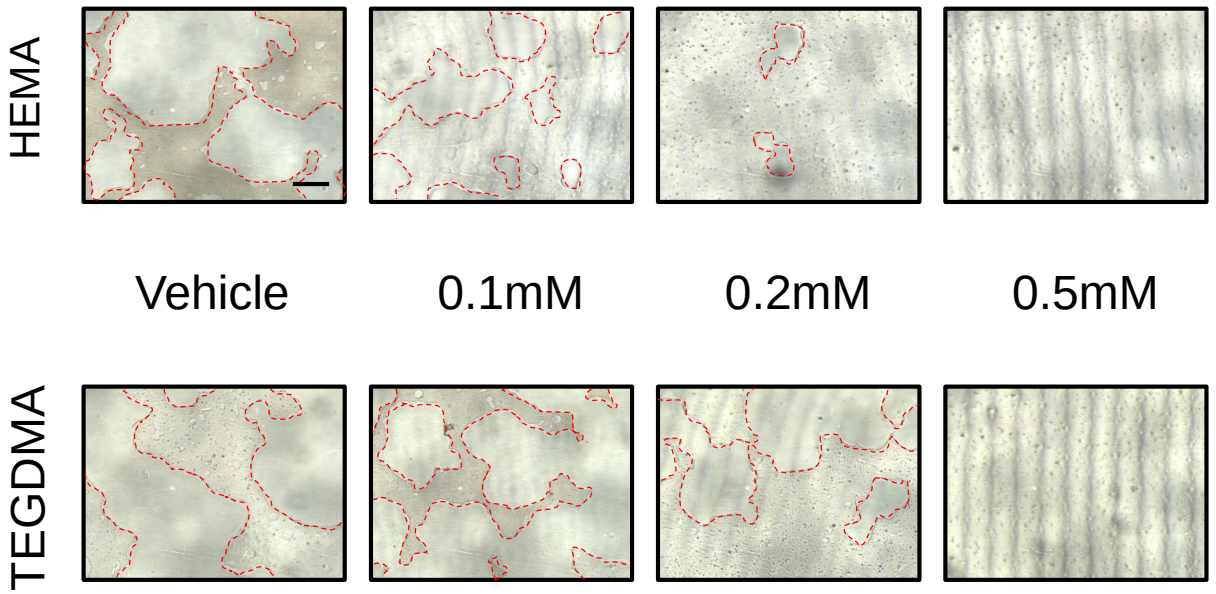

A
0mM
0.1mM
0.2mM
0.5mM
B
(cell)
TRAP(+)BMMs
1000
800
600
400
200
0
0 0.1 0.2 0.5 1.0 1.5 2.0
HEMA(mM)
C
OD450
0.6
0.4
0.2
0.0
0 0.1 0.2 0.5 1.0 1.5 2.0
HEMA (mM)
D
0mM
0.1mM
0.2mM
0.5mM
E
(cell)
TRAP(+)BMMs
1000
800
600
400
200
0
0 0.1 0.2 0.5 1.0 1.5 2.0
TEGDMA(mM)
F
OD450
0.6
0.4
0.2
0.0
0 0.1 0.2 0.5 1 1.5 2.0
TEGDMA (mM)

Figure 2

A


HEMA
TEGDMA
Vehicle
0.1mM
0.2mM
0.5mM

B

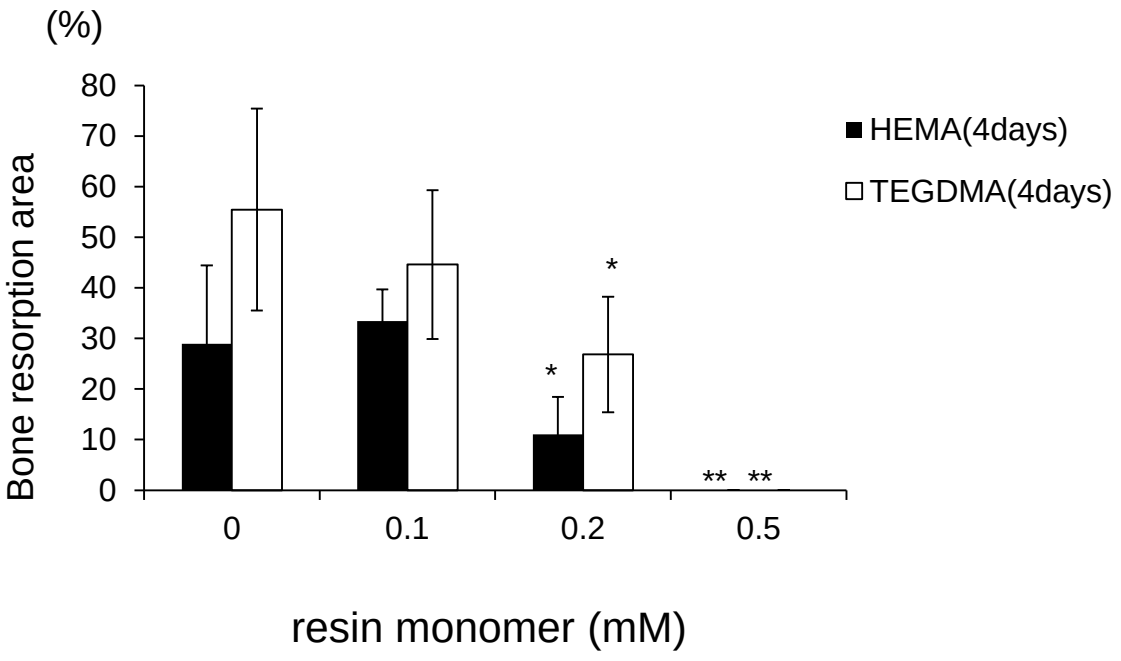

(%)
Bone resorption area
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.5
resin monomer (mM)
HEMA(4days)
TEGDMA(4days)
*
*
**
**

Figure 3

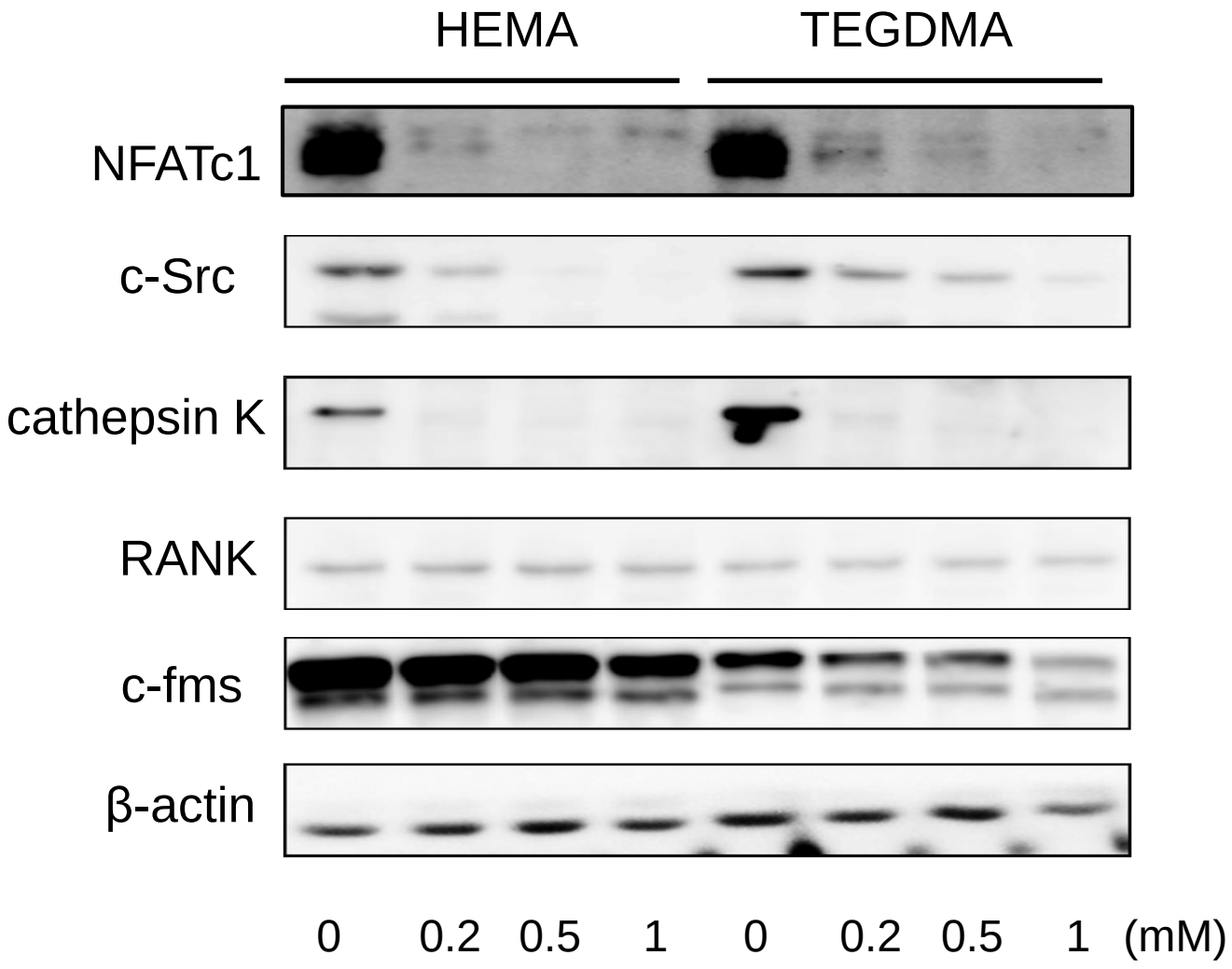

HEMA
TEGDMA
NFATc1
c-Src
cathepsin K
RANK
c-fms
β-actin
0 0.2 0.5 1 0 0.2 0.5 1 (mM)

Figure 4

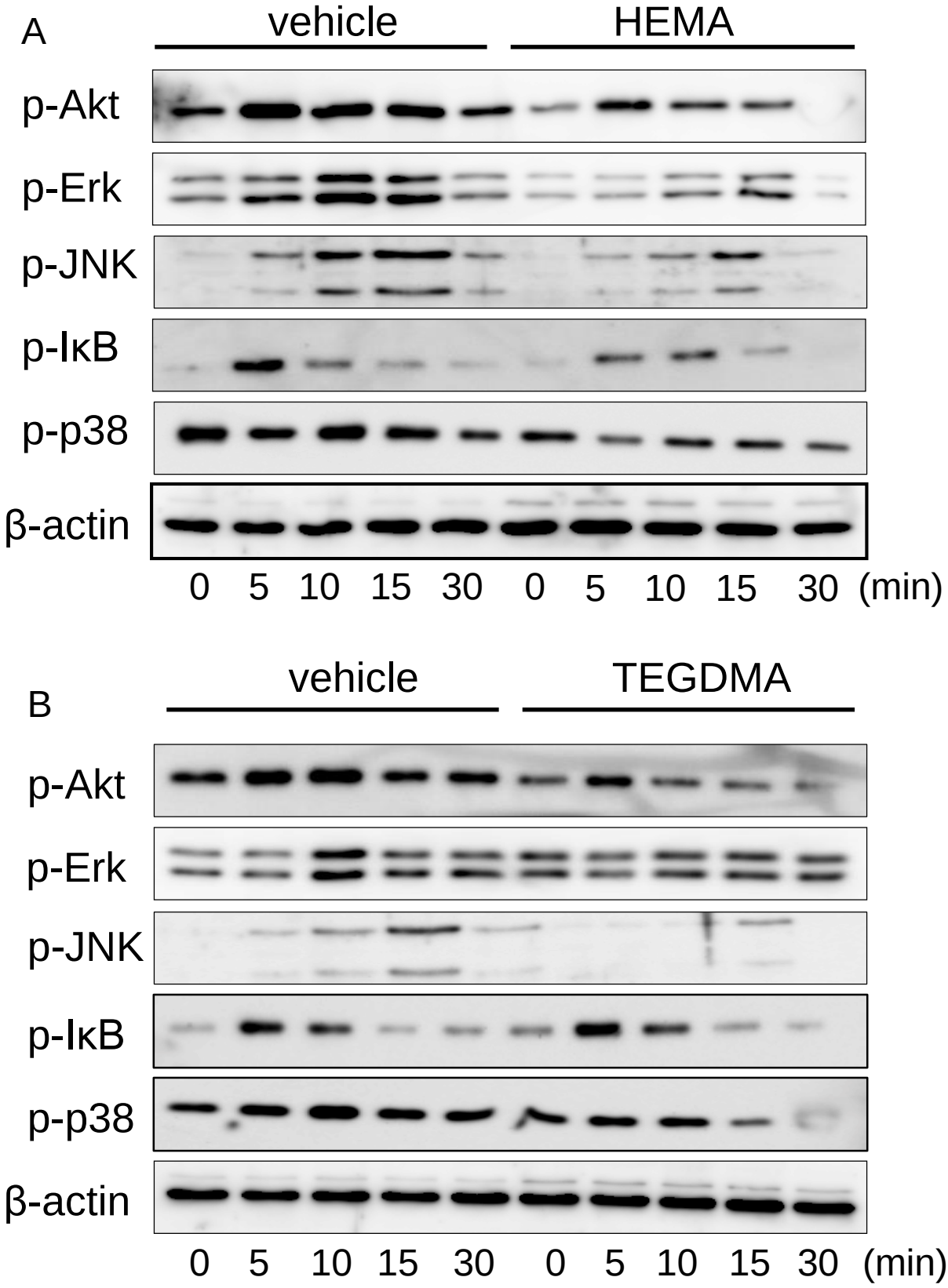

A
vehicle
HEMA
p-Akt
p-Erk
p-JNK
p-IκB
p-p38
β-actin
0 5 10 15 30 0 5 10 15 30 (min)
B
vehicle
TEGDMA
p-Akt
p-Erk
p-JNK
p-IκB
p-p38
β-actin
0 5 10 15 30 0 5 10 15 30 (min)

Figure 5