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Original article:

LINEAR CHAIN ALDEHYDES EVOKE CALCIUM RESPONSES IN B16 MELANOMA CELLS

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ABSTRACT

Oxidative stress is involved in various physiological impairing stages, such as aging, diabetes, atherosclerosis, cirrhosis, and neurological disorders. Recent research indicates that aldehyde compounds derived from oxidized lipids increase in cancer patients compared to healthy individuals. Among of them, hexanal, a six-carbon liner chain aldehyde, is commonly found in cancer patients. Lipid oxidation products including aldehydes are in general chemically unstable and react with biological molecules such as proteins. The purpose of this study is to investigate effects of lipid-derived aldehydes and the related compounds on intracellular Ca^{2+} responses in B16 melanoma cells. Hexanal-induced $[Ca^{2+}]_i$ elevation is observed in B16 cells in a dose dependent manner, but $[Ca^{2+}]_i$ changes were observed neither in 3T3-L1 cells nor Caco-2 cells. Propanal, a chain length analogue of hexanal, elicited no change in $[Ca^{2+}]_i$, but nonanal initiated $[Ca^{2+}]_i$ increases. Analogue compounds of hexanal failed to induce $[Ca^{2+}]_i$ elevation. Furthermore, unsaturated aldehydes known as TRPA1 channel agonists also failed to alter $[Ca^{2+}]_i$ levels in B16 melanoma cells. Pharmacological spectra using inhibitors against intracellular Ca^{2+} signaling suggest that hexanal-induced $[Ca^{2+}]_i$ responses in B16 cells might be involved in TRP channels other than TRPA1. Our results suggest that saturated aliphatic chain aldehydes would be novel compounds for initiating $[Ca^{2+}]_i$ increases through very strict recognitions of chain saturation, aldehydic base structures, and chain lengths in B16 melanoma cells. B16 cells would have sensing mechanisms for oxidative status and/or metabolic activities in their growth environment.

Keywords: B16 melanoma, oxidative stress, lipid peroxidation, hexanal, calcium response

INTRODUCTION

Oxidative stress is involved in various physiological impairing stages, such as aging, diabetes, atherosclerosis, cirrhosis, and neurological disorders. Lipid oxidation products are also observed under these conditions (Dmitriev and Titov, 2010; Ando et al., 1997; Sorrentino et al., 2010; Colas et al., 2010). Many researchers reported the relation between the generation of lipid oxidation products and cancer developments. Yazdanpanah and his co-workers found the generation of aldehydic compounds in patient plasma from various forms of cancer (Yazdanpanah et al., 1997). Correlation between increase in formaldehyde level and tumor progression in mice has been also reported (Ebeler et al., 1997). High levels of hexanal and heptanal were also reported in breath, blood and urine of lung cancer patients (Chen et al., 2007; Li et al., 2005; Guadagni et al., 2011). Hex-

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anal, 1-octen-3-ol and octane showed significant increases in liver cancer patients compared to healthy individuals (Xue et al., 2008). Evidences of the generation of aldehydic volatile compounds in cancer cells were recently reported (Shin et al., 2009; Bartolazzi et al., 2010). Many reports thus suggested that aldehydes derived from oxidized lipids were often found in cancer development. Melanoma is one of the most fatal types of skin cancer because of its high metastatic property. This kind of cancer is not highly frequent, but an increase in the number of cases has been recently observed in the last 30 years (Geller et al., 2007). In the case of melanoma, volatile compound generation was detected by trained dogs that used their olfaction to locate tumors (Pickel et al., 2004). On the other hand, lipid oxidation products including aldehydes are in general chemically unstable and react with biological molecules such as proteins. The purpose of this study is to investigate effects of the lipid-derived aldehydes and the related compounds on intracellular Ca^{2+} signaling in B16 melanoma cells.

MATERIALS AND METHODS

Chemicals

Calcium Green-1-AM was obtained from Life Technologies Japan (Tokyo, Japan). Hexanal, Cremophor EL, and ionomycin were from Sigma-Aldrich Japan (Tokyo, Japan). L-Glutamine, dimethyl sulfoxide (DMSO) were from Wako Pure Chemical industries (Osaka, Japan). Dulbecco's Modified Eagle's Medium (DMEM) was from Nissui Phermaceutical Co., Ltd. (Tokyo, Japan). Special grades were used for all other chemicals.

Cell culture

B16 melanoma cells were obtained from ATCC via Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). B16 melanoma cells were cultured in DMEM supplemented with 10 % heat inactivated fetal bovine serum and 4 mM L-glutamine. B16 melanoma cells were grown and maintained at 37 °C and 5 % CO_2, humidified atmosphere in flask. For measurement of calcium responses, cells were seeded at 1×10^4 cells/cm^2 on round glass coverslips coated with collagen and fibronectin and incubated for 12 h before each experiment.

Cell viability assay

B16 melanoma cells were seeded at 5×10^3 cells/well in 96-well plate 24 h before experiment. Medium was then replaced 200 μM of fresh medium containing 10-1000 μM hexanal. After 24 h, the cell viability was assayed with WST-1 (Dojindo, Kumamoto, Japan). The reduction of WST-1 to its formazan product was monitored by a multi-well plate reader at a wavelength of 450 nm. The viability was calculated as an absorbance percentage of treated cells based on that of control cells.

Calcium imaging

The changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) were monitored using Calcium Green-1-AM. The coverslips seeded with cells were rinsed three times with Hepes buffered Krebs Ringer solution (HBKR, in mM, 130 NaCl, 4.7 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 4 $NaHCO_3$, 1.2 KH_2PO_4, 10 HEPES, 11.5 glucose. Adjusted to pH 7.4 by NaOH). Subsequently, the calcium indicator was loaded into cells with HBKR solution containing 1 μM Calcium Green-1-AM, 0.1 % DMSO and 0.03 % Cremophor EL for 30 min at 37 °C. The live cell imaging was performed at room temperature in a flow chamber. The flow chamber was constructed with a cell culture dish with a hole of 10 mm in diameter at the bottom. The coverslip seeded with indicator-loaded cells was attached to the bottom of the dish with a silicon grease sealant. The fluid in the flow chamber was perfused at the rate of 2.0 ml/min by utilizing a peristaltic pump (AC-2120, Atto, Tokyo, Japan). The flow chamber was mounted on the stage of microscope (IX-70, Olympus, Tokyo, Japan) to acquire real-time fluorescent images of the cells. The microscope was equipped with Xenon lamp (AH2-RX-T, Olympus, Tokyo, Japan) and mirror unit

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(U-MWIBA3, Olympus, Tokyo, Japan). Fluorescent images were acquired via a CCD camera and the bundled software (C7780-20, Hamamatsu Photonics, Shizuoka, Japan). Cells were conditioned with HBKR solution and stimulated with HBKR solutions containing various concentrations of reagents. In the experiment with $LaCl_3$, we used a HEPES buffered salt solution that did not contain bicarbonate and phosphate in order to avoid precipitation of La^{3+}. In the end of perfusion sequences, cells were treated with 1 μM ionomycin to get the maximum fluorescent intensity for normalizing the fluorescent intensity of each experiment.

Statistical analysis

Results are shown as mean ± SD. Statistical comparisons among groups were performed by one-way analysis of variances, followed by Dunnett's post hoc comparisons.

RESULTS

Ca^{2+} response by hexanal in some culture cell lines

Hexanal at the concentrations of 10-1000 μM induced Ca^{2+} response in B16 melanoma cells (Figure 1A) but not in mouse preadipocyte 3T3-L1 (Figure 1B) and human colon carcinoma Caco-2 cell lines (Figure 1C). In order to assess the toxicity of the concentration range of hexanal, the viability of B16 melanoma cell was evaluated 24 h after hexanal challenge at the concentrations of 10-1000 μM, resulting that no significant difference was observed between experimental and control groups (Figure 2).

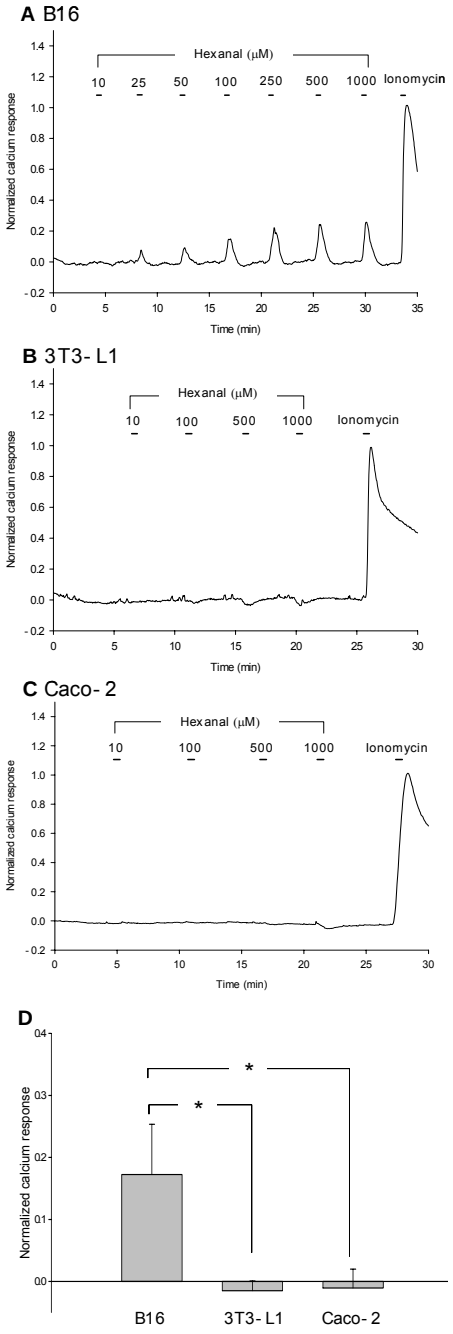

Figure 1: Effects of hexanal on intercellular calcium concentration changes in culture cell lines. B16 mouse melanoma (A), 3T3-L1 mouse pre-adipocyte fibroblast (B), and Caco-2 human colon carcinoma (C). 1 μM ionomycin was administrated for normalization of each fluorescent intensity in the end of sequence. $[Ca^{2+}]_i$ changes in the administration of 1000 μM hexanal for cell lines (D). Response values were 0.172 ± 0.08 (B16), -0.01 ± 0.01 (3T3-L1), -0.01 ± 0.03 (Caco-2). Values are represented as the mean value ± SD (n=3). * P ≤ 0.05.

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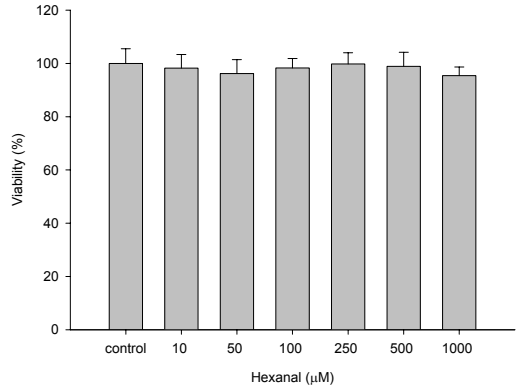

Figure 2: Effects of hexanal on B16 cell viability 24 h after hexanal exposure in a range of 10-1000 μM. Viability was represented in normalization against the control value as 100 %. Values are represented as the mean value ± SD (n=5).

Effects of aliphatic aldehyde chain lengths on Ca^{2+} responses in B16 melanoma cells

The effects of chain length and dose dependency of linear chain aldehydes on the calcium responses of B16 cells were evaluated (Figure 3). B16 cells were stimulated with propanal and nonanal at the concentrations of 10-1000 μM which were main oxidative products derived from n-3 and n-9 fatty acids. Hexanal and nonanal initiated intracellular calcium responses in a concentration-dependent manner, where maximal responses were 0.18 ± 0.07 and 0.25 ± 0.09, respectively, and reached half maximal values (EC_{50}) at 84.5 μM and 142.69 μM, respectively. On the other hand, administration of propanal did not elicit $[Ca^{2+}]_i$ changes (the maximal response was less than 0.01).

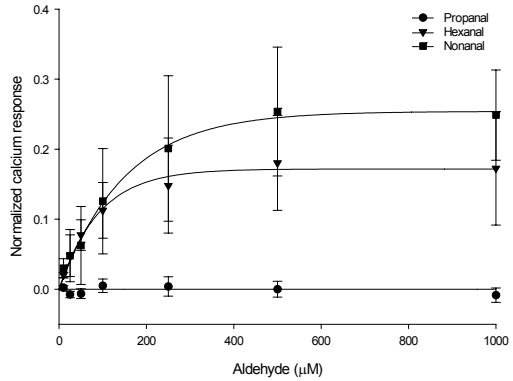

Figure 3: Effects of chain lengths of aliphatic aldehydes on intracellular calcium concentrations in B16 cells. Values are represented as the mean value ± SD (n=3).

Effects of aliphatic aldehyde analogue compounds on B16 cell Ca^{2+} responses

We then examined the effects of fatty acids with chain lengths similar to the aldehydes, propionic acid, hexanoic acid and nonanoic acid, on the $[Ca^{2+}]_i$ responses. No increase in $[Ca^{2+}]_i$ was observed for the fatty acid analogues in the range of 10-1000 μM (Figure 4), while nonanoic acid reduced $[Ca^{2+}]_i$ slightly (–0.15 ± 0.04 at 1000 μM). 1-Hexanol, 1-hexanoic acid ethyl ester and hexane with a six-carbon chain similar to hexanal did not evoke $[Ca^{2+}]_i$ changes (Figure 5). Unsaturated analogues of aliphatic aldehydes, 2-hexenal, 2-nonenal, 4-hydroxy-hexenal (4-HHE) and 4-hydroxy-nonenal (4-HNE) known as transient receptor potential (TRP) A1 calcium channel agonists were also tested. The TRPA1 agonists other than 4-HHE failed to increase $[Ca^{2+}]_i$ in B16 cells, while 4-HHE elicited slight $[Ca^{2+}]_i$ increase (0.03 ± 0.01 at 1000 μM), much lower than that for hexanal (Figure 6).

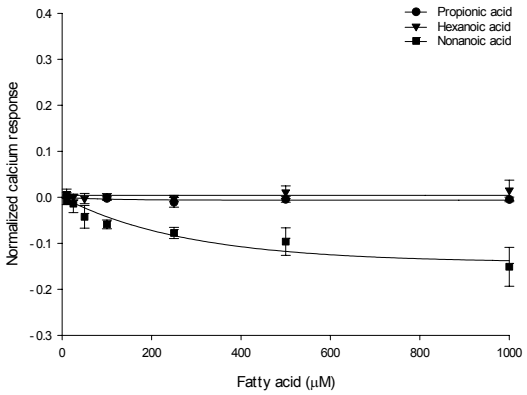

Figure 4: Effects of fatty acids on intracellular calcium concentrations in B16 cells. Values are represented as the mean value ± SD (n=3).

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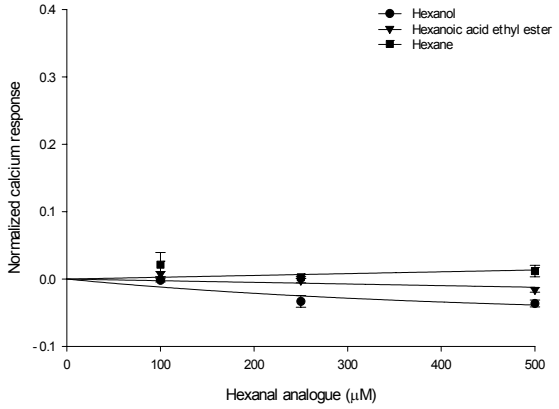

Figure 5: Effects of analogue compounds of hexanal on intracellular calcium concentrations in B16 cells. Values are represented as the mean value ± SD (n=3).

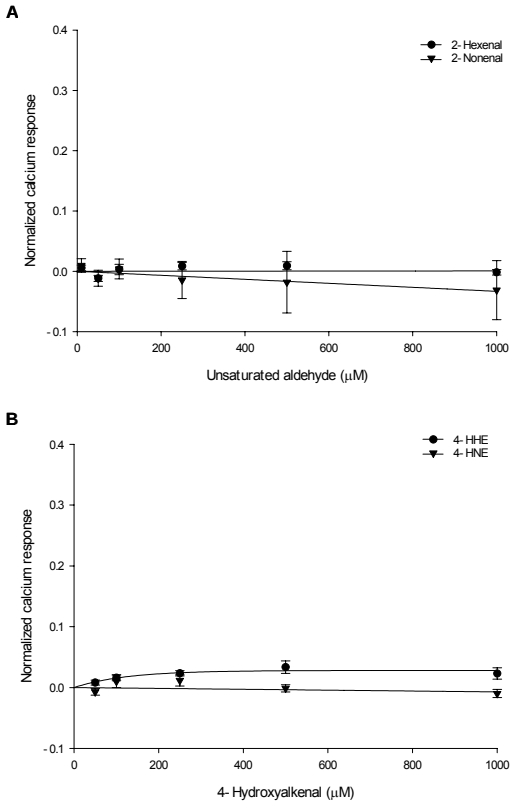

Figure 6: Effects of TRPA1-agonistic aldehydes, unsaturated aldehydes (A) and 4-hydroxyalkenals (B), on intracellular calcium concentrations in B16 cells. Values are represented as the mean value ± SD (n=3).

Influences of ion channel inhibitors on hexanal-induced Ca^{2+} responses

We investigated $[Ca^{2+}]_i$ regulation properties of B16 cells using ion channel inhibitors and blockers in order to elucidate the calcium signaling manner in the hexanal-induced $[Ca^{2+}]_i$ responses. Figure 7 shows effects of the removal of extracellular Ca^{2+} on basal levels of $[Ca^{2+}]_i$, together with the effects of nonselective calcium channel inhibitors Cd^{2+} and La^{3+}, 2-aminoethoxydiphenylborate (2-APB), and a L-type calcium channel inhibitor, nimodipine. Observed were drastic $[Ca^{2+}]_i$ decreases in the absence of extracellular Ca^{2+}, in the presences of 100 μM Cd^{2+} and 100 μM 2-APB, whereas 100 μM La^{3+} and 1 μM nimodipine failed to alter $[Ca^{2+}]_i$ levels. Ca^{2+} responses induced by 500 μM hexanal were significantly reduced in the removal of extracellular Ca^{2+} (89.9 % decrease), in the presence of 100 μM Cd^{2+} (89.6 % decrease) and 100 μM 2-APB (94.8 % decrease), while no effect was observed in the presence of 100 μM La^{3+} and 1 μM nimodipine (Figure 8).

DISCUSSION

Oxidative stress occurs in cancer and other diseases, inducing lipid peroxidation and generating many types of secondly oxidized products including aldehydes (Dmitriev and Titov, 2010; Ando et al., 1997; Sorrentino et al., 2010; Colas et al., 2010). Among many kinds of lipid oxidized compounds, hexanal occurs well in some types of cancers (Ebeler et al., 1997; Li et al., 2005; Guadagni et al., 2011; Yazdanpanah et al., 1997). The molecular mechanism of hexanal generation is explained with β-cleavage of terminal double bond of n-6 lipid hydroperoxide (Artz et al., 1993; Lin et al., 2001). Pickel and co-workers demonstrated that volatile compounds generated in melanoma patients were precisely detected by olfaction of the trained dogs (Pickel et al., 2004). Occurrences of volatile compounds derived from lipid oxidation were also reported in melanoma tissues and primary cultures (Bartolazzi et al., 2010; Pennazza et al., 2009; D'Amico et al., 2008). In these cancer cells, oxidative stress is thought accelerated by their high metabolic rates. Hexanal and nonanal evoked $[Ca^{2+}]_i$ increases in a dose dependent manner, whereas propanal failed to change $[Ca^{2+}]_i$ in this study, suggesting that B16 cell might

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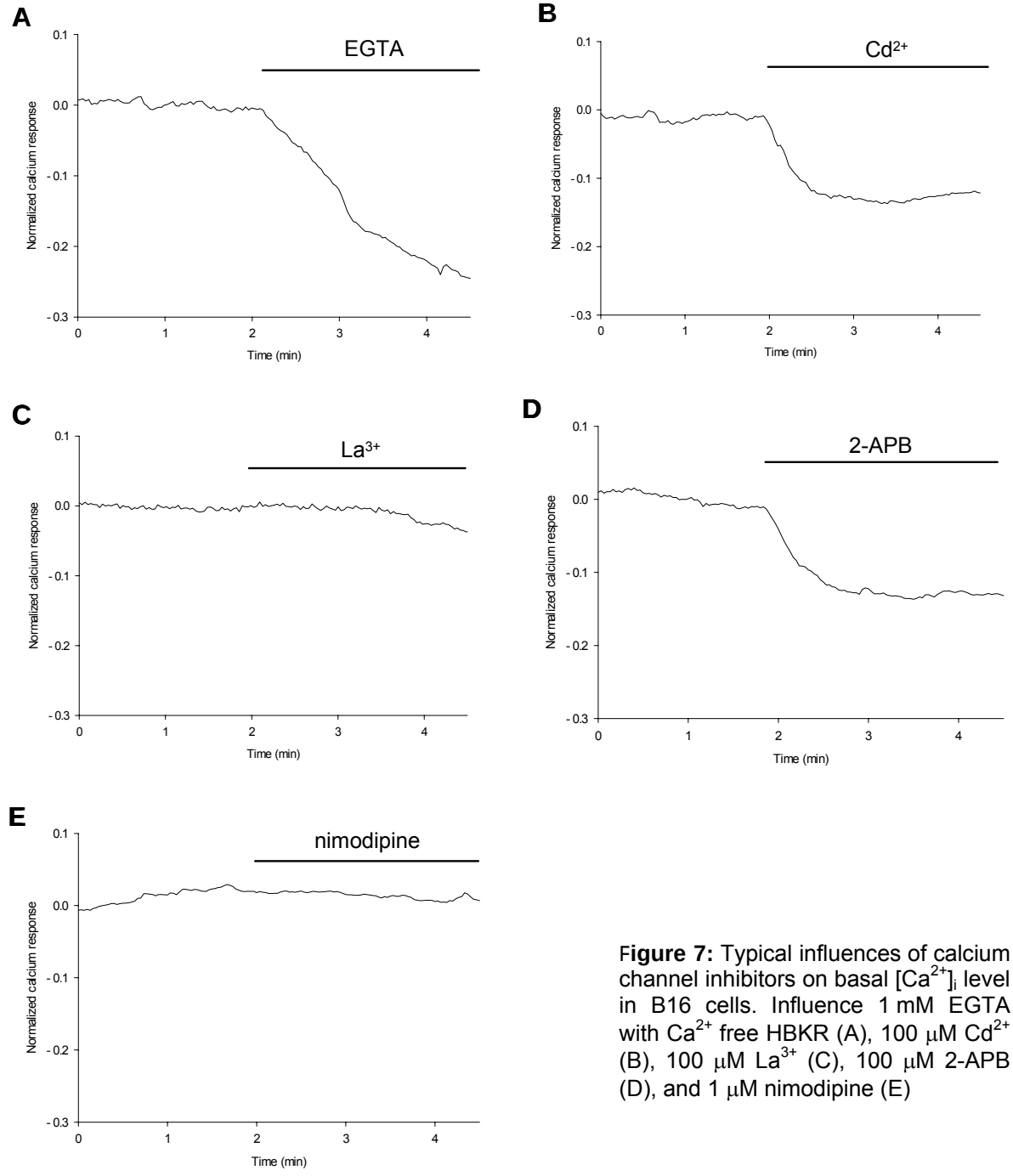

Figure 7: Typical influences of calcium channel inhibitors on basal $[Ca^{2+}]_i$ level in B16 cells. Influence 1 mM EGTA with Ca^{2+} free HBKR (A), 100 μM Cd^{2+} (B), 100 μM La^{3+} (C), 100 μM 2-APB (D), and 1 μM nimodipine (E)

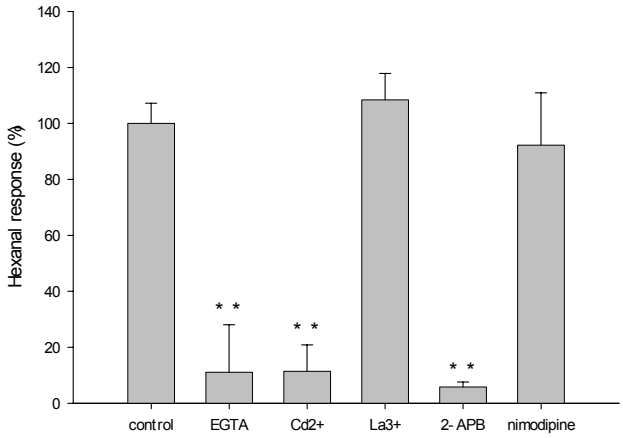

Figure 8: Effects of inhibitors for hexanal induced calcium responses. 500 μM hexanal was used as the control response. inhibitors were used at concentration of 1 mM EGTA with Ca^{2+} free HBKR, 100 μM Cd^{2+}, 100 μM La^{3+}, 100 μM 2-APB and 1 μM nimodipine inhibitors containing HBKR buffer were applied before 4 min of hexanal administration. Responses ware normaliezed to the control hexanal response. Values are represented as the mean value ± SD (n=3). * * P ≤ 0.01.

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possess specific recognition mechanisms for linear aldehydes longer than six-carbon chain. Melanoma cells might recognize the oxidative stress status via the levels of hexanal and nonanal derived from n-6 and n-9 fatty acids, respectively. Hexanal-induced $[Ca^{2+}]_i$ increase was also described in PC12 cells at the concentration of 0.1 % (approx. 8 mM) (Kobayashi et al., 2010), much higher than our result of EC_{50} 84.5 μM in melanoma cell. Hexanal-induced $[Ca^{2+}]_i$ elevation was observed neither in 3T3-L1 pre-adipocyte fibroblast nor Caco-2 colon carcinoma cells. $[Ca^{2+}]_i$ alteration was not observed in other different characteristic culture cell lines, human hepatoma cells (HepG2), murine myeloma cells (P3×63-Ag8U.1, P3U1), murine macrophage cells (RAW264.7), and Chinese hamster ovary cells (CHO-K1) (data not shown). The cell-type differences in the hexanal-induced response might come from oxidative stress levels of cell origins and/or metabolic activities. Cell viability of B16 cells under the 24 h hexanal exposure in the range of 10-1000 μM was not different from that of the control. Hexanal had no effect on the cell viability also in PC12 cell in the concentration higher than our results (Kobayashi et al., 2010). Even in the challenge at 80-folds of our maximal dose, the cell viability of human monocyte-macrophage was over 70 % (Mullar et al., 1996). These results suggest that the hexanal-induced $[Ca^{2+}]_i$ elevation in B16 cells would not be caused by cell damage or injury through chemical reactivity of aldehydes. The suggestion is also confirmed by the result that propanal with similar chemical reactivities failed to induce $[Ca^{2+}]_i$ changes in this study. Because neither linear fatty acids of the same chain length nor other analogues evoked $[Ca^{2+}]_i$ rise, aldehydic base would be important for the recognition of aldehydes by B16 cells. Alkenals and hydroxyalkenals did not induce $[Ca^{2+}]_i$ increases in B16 cells. It has been reported that α,β-unsaturated hydroxyalkenals activate TRPA1 channel and that C4-6 chain length α,β-unsaturated aldehydes activate TRPA1 channel but not saturated analogues (Andersson et al., 2008; Trevisani et al., 2007; Macpherson et al., 2007). The Ca^{2+} response in B16 cells would be thus induced in a TRPA1-independent manner. The removal of extracellular Ca^{2+} markedly reduced the basal $[Ca^{2+}]_i$ level and calcium channel inhibitors Cd^{2+} and 2-APB also decreased the levels. These compounds also reduced the hexanal-induced $[Ca^{2+}]_i$ responses significantly. L-type channel specific inhibitors, La^{3+} and nimodipine, did not affect the basal $[Ca^{2+}]_i$ level and the hexanal-induced $[Ca^{2+}]_i$ responses. Extracellular Ca^{2+} influx via L-type channels would be not important for the $[Ca^{2+}]_i$ changes in B16 cells. It was reported that TRP channels were responsible for a large decrease in basal $[Ca^{2+}]_i$ levels in the removal of extracellular Ca^{2+} and the presence of 2-APB (Wimmers and Strauss, 2007; Ng et al., 2009; Bodding and Flockerzi, 2004). 2-APB is a blocker for capacitative Ca^{2+} entry channels such as TRP channels (Bootman et al., 2002). Since some TRP channels were not inhibited by lanthanides in micromolar concentration (Schaefer et al., 2002; Jung et al., 2003). Saturated aliphatic chain aldehydes would be novel compounds for initiating $[Ca^{2+}]_i$ increases with very strict recognizations for chain saturation, aldehydic base structures, and chain lengths in B16 melanoma cells. The present pharmacological spectra suggest that the hexanal-induced $[Ca^{2+}]_i$ responses in B16 cells might be involved in TRP channels other than TRPA1. The relationship between the hexanal-induced calcium entry in B16 cells and their sensing systems for oxidative status and/or metabolic activity should be disclosed in future.

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