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Acrolein induces Hsp72 via both PKCδ/JNK and calcium signaling pathways in human umbilical vein endothelial cells

YOSHIKO MISONOU, MOTOKO TAKAHASHI, YONG SEEK PARK, MICHIO ASAHI, YASUHIDE MIYAMOTO, HARUHIKO SAKIYAMA, XINYAO CHENG, & NAOYUKI TANIGUCHI

Department of Biochemistry, Osaka University Graduate School of Medicine, B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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Abstract
Acrolein is a highly electrophilic α,β-unsaturated aldehydes to which humans are exposed in a variety of environment situations and is also a product of lipid peroxidation. Increased levels of unsaturated aldehydes play an important role in the pathogenesis of a number of human diseases such as Alzheimer's disease, atherosclerosis and diabetes. A number of studies have reported that acrolein evokes downstream signaling via an elevation in cellular oxidative stress. Here, we report that low concentrations of acrolein induce Hsp72 in human umbilical vein endothelial cells (HUVEC) and that both the PKCδ/JNK pathway and calcium pathway were involved in the induction. The findings confirm that the production of reactive oxygen species (ROS) is not directly involved in the pathway. The induction of Hsp72 was not observed in other cells such as smooth muscle cells (SMC) or COS-1 cells. The results suggest that HUVEC have a unique defense system against cell damage by acrolein in which Hsp72 is induced via activation of both the PKCδ/JNK and the calcium pathway.

Keywords: *Acrolein, Hsp72, calcium, PKCδ, JNK*

Introduction

Acrolein produced by the thermal degradation of certain organic compounds, such as glycerol, fuels, wood, food and tobacco, occurs as a ubiquitous pollutant in the environment, especially as an incomplete combustion product of cigarette smoking [1,2]. It is also produced from cellular lipid peroxidation [3]. Among all, α, β-unsaturated aldehydes including 4-hydroxy-2-nonenal, acrolein is one of the strongest electrophiles and, therefore, shows the highest reactivity with nucleophiles such as the sulfhydryl groups of cysteine, imidazole groups of histidine and amino groups of lysine [4,5]. A number of studies indicate that acrolein exerts downstream signaling via cellular oxidative stress [6,7]. It has been reported that the treatment of cells with acrolein decreases glutathione (GSH) levels and subsequently, induces the production of reactive oxygen species (ROS) [8,9]. We recently found that acrolein also inactivates thioredoxin reductase, a major antioxidative enzyme, both *in vitro* and in human umbilical vein endothelial cells (HUVEC) and causes an increase in cellular hydrogen peroxide levels [10].

Heat shock protein 72 (Hsp72) is a major inducible heat shock protein [11,12]. It contains two conserved domains; an ATP-binding domain (ABD) and a peptide-binding domain (PBD), which are important for its chaperon function [13–15]. Hsp72 plays a role in a variety of cellular activities including protein synthesis, protein folding and protein translocation into organelles as well as the assembly of multiprotein

Correspondence: N. Taniguchi, Department of Biochemistry, Osaka University Graduate School of Medicine, B1, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel: 81 6 6879 3421. Fax: 81 6 6879 3429. E-mail: proftani@biochem.med.osaka-u.ac.jp

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complexes [11,16,17]. It also prevents cell death initiated by various apoptotic stresses, including heat shock, ceramide, ionizing irradiation, TNF-α and ischemia [18–22].

In the present study, we report that acrolein induces Hsp72 expression in HUVEC and that both the activation of the PKCδ/JNK pathway and the upregulation of calcium levels are involved in the pathway.

Materials and methods

Materials

Acrolein was obtained from Tokyo Kasei Kogyo (Japan). Methylglyoxal (MG) and anti-β-actin antibody were purchased from Sigma (St. Louis, MO, USA). 3-Deoxyglucosone (3-DG) was custom synthesized by the Peptide Institute (Japan). PD98059, SB203580, BAPTA-AM, rottlerin and Ro-32-0432 were obtained from Calbiochem (San Diego, CA, USA). SP600125 was purchased from Biomol (Plymouth Meeting, PA, USA). Anti-phosphorylated JNK, ERK1/2, p38 MAPK, PKCα/βII, PKCδ antibodies and anti-JNK, ERK, p38 and PKC (pan) antibodies were obtained from Cell Signaling (Beverly, MA, USA), and the anti-Hsp72 antibody was purchased from StressGen (Canada).

Cell culture

HUVEC, smooth muscle cells (SMC) and COS-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown at 37°C in 100-mm Petri dishes in MCDB131 medium (Nikken Bio Med Lab, Japan) containing 10% fetal calf serum, 10 ng/ml recombinant human basic fibroblast growth factor, 1 mg/ml hydrocortisone, 100 U/ml penicillin G, 100 mg/ml streptomycin sulfate and 0.25 mg/ml amphotericin B, under a humid atmosphere containing 5% CO_2. The cells were passaged every 3–4 days. Throughout the experiments, the cells were used within passages 6–9.

Western blotting analysis

HUVEC (60–80% confluent) were rinsed with phosphate-buffered saline (PBS), harvested in lysis buffer (20 mM Tris–HCl, pH7.4, 150 mM NaCl, 5 mM EDTA, 1% (w/v) Nonidet P-40, 10% (w/v) glycerol, 5 mM sodium pyrophosphate and 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate and 1 mM dithiothreitol) with protease inhibitors. A measured quantity of 2–20 μg of protein was separated on a 10% SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). After protein transfer, the membranes were blocked with 5% BSA in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature and then incubated with the primary antibody diluted 1:1000 with TBST at 4°C overnight. The membranes were incubated for 1 h with the secondary antibody diluted 1:1000 with TBST. Detection was performed using an ECL kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Isolation of RNA and northern blot analysis

Total RNA was extracted with acid guanidium thiocyanate–phenol–chloroform, as reported previously [23]. Twenty micrograms of total RNA were run on a 1% agarose gel containing 2.2 mol/l formaldehyde. The size-fractionated RNAs were transferred to Zeta-Probe membranes (Bio-Rad) overnight by capillary action. Human Hsp72 cDNA was labeled with [α-^{32}P] dCTP (Amersham Pharmacia Biotech) using random hexanucleotide primers (Multiprime DNA labeling system; Amersham Pharmacia Biotech). After hybridization with the labeled probes at 42°C in the presence of 50% formamide, the membrane was washed twice with 2 × sodium chloride–sodium citrate (SSC; 1 × SSC, 15 mM sodium citrate, 150 mM NaCl, pH 7.5) with 0.1% sodium dodecyl sulfate (SDS) at 50°C for 30 min, and then washed with 0.2 × SSC with 0.1% SDS at 50°C for 10 min. The Fuji X-lay films were exposed for 1–2 days to an intensifying screen at −80°C. The intensities of the bands on X-ray films were quantified with a CS-9000 gel scanner (Shimadzu, Japan).

Measurement of intracellular production of reactive oxygen species (ROS)

After treating the cells were with FCS-free medium for 1 h, the culture medium was exchanged to phosphate-buffered saline (PBS), followed by incubation with 5 μM CM-H_2DCF-AM, a cell membrane-permeable ROS sensitive fluorescence dye, for 30 min at room temperature. After washing the cells twice with PBS, various concentrations of acrolein or other reagents were added to the PBS. The relative 530 nm fluorescent intensity was recorded by means of a ratio-imaging system, Aquacosmos/Ratio (Hamamatsu Photonics).

Measurement of intracellular calcium levels

Intracellular calcium concentrations were determined using a cell membrane-permeable calcium sensitive fluorescence dye, Fura-2-AM. After treating the cells with FCS-free medium for 1 h, the culture medium was exchanged with HEPES-tyrode buffer. The cells were loaded with 5 μM Fura-2-AM for 30 min at room temperature and then washed three times with HEPES-tyrode buffer. Various concentrations of acrolein or other reagents were then added to the HEPES-tyrode buffer with 1 mM $CaCl_2$ and changes in Ca^{2+} concentration were measured by recording the ratio of the fluorescence intensities between the excitation wavelengths of 340 and 380 nm by Aquacosmos/Ratio (Hamamatus Photonics). The data were analyzed using the Aquacosmos V.2.5 software program.

Results

Induction of Hsp72 by acrolein in HUVEC

HUVEC were incubated with various levels (1–25 μM) of acrolein for 24 h and Hsp72 protein levels were determined by Western blot analyses. Hsp72 protein levels were increased in a dose-dependent manner (Figure 1A). It was also found that Hsp72 is induced in a time-dependent manner, reaching a maximum level after 12 h as a result of treatment with 25 μM acrolein (Figure 1B, upper panel). A Northern blot analysis revealed that the mRNA levels of Hsp72 reached a maximum level after 6 h as the result of treatment with 25 μM acrolein (Figure 1B, lower panels).

Hsp72 is not induced by methylglyoxal and 3-DG in HUVEC

The effect of other carbonyl compounds on Hsp72 levels in HUVEC was also examined. HUVEC were incubated with 0.5 mM methylglyoxal or 0.5 mM 3-DG for 24 h, and subjected to the Western blotting by using anti-Hsp72 antibody. The expression levels of Hsp72 were not increased by treatment with either of these compounds (Figure 2A). We have also observed that at those concentrations, the morphology and the survival of the HUVEC were not affected.

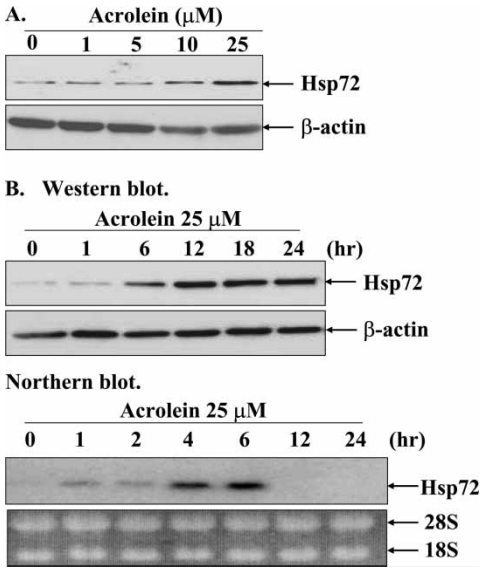

Figure 1. Dose and time dependent induction of Hsp72 by acrolein in HUVEC. (A) HUVEC were treated with the indicated concentrations of acrolein for 24 h. Whole cell lysates were prepared and 2 μg samples of proteins were subjected to Western blotting using anti-Hsp72 antibody and anti-β-actin antibody. (B, upper panel) HUVEC were treated with 25 μM acrolein for the indicated periods. Whole cell lysates were prepared and subjected to Western blotting, the same as in (A). (B, lower panels) HUVEC were treated with 25 μM acrolein for the indicated periods. Twenty micrograms of RNA were subjected to Northern blotting using Hsp72 as a probe.

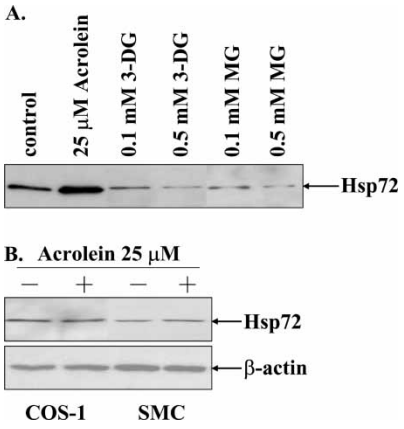

Figure 2. (A) Effect of MG and 3-DG on the induction of Hsp72 in HUVEC. HUVEC were stimulated with 25 μM acrolein, 0.5 mM MG or 0.5 mM 3-DG for 24 h. Lysates were prepared and 2 μg samples of proteins were analyzed by Western blotting using an anti-Hsp72 antibody and anti-β-actin antibody. (B) Effect of acrolein on the induction of Hsp72 in SMC and COS-1. SMC and COS-1 cells were treated with 25 μM acrolein for 24 h. Lysates were prepared and 2 μg samples of proteins were analyzed by Western blotting using anti-Hsp72 antibody and anti-β-actin antibody.

Hsp72 is not induced by acrolein in SMC and COS-1 cells

We tested the effect of acrolein on the expression level of Hsp72 in other cell types, such as SMC and COS-1 cells. In these experiments, the cells were incubated with 25 μM acrolein for 24 h, and subjected to the Western blotting for Hsp72. Induction of Hsp72 was not found in any of the other cells examined (Figure 2B).

Activation of JNK, ERK and p38 MAPK by acrolein and its relevance to Hsp72 induction

In order to understand the mechanism of how acrolein induces Hsp72, we examined MAPKs activation in HUVEC, since various members of the MAPK family have been implicated in the response to carbonyl stress. MAPKs activation in HUVEC, as a result of acrolein treatment is shown in Figure 3A. We used specific antibodies to phosphorylated JNK, ERK and p38 MAPK. The results indicate that acrolein stimulated JNK phosphorylation at 30 min, ERK phosphorylation at 5 min and p38 MAPK phosphorylation at 10 min. HUVEC were next preincubated with the JNK inhibitor SP600125, the MEK inhibitor PD98059, and the p38 MAPK inhibitor SB203580 for 30 min. The cells were then treated with 25 μM acrolein for 24 h and then subjected to Western blotting for Hsp72. Figure 3B shows that Hsp72 induction by acrolein was suppressed by pretreatment of the HUVEC with SP600125, indicating that JNK is involved in the pathway. On the other hand, no effect was found in the case of treatment with PD93059 or SB203580.

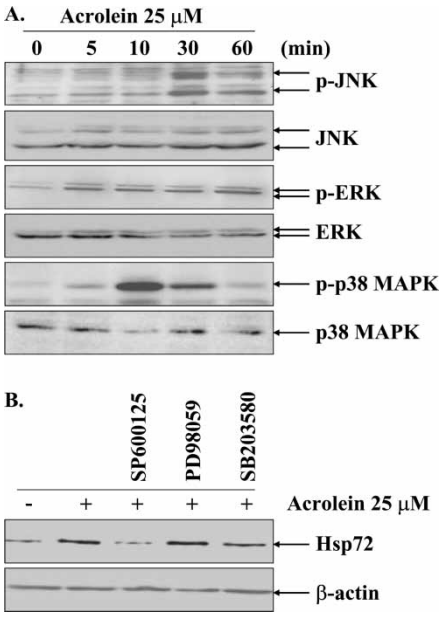

Figure 3. Activation of JNK, ERK and p38 MAPK in HUVEC by acrolein and the effect of MAPKs inhibitor on Hsp72 induction in HUVEC. (A) HUVEC were stimulated with 25 μM acrolein for the indicated periods. Lysates were prepared and 20 μg samples of proteins were analyzed by Western blotting using anti-phospho-JNK, ERK1/2 or p38 MAPK antibodies and anti-JNK, ERK, p38 MAPK antibodies as indicated. (B) HUVEC were preincubated with the JNK inhibitor SP600125 (20 μM), the MEK inhibitor PD98059 (20 μM), and the p38 MAPK inhibitor SB203580 (10 μM) for 30 min. The cells were then treated with 25 μM acrolein for 24 h and 2 μg samples of whole cell lysates were subjected to the Western blotting using anti-Hsp72 antibody and anti-β-actin antibody.

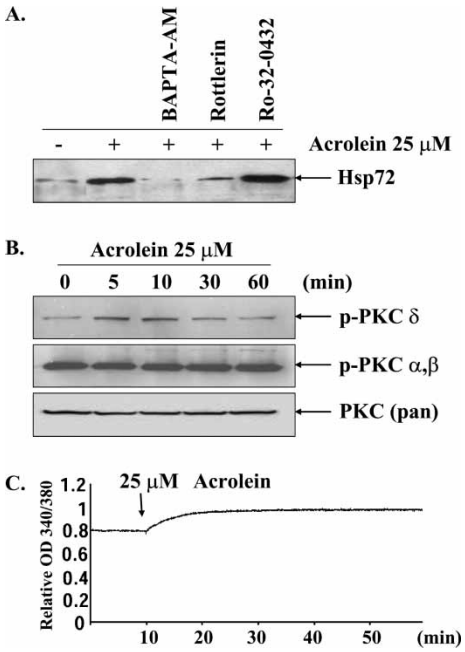

Figure 4. Effect of Ca^{2+} and PKC on the induction of Hsp72 by acrolein. (A) HUVEC were preincubated with calcium chelator, BAPTA-AM (10 μM), PKCδ inhibitor, rottlerin (30 μM) or PKCα,β inhibitor, Ro-32-0432 (1 μM) for 30 min. The cells were then treated with 25 μM acrolein for 24 h and subjected to the Western blotting for Hsp72. (B) HUVEC were stimulated by treatment with 25 μM acrolein for the indicated periods. Lysates were prepared and analyzed by Western blotting using anti-phospho-PKCα,β, PKCδ antibodies and anti-PKC (pan) antibody as indicated. (C) HUVEC were loaded with 5 μM Fura-2-AM for 30 min at room temperature. After washing with HEPES-tyrode buffer, 25 μM acrolein were added to the HEPES-tyrode buffer with 1 mM $CaCl_2$ and changes in Ca^{2+} concentration were measured by recording the ratio of the fluorescence intensities between the excitation wavelengths of 340 and 380 nm by Aquacosmos/Ratio (Hamamatus Photonics).

Effect of intracellular calcium levels and PKC on the induction of Hsp72 by acrolein

We investigated the issue of whether protein kinase C (PKC) and calcium are involved in the induction of Hsp72 by acrolein, since PKC and intracellular calcium levels have been reported to be involved in JNK activation by various cellular stress conditions [24,25]. The cells were preincubated with a membrane-permeable calcium chelator BAPTA-AM, the PKCδ inhibitor rottlerin and the PKCα,β inhibitor Ro-32-0432 for 30 min and then treated with 25 μM acrolein. As shown in Figure 4A, Hsp72 induction by acrolein was decreased by the addition of BAPTA-AM and rottlerin. No effect was found in the case of treatment with Ro-32-0432. When PKCs' activation was examined using specific antibodies to phosphorylated PKCα,β and -δ, it was observed that acrolein stimulated PKCδ phosphorylation at 5 min but not PKCα,β phosphorylation (Figure 4B). We also measured intracellular calcium levels using the membrane-permeable calcium sensitive dye, Fura-2-AM and found that calcium levels were increased immediately after acrolein treatment (Figure 4C).

Hsp72 induction by acrolein is not inhibited by N-acetyl cysteine (NAC) or curcumin

Since acrolein has been considered to exert intracellular signaling via oxidative stress [6,7], we measured the time course for intracellular ROS levels after acrolein treatment using CM-H_2DCF-AM, a cell membrane-permeable ROS sensitive fluorescence dye. In our previous studies, major ROS produced in these conditions are found to be peroxides [26]. Intracellular peroxide levels were increased immediately after acrolein treatment (Figure 5A). This increase was reversed by preincubation with NAC. We also investigated whether ROS are involved in the induction of acrolein-induced Hsp72. The cells were preincubated with NAC or curcumin for 30 min, treated with 25 μM acrolein for 24 h and then subjected to Western blotting for Hsp72. As shown in Figure 5B, NAC or curcumin treatment had no effect, suggesting that ROS are not directly implicated in the induction of Hsp72 by acrolein.

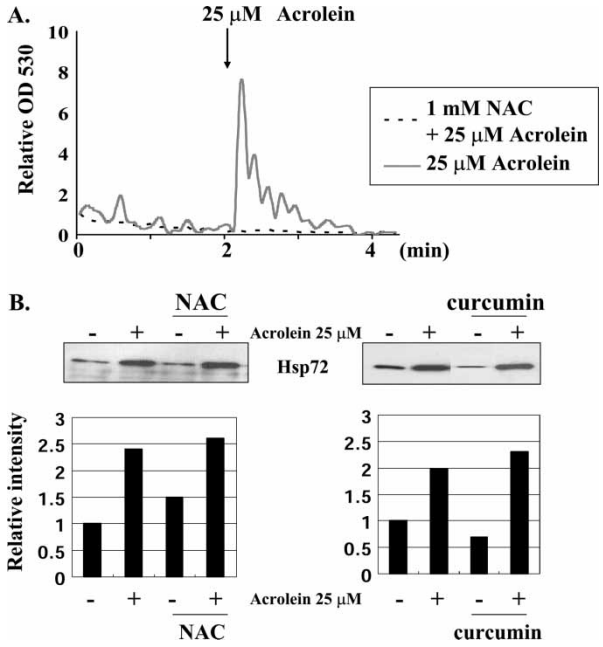

Figure 5. (A) Generation of ROS in HUVEC treated with acrolein and the effect of antioxidants. After treating the cells in FCS-free medium for 1 h, the culture medium was exchanged to PBS and 5 μM of CM-H_2DCF-AM was loaded for 30 min at room temperature. After washing the cells twice with PBS, they were preincubated with or without 1 mM NAC for 30 min and then treated with 25 μM acrolein. Relative 530 nm fluorescent intensity was recorded by ratio-imaging system, Aquacosmos/Ratio (Hamamatsu Photonics). (B) The effect of antioxidants on Hsp72 induction by acrolein in HUVEC. Cells were preincubated with 1 mM NAC or with 20 μM curcumin for 30 min and/or treated with 25 μM acrolein for 24 h. Samples of 2 μg of lysates were separated by SDS-PAGE and analyzed by Western blotting using anti-Hsp72 antibody. The lower panels indicate the densitometric analysis of upper panels.

Discussion

Acrolein is one of the strongest known electrophiles and exerts a variety of intracellular signaling [27–29]. The findings herein show that acrolein induces Hsp72 in HUVEC in a dose- and time-dependent manner. Since the intensity of upregulation of Hsp72 transcript does not necessarily correspond to that of protein level, it is possible that acrolein also stabilizes the Hsp72 protein. We examined other cells such as SMC and COS-1 cells, but Hsp72 induction was only observed in HUVEC among the cell lines we have tested. The findings also indicate that PKCδ/JNK activation and the upregulation of calcium levels are involved in the pathway. Finally, we confirmed that ROS is not directly involved in the pathway. Consistent with this observation, 3-DG and MG did not induce Hsp72 although they induced intracellular ROS production. We conclude that acrolein increases the cellular levels of calcium, and activates the PKCδ/JNK pathway, and that both pathways are involved in Hsp72 induction. The requirement of both pathways was confirmed by the observation that Hsp72 induction was suppressed by individually treating HUVEC with a calcium chelator, and a PKCδ specific inhibitor or JNK inhibitor. The relation between JNK activation and Hsp72 induction has not been reported so far. We assume that phosphorylation of one of JNK substrates is involved in the activation of heat shock factors. Since is has been reported that the total aldehydes including acrolein generated by smoking one cigarette, if completely dissolved in the lung lining fluid, would be present at 2–3 mM [30], the concentration of acrolein in this study is considered to be physiological level.

Acrolein has been considered to exert intracellular signaling via oxidative stress or by modifying cellular molecules [5,6,28,31]. It has been reported that the treatment of cells with acrolein decreases GSH levels and subsequently induces intracellular ROS production [8,9]. We recently found that acrolein inactivates TR and induces an increase in cellular hydrogen peroxide levels [10]. Therefore, we examined the issue of whether ROS are involved in the induction of Hsp72, but the findings show that there is no direct implication of oxidative stress in the pathway, since NAC or curcumin, which suppress intracellular ROS levels were ineffective in inhibiting the induction of Hsp72 by acrolein.

A pathway in which acrolein increases intracellular calcium levels in HUVEC was found. The upregulation of calcium levels were not observed in other cells such as SMC and COS-1. Although the precise mechanisms by which acrolein causes this increase is not known, we assume that the pathway is specific for HUVEC, and thus the induction of Hsp72 is only seen in HUVEC. Since endothelial cell injury is involved in AIDS, Alzheimer's disease, and some kind of neurodegenerative diseases, HUVEC might play a role in defending against various stresses in the blood [32,33]. Other carbonyl compounds, such as MG and 3-DG, did not induce Hsp72 expression in HUVEC. This might be due to the lack of the upregulation of calcium levels by these compounds in HUVEC.

As shown in "Materials and Methods", HEPES-tyrode buffer/1 mM $CaCl_2$ was used to measure intracellular calcium levels. To test the effect of extracellular calcium, HEPES-tyrode buffer, without $CaCl_2$ was also used. The result showed that the removal of extracellular calcium had no effect on the Ca^{2+} increase (data not shown). Thus, the elevation in Ca^{2+} levels by acrolein appears to be mediated by calcium mobilization from intracellular stores rather than by calcium influx.

In conclusion, we report that acrolein induces Hsp72 in HUVEC via both the PKCδ/JNK and calcium signaling pathways. The expression of Hsp72 is considered to play an important role in cytoprotection against various cellular stresses, and therefore, the upregulation of Hsp72 is considered to be a unique defense system of HUVEC, which is more likely to be exposed to exogenous reactive chemical compounds. In this study, the upstream signaling causing the

activation of PKCδ and the upregulation of calcium was not identified, but the receptor tyrosine kinase represents a potential candidate, since the phosphorylation of EGFR by acrolein has been reported in keratinocytes [29]. Further investigation should be done for the elucidating of the upstream of this signaling pathway.

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