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Stress-induced nuclear export of 5-lipoxygenase

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Abstract

A key enzyme for leukotriene biosynthesis is 5-lipoxygenase (5-LO), which we found is exported from the nucleus when p38 MAPK is activated. CHO-K1 cells stably express green fluorescent protein-5-lipoxygenase fusion protein (GFP-5LO), which is located predominantly in the nucleus, and is exported by anisomycin, hydrogen peroxide, and sorbitol, with activation of p38 MAPK. SB203580, an inhibitor of p38 MAPK, and Leptomycin B, an inhibitor of the nuclear export, blocked the anisomycin-induced export of GFP-5LO. When HEK293 cells were transformed with plasmids for wild-type GFP-5LO, GFP-5LO-S271A or GFP-5LO-S271E mutants, most wild-type GFP-5LO and GFP-5LO-S271A localized in the nucleus, but GFP-5LO-S271E localized in the cytosol. Thus, phosphorylation at Ser-271 of 5-LO is important for its export. Endogenous 5-LO in RBL cells stimulated with anisomycin was also exported from the nucleus. These results suggest that the nuclear export of 5-LO depends on the stress-induced activation of the p38 MAPK pathway.

Keywords: 5-Lipoxygenase; Stress stimulus; p38 MAPK; MK; Phosphorylation of Ser-271; Nuclear export

Arachidonate 5-lipoxygenase (5-LO) is the key enzyme in the biosynthesis of leukotrienes (LTs) from arachidonic acid. LTs are potent lipid mediators involved in normal cell functions and in excess in pathological processes. However, little is known regarding how 5-LO is regulated inside the cell. 5-LO was first isolated from cytosolic fractions of various cells [1–3]. It was reported that 5-LO translocates from the cytosol to phospholipid membranes, when cells are activated [4]. However, by immunohistochemical analysis, it was found that subcellular localization of 5-LO differs among cell types [5–7]. Localization and translocation of 5-LO are the determining factors in the production of LTs and so must be tightly controlled [8–10]. In a previous study, we proposed that the localization of 5-LO depends on an import-export balance of the nuclear transport system [11]. Using 5-LO fused with green fluorescent protein (GFP-5LO) expressed in Chinese hamster ovary (CHO)-K1 cells, we demonstrated that a putative bipartite nuclear localization signal (NLS), amino acids 638–655, is important for the nuclear localization of 5-LO, as earlier reported [12], and also that a functional nuclear export signal (NES)-dependent transport may work to determine the cytosolic localization of 5-LO [11].

Signaling pathways that activate the mitogen-activated protein kinases (MAPKs) are involved in relaying extracellular stimulations to intracellular responses. MAPKs regulate cell proliferation, differentiation, motility, and survival, which are mediated by members of a growing family of MAPK-activated protein kinases (MKs; formerly known as MAPKAP kinases) [13]. Among MAPK families, p38 MAPK is activated when cells are exposed to cytokines or various forms of cellular stress resulting in activation of MKs [14]. It was reported that the activation of p38 MAPK in human polymorphonuclear leukocytes and MM6 cells leads to the activation of MKs, which can subsequently phosphorylate 5-LO in vitro [15]. It was also reported that arachidonic acid promotes phosphorylation of 5-lipoxygenase at Ser-271 by MK2 resulting in increase of 5-LO activity [16]. However, the molecular mechanism of how phosphorylation of Ser-271 modulates

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enzyme activity of 5-LO has not been clarified. In this paper, we found that 5-LO is exported from the nucleus by stress stimuli via the p38 MAPK signaling pathway and results in a bi-directional regulation of enzyme activity.

Materials and methods

Construction of expression vectors. The cDNA of human 5-LO (GenBank Accession No. J03571) was cloned by PCR and ligated with *Eco*RI-cut pEGFP-C1 (Clontech, Palo Alto, CA). The PCR product was obtained with the primers 5′-CGGAATTCCCCCTCCTACACGGTCACC-3′ (sense) and 5′-CGGAATTCCGGTCAGATGGCCACACTGTTC-3′ (antisense) using Amply Taq Gold (Perkin-Elmer Biosystems, Foster City, CA). A cDNA (pEGFP-5LO) encoding 5-LO fused with GFP at the N-terminus of 5-LO was obtained with pEGFP-C1. To verify the correct insertion and introduced mutations, DNA sequencing was done with an ABI 373 sequencer using a Big Dye Terminator Ready Reaction Kit (Perkin-Elmer Biosystems).

Cell culture and DNA transfection. CHO cells were cultured in Ham's F-12 medium (Sigma) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 incubator. Rat basophilic leukemia (RBL) cells were grown in RPMI-1640 medium (Sigma, St. Louis, MO) with 10% FBS, Human embryonic kidney (HEK) 293 cells in Dulbecco's modified Eagle's medium (Sigma) with 10% FBS. Cells growing in 35 mm Tissue Culture Dish (Corning, Corning, NY) were transfected with plasmid DNAs by Lipofectamine 2000 (Life Technologies, Rockville, MA). For confocal microscopic analyses of GFP localization, cells were observed 16 h after transfection, and for the analyses of 5-LO activity, cell lysates were obtained 24 h after transfection.

Stimulation of stable transformant CHO-GFP-5LO cells. pEGFP-C1 or pEGFP-5LO was transfected into CHO-K1 cells. Among more than 20 clones for each plasmid DNA resistant to Geneticin (1 mg/ml), we selected two cell lines for each DNA expressing pEGFP-C1 (CHO-GFP cells) or pEGFP-5LO (CHO-GFP-5LO cells), respectively. These cells were maintained in the presence of 0.3 mg/ml geneticin. To observe the translocation of fluorescent signals, cells were incubated for 10 min at 37 °C in a Hepes–Tyrode's buffer (25 mM Hepes–NaOH, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.37 mM NaH_2PO_4, and 0.49 mM $MgCl_2$) with 1% fatty acid-free BSA, and then stimulated with 10 μg/ml anisomycin, 10 μM ionomycin, 1 mM H_2O_2 or 0.4 M sorbitol. To observe the effects of a p38 MAPK inhibitor on the localization of GFP signals, cells were pre-treated with 100 μM SB203580 (Calbiochem, San Diego, CA) for 1 h before stimulation.

Immunoblot analysis. Cells were dissolved in M-PER Mammalian Protein Extraction Reagent (PIERCE, Rockford, IL), and protein concentrations of the homogenates were determined with a Protein Assay Kit II (Bio-Rad, Hercules, CA). Samples containing 10 μg protein were separated on a SDS–polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad). Membranes were probed with an anti-GFP polyclonal antibody (Clontech) (1: 2000 dilution), an anti-human 5-LO antiserum (1: 1000 dilution), an anti-p38 MAPK antiserum (Santa Cruz, CA) (1: 200 dilution) or an anti-phospho-p38 MAPK antiserum (Santa Cruz, CA) (1: 200 dilution) followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (Zymed, San Francisco, CA) (1: 2000 dilution) and treated with an ECL plus chemiluminescence detection system (Amersham–Pharmacia Biotech, Bucks, UK). The anti-human 5-LO antiserum was a generous gift of Dr. J. Evans, Department of Pharmacology, Merck Research Laboratories, West Point, PA.

Calcium mobilization assay. CHO cells (3×10^5) were cultured on glass-bottomed dishes for 16 h and loaded with 3 μM Fura2/AM (Dojin, Kumamoto, Japan) in Hepes–Tyrode's BSA buffer with 0.1% (w/v) protease-free BSA (Wako, Osaka, Japan) and 0.01% (w/v) cremophor EL (Sigma) at 37 °C for 1 h, and then further incubated at room temperature for 1 h. Cells were stimulated with reagents at room temperature and fluorometric calcium cell images were recorded with an ICCD camera/image analysis system (ARGUS-50, Hamamatsu Photonics, Japan).

Fluorescent microscopy. RBL cells were fixed with methanol at −25 °C for 60 min. Cells were incubated with an anti-human 5-LO antiserum (1:200 dilution) and then a goat FITC-conjugated anti-rabbit IgG (Zymed) (1:200 dilution) for 1 h at 37 °C. Nuclear DNA was counterstained with SYTO 82 orange fluorescent nucleic acid. After three washes with PBS, cells were treated with SlowFade Antifade Kit (Molecular Probes, Eugene, OR). The fluorescent signal was observed with an LSM 510 Laser Scanning Microscope System (Carl Zeiss, Oberkochen-Jena, Germany).

Assay of 5-lipoxygenase. 5-LO activity was measured as previously reported [11] with minor modifications. Cell lysates from HEK293 cells at 24 h after DNA transfection or RBL cells were prepared by sonication and then incubated in an assay buffer (1 mM ATP, 2 mM $CaCl_2$, and 50 mM Tris–HCl, pH 8.0) containing 40 μM arachidonic acid, at 37 °C for 10 min. The reactions were terminated with 2 volumes of an ice-cold stop solution (acetonitrile:methanol:acetic acid, 350:150:3, v/v/v), and the mixtures were spun down at 10,000*g* for 10 min. 5-LO metabolites from arachidonic acid (5-HPETE and 5-HETE) in the supernatants were analyzed by reversed-phase-HPLC in a mobile phase consisting of acetonitrile: methanol: water: acetic acid (350:150:250:1, v/v/v/v), with UV detection at 235 nm and at a flow rate of 1 ml/min on an Agillent 1100 series equipped with Polarity dC18 column (150 × 4.6 mm, 5 μm; Waters, Milford, MA). In some cases, two all *trans*-isomers of LTB_4, non-enzymatic products of LTA_4, were measured at 270 nm.

Results

Nuclear export of GFP-5LO is dependent on the phosphorylation of p38 MAPK

While GFP alone is distributed diffusely in both the cytoplasm and nucleus in CHO-GFP cells (data not shown), GFP-5LO is located mainly in the nucleus (nucleoplasm) with a much weaker signal in the cytosol in CHO-GFP-5LO cells. When CHO-GFP-5LO cells were treated with anisomycin (10 μg/ml), a specific activator of p38 MAPK, GFP-5LO was almost completely exported to the cytoplasm within 3 min after stimulation, and localized in the nuclear membrane and perinuclear area (Fig. 1A) accompanied by the phosphorylation of p38 MAPK (Fig. 1D, upper panel). The mobilization of intracellular calcium was not evoked by anisomycin (data not shown). As p38 MAPK is thought to be involved in a stress-induced signaling pathway [17], we tested the effects of other stress stimuli like H_2O_2 (1 mM) or sorbitol (0.4 M) on the translocation of GFP-5LO. Both stimuli led to the nuclear export of GFP-5LO within 30 min with the phosphorylation of p38 MAPK (Fig. 1E). On the one hand, when CHO-GFP-5LO cells were stimulated with ionomycin (10 μM), GFP-5LO moved from the nucleus to the nuclear membrane in 1 min without nuclear export (Fig. 1B). Furthermore, this ionomycin-induced translocation of GFP-5LO was not accompanied by the phosphorylation of p38 MAPK (Fig. 1D, middle panel). These data strongly suggest that the nuclear export of 5-LO is induced by the activation of p38 MAPK.

Next, we examined the effects of inhibitors on the nuclear export of GFP-5LO-induced by anisomycin. SB203580, a specific inhibitor of the p38 MAPK, completely blocked the nuclear export of GFP-5LO (Fig. 1C, left panels) and

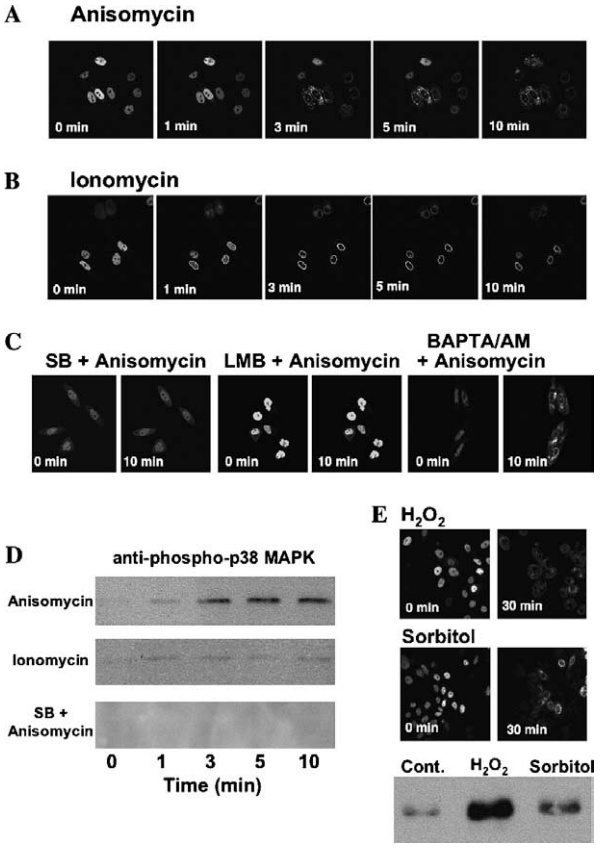

Fig. 1. GFP-5LO translocated by stress or calcium stimuli. CHO-GFP-5LO cells were treated with 10 μg/ml anisomycin (A), 10 μM ionomycin (B), 1 mM hydrogen peroxide or 0.4 M sorbitol (E), and the fluorescent signal of GFP-5LO was observed sequentially at the indicated times with a confocal microscope. CHO-GFP-5LO cells were pre-treated with inhibitors, and the fluorescent signal was observed after stimulation with 10 μg/ml anisomycin (C). SB, 100 μM SB203580 for 10 min; LMB, 10 ng/ml leptomycin B for 10 min; BAPTA/AM, 10 μM BAPTA/AM for 1 h. Immunoblot analysis for phosphorylated p38 MAPK in CHO-GFP-5LO cells stimulated with anisomycin, ionomycin, or anisomycin after pre-treatment with 100 μM SB203580 is shown with an antibody against phospho-p38 MAPK (D). Immunoblot analysis for phosphorylated p38 MAPK in CHO-GFP-5LO stimulated with 1 mM hydrogen peroxide or 0.4 M sorbitol is also shown with an antibody against phospho-p38 MAPK (E).

prevented the phosphorylation of p38 MAPK (Fig. 1D, lower panel). Leptomycin B (LMB) is a specific inhibitor of nuclear export [18,19] and interferes with the binding of the leucine-rich Rev-type NES to exportin 1, CRM1. After addition of LMB for 14 h, GFP-5LO localized exclusively in the nucleus and did not translocate anywhere upon stimulation with anisomycin (10 μg/ml) (Fig. 1C, middle panels). Next, CHO-GFP-5LO cells were treated with bis-(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA/AM) to deduce the intracellular concentration of Ca^{2+} and stimulated with anisomycin (10 μg/ml). BAPTA loading did not change the anisomycin-induced nuclear export of GFP-5LO (Fig. 1C, right panels). These data indicate that the nuclear export of 5-LO is dependent on the p38 MAPK pathway and the NES-CRM1 system, but does not require calcium signaling.

Expression of GFP-5LO mutants at Ser-271 in HEK 293 cells

5-LO has a MK2 phosphorylation motif (hyd-Xaa-Arg-Xaa-Xaa-Ser) in the primary sequence with Ser-271 as the putative phosphorylation site [16]. To understand the roles of the p38 MAPK pathway in the subcellular distribution of 5-LO, vectors for two kinds of mutated GFP-5LO at Ser-271 of 5-LO were constructed and transfected into HEK293 cells. In one vector (pEGFP-5LO-S271A), Ser-271 is replaced by alanine to delete the phosphorylation site, and in the other (pEGFP-5LO-S271E) is replaced by glutamic acid to mimic phosphorylated serine. Fluorescent signals of mutated GFP-5LO transiently expressed in these cells were observed using confocal microscopy (Fig. 2). Wild-type GFP-5LO and GFP-5LO-S271A were observed mainly in the nucleus, while in pEGFP-5LO-S271E transformed cells the cytosolic expression of GFP-5LO-S271E was greatly increased (Fig. 2). Cells in which the cytosolic fluorescent signal was stronger than the nuclear fluorescent signal were rarely observed in each case. The results suggest that the phosphorylation of Ser-271 is associated with cytosolic localization of 5-LO and support the idea that phosphorylation of Ser-271 is necessary for the nuclear export of 5-LO.

The cell lysates of each of the transformed HEK293 cells were obtained by sonication and incubated with arachidonic acid (40 μM) in the presence of 1 mM ATP, 2 mM $CaCl_2$ for 10 min at 37 °C. The 5-LO products in the reactants

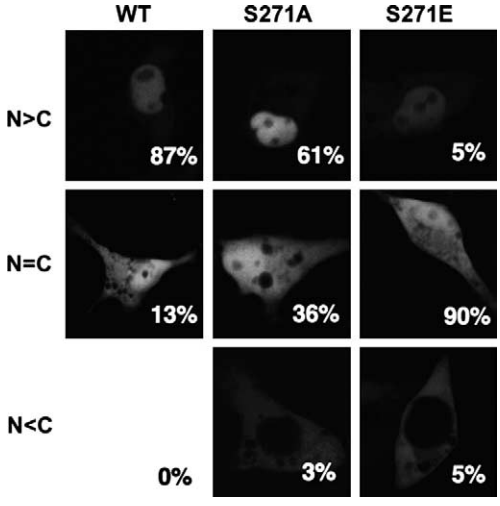

Fig. 2. Analysis of subcellular localization of GFP-5LO mutants at Ser-271. The subcellular localization of wild-type GFP-5LO (WT), GFP-5LO-S271A mutant (S271A), and GFP-5LO-S271E mutant (S271E) in HEK293 was analyzed by confocal microscopy. The percentage in each photo shows the ratio of the expressing pattern in the HEK 293 cells. "N" means nuclear fluorescent signal and "C" means cytosolic fluorescent signal.

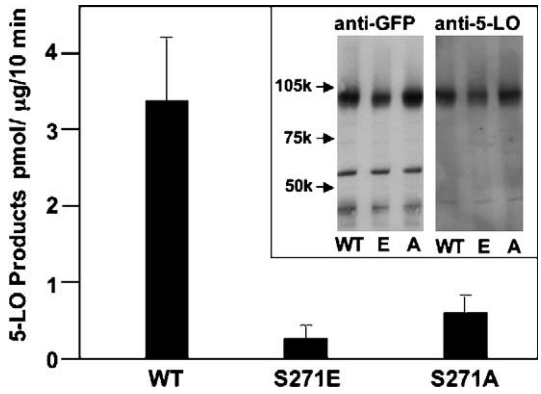

Fig. 3. Enzyme activities of GFP-5LO mutants at Ser-271 expressed in HEK293 cells. Cell lysates of HEK293 cells transfected with pEGFP-5LO, pEGFP-5LO-S271A or pEGFP-5LO-S271E were incubated with arachidonic acid (40 μM), and the resulting products were analyzed by HPLC as described in Materials and methods. (Inset) Immunoblot analyses of wild-type and mutant GFP-5LO proteins transiently expressed in HEK293 cells are shown with antibody against GFP or 5-LO (WT, GFP-5LO; A, GFP-5LO-S271A; and E, GFP-5LO-S271E).

were analyzed by HPLC. As shown in Fig. 3, compared with the activities of wild-type GFP-5LO expressing cells (3.38 pmol/μg/10 min), those of the Ser-271 mutants were low, but significantly higher than that of vector-transformed cells (less than 0.1 pmol/μg/10 min). The activity for GFP-5LO-S271A was 0.27 pmol/μg/10 min (about 8% of wild-type GFP-5LO), and that for GFP-5LO-S271E was 0.61 pmol/μg/10 min (about 18% of wild-type GFP-5LO). Expressions of GFP-5LO, GFP-5LO-S271A, and GFP-5LO-S271E were similar, as determined by Western blot using anti-GFP antibody or anti-5-LO antibody (Fig. 3 inset). Though precise enzymatic analyses of purified 5-LO in the HEK293 transformants were not performed, these data may indicate that mutation at Ser-271 is a disadvantage for enzymatic activity of 5-LO.

Nuclear export of endogenous 5-LO in RBL cells

To analyze the translocation of endogenous 5-LO, RBL cells were stimulated with anisomycin or ionomycin. In RBL cells, the fluorescent signal of 5-LO was observed in both the nucleus and the cytosol, but the nuclear signal was stronger than the cytosolic signal (Figs. 4A and B, time = 0). When RBL cells were treated with anisomycin, 5-LO moved from the nucleus to the cytosol within 10 min, and localized in the nuclear membrane and perinuclear area (Fig. 4A). Before the nuclear export of 5-LO, obvious phosphorylation of p38 MAPK was observed 3 min after stimulation (Fig. 4C, left panel). In contrast, when RBL cells were stimulated with ionomycin, 5-LO rapidly moved to the nuclear membrane within 1 min after stimulation without the export from the nucleus. Strong phosphorylation of p38 MAPK was observed 10 min after stimulation with ionomycin, but much later than the rapid translocation of 5-LO to the nuclear membrane (Fig. 4C, right panel). Thus, the late

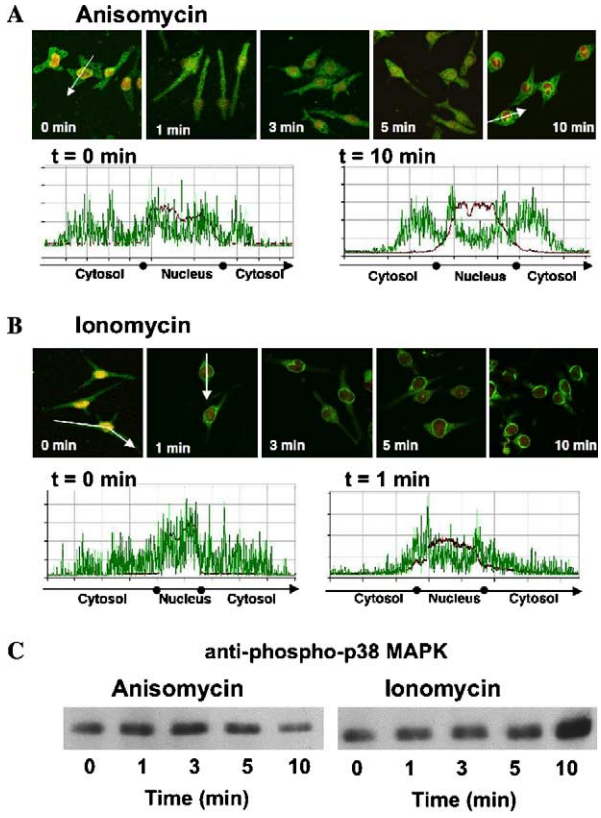

Fig. 4. Translocation of endogenous 5-LO and phosphorylation of p38 MAPK in RBL cells stimulated with anisomycin or ionomycin. RBL cells were stimulated with 10 μg/ml anisomycin (A) or 10 μM ionomycin (B) for 1, 3, 5, and 10 min. Fixed RBL cells were treated with an anti-5-LO antiserum, followed with FITC-conjugated second antibody and SYTO 82 orange fluorescent nucleic acid stain. Fluorescence was observed with a confocal microscope. Fluorescent profiles of the cross-sections are indicated by white lines. Small circles indicate the edge of the nucleus. Immunoblot analysis of phosphorylated p38 MAPK stimulated with anisomycin or ionomycin in RBL cells is shown with an antibody against phospho-p38 MAPK (C).

activation of p38 MAPK-induced by ionomycin was not involved in the rapid translocation of 5-LO to the nuclear membrane.

Activity of endogenous 5-LO in intact RBL cells induced by stress stimuli

To analyze the effects of the translocation of 5-LO on its enzyme activity, the enzyme activity of intact RBL cells stimulated with anisomycin or/and ionomycin in the presence of exogenous arachidonic acid (40 μM) was determined by HPLC. As shown in Table 1, the basic 5-LO activity of intact RBL cells was 0.69 pmol/μg/10 min, while without arachidonic acid no 5-LO products were observed (data not shown). When RBL cells were stimulated with anisomycin, the enzyme activity became about 1.3 times that of the basic activity. On the other hand, when RBL cells were stimulated with ionomycin, enzyme activity was enhanced 11 times. In the case of simultaneous stimulation with anisomycin and ionomycin, the activity was

Table 1
The 5-LO activity in intact RBL cells treated with anisomycin or/and ionomycin

Anisomycin	Ionomycin	5-LO activity (pmol/mg/10 min)
−	−	0.69 ± 0.02
+	−	0.88 ± 0.14
−	+	7.60 ± 2.42
+	+	3.33 ± 0.45

RBL cells were incubated with arachidonic acid (40 μM) in the presence of anisomycin (10 μg/ml) or/and ionomycin (10 μM) for 10 min as described in Materials and methods. 5-LO products were analyzed by HPLC. Values are given as means ± SD ($n = 3$).

about 4.8 times that of the basic activity, between stimulation with anisomycin or ionomycin.

Discussion

5-Lipoxygenase (5-LO) is predominantly localized in the cytosol of peripheral blood polymorphonuclear cells [6] and peritoneal macrophages [5], whereas it is found in both the nucleus and the cytosol of alveolar macrophages [20], mast cells [7], and RBL cells [21]. The intracellular localization of 5-LO varies according to cell type and might depend on how nuclear import and export systems work on 5-LO. Many NLSs were reported in 5-LO [22], while NES was not identified yet. In this report, we showed that GFP-5LO and 5-LO, predominantly localized in the nucleus in CHO-K1 and HEK293 cells at resting states, are exported from the nucleus when cells are treated with anisomycin and stress stimuli. The nuclear export of 5-LO is accompanied by the activation of p38 MAPK (Figs. 1 and 4). This translocation of 5-LO was blocked by SB202380, a p38 MAPK inhibitor, and by LMB, an inhibitor for CRM1-dependent nuclear export (Fig. 1C). These data suggest that nuclear export systems begin to work on 5-LO by stress stimuli, possibly by the unmasking the NES of 5-LO.

P38 MAPK is rapidly activated by diverse stresses (hyperosmolarity, UV light, heat shock, arsenite, and anisomycin) as well as by endotoxins and cytokines. Activated p38 MAPK phosphorylates and regulates downstream protein kinases and certain transcription factors [23]. Multiple p38-dependent inflammatory responses are mediated by a serine/threonine kinase, MK2 [24,25]. In our hypothesis, p38 MAPK is phosphorylated with stress stimuli, and activated p38 MAPK is imported into the nucleus to phosphorylate MK2. MK2 in its inactive form is mainly in the nucleus, and its stress-induced activation is paralleled by an export to the cytoplasm [26]. As a result, 5-LO could be phosphorylated by the p38 MAPK-regulated MK2 both in the nucleus and the cytoplasm. The phosphorylation of nuclear 5-LO may cause the NES to be unmasked, and the CRM1-dependent export system translocates 5-LO to the cytoplasm. These ideas might be supported by the results obtained from the mutation of S271E to mimic the phosphorylation, as the cytosolic expression of GFP-5LO-S271E was greatly increased (Fig. 2). The effects of phosphorylation of Ser-271 on enzyme activity are not straightforward. The 5-LO activities of GFP-5LO-S271A and GFP-5LO-S271E expressed in HEK 293 cells were lower than that of wild-type GFP-5LO. In intact RBL cells, anisomycin slightly stimulated 5-LO activity compared with resting cells, but to a much lower extent than stimulation with ionomycin. Simultaneous stimulation with ionomycin and anisomycin caused a decrease of 5-LO activity compared with the stimulation with ionomycin only. These data here may indicate that the stress-induced phosphorylation of Ser-261 and the subsequent nuclear export of 5-LO have bi-directional effects on the 5-LO activity. The transportation of 5-LO from the nucleoplasm to the cytosol destined to the nuclear and perinuclear membranes might be preferable for enzyme activity to reach the substrate, arachidonic acid, liberated from membrane phospholipids. However, the phosphorylation itself of 5-LO at Ser-261 decreases enzyme activity. There are other serine residues of 5-LO reported to be phosphorylated by MAPK (Ser-663) [27] and by protein kinase A (Ser-523) [28]. Whether these serine residues are phosphorylated or not under each cell condition needs to be investigated to better understand the molecular mechanisms involved if the control of the 5-LO activity was regulated by extracellular stimuli.

In this report, we showed that stress stimuli causes the transportation of 5-LO from the nucleus to the cytoplasm in a p38 MAPK-dependent manner, and that phosphorylation of Ser-271 might be important for this nuclear export. However, the molecular mechanisms and the effects on 5-LO activity of this nuclear export remain to be elucidated.

Acknowledgments

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