

Rapid degradation of cyclooxygenase-1 and hematopoietic prostaglandin D synthase through ubiquitin–proteasome system in response to intracellular calcium level

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ABSTRACT Cyclooxygenase (COX)-1 and hematopoietic prostaglandin (PG) D synthase (H-PGDS) proteins, which are both involved in the arachidonate cascade, were stable in human megakaryocytic MEG-01 cells. In contrast, once the intracellular calcium level was increased by treatment with a calcium ionophore, both protein levels rapidly decreased with a half-life of less than 30 and 120 min for COX-1 and H-PGDS, respectively. In the presence of a proteasome inhibitor, COX-1 and H-PGDS proteins accumulated within 10 and 30 min, respectively, and concurrently appeared as the high-molecular-mass ubiquitinated proteins within 30 and 60 min, respectively, after an increase in the intracellular calcium level. The ubiquitination of these proteins was also observed when ADP, instead of a calcium ionophore, was used as an inducer to elevate the intracellular calcium level. When the entry of calcium ion into the cells was inhibited by ethylene glycol tetraacetic acid (EGTA), the ubiquitination of COX-1 and H-PGDS was clearly suppressed; and the addition of CaCl₂ to the medium cleared the EGTA-mediated suppression of the ubiquitination. These results indicate that COX-1 and H-PGDS were rapidly ubiquitinated and degraded through the ubiquitin–proteasome system in response to the elevation of the intracellular calcium level.

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INTRODUCTION

The production of prostaglandins (PGs) is restricted to certain areas, where they function as local hormones with short half-lives and play a number of physiological roles. PGs are synthesized through the following three enzymatic steps: 1) cytosolic phospholipase A₂ (cPLA₂) releases arachidonic acid from membrane phospholipids; 2) the arachidonic acid is then converted to PGH₂ by the action of ei-

ther cyclooxygenase (COX)-1 or COX-2; and 3) PGH₂, the common precursor of all prostanoids, is converted to various PGs, including PGD₂, by the specific terminal PG synthases (Urade and Hayaishi, 2000a, 2000b; Helliwell *et al.*, 2004; Ueno *et al.*, 2005).

COXs are the rate-limiting enzymes in the arachidonate cascade (Smith *et al.*, 2000; Kang *et al.*, 2007). COX-1 and COX-2 are highly homologous, but their expression profiles are quite different. COX-1 is constitutively expressed in a variety of cells, and its expression in megakaryocytic cells is enhanced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In contrast, COX-2 expression is inducible in response to various stimuli, such as proinflammatory cytokines (Smith *et al.*, 2000; Kang *et al.*, 2007). The transcriptional regulatory mechanisms of these COXs have been well investigated (Kang *et al.*, 2007). Post-transcriptional regulation of COX-2, but not that of COX-1, has been well studied. COX-2 mRNA is rapidly degraded through AU-rich, sequence element-mediated degradation (Dixon, 2004). Moreover, the COX-2 protein is degraded through the ubiquitin–proteasome system via the endoplasmic reticulum (ER)-associated degradation system (Mbonye *et al.*, 2006, 2008; Mbonye and Song, 2009).

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Abbreviations used: CHX, cycloheximide; COX, cyclooxygenase; cPLA₂, cytosolic phospholipase A₂; cPLA₂i, cPLA₂ inhibitor; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; HBSS, Hank's balanced salt solution; H-PGDS, hematopoietic PGDS; PBS, phosphate-buffered saline; PG, prostaglandin; PGDS, prostaglandin D synthase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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PGD₂ is produced in a variety of cells by two distinct types of PGD synthases (PGDS; EC 5.3.99.2; Urade and Hayaishi, 2000a). One is the lipocalin-type PGDS, and the other is hematopoietic PGDS (H-PGDS). H-PGDS is the 26-kDa cytosolic enzyme responsible for the glutathione-dependent biosynthesis of PGD₂ and is the only vertebrate member of the sigma class of cytosolic glutathione S-transferases (Kanaoka and Urade, 2003). It is also called GSTS1 (Hayes *et al.*, 2005). H-PGDS has been isolated from human, mouse, rat, and chicken sources, and its molecular properties have been extensively studied (Kanaoka *et al.*, 1997; Thomson *et al.*, 1998; Kanaoka *et al.*, 2000). H-PGDS is expressed in antigen-presenting cells (Urade *et al.*, 1989), megakaryocytes (Fujimori *et al.*, 2000), type 2 helper T lymphocytes (Tanaka *et al.*, 2000), microglia (Mohri *et al.*, 2003), and dendritic cells (Shimura *et al.*, 2010). Moreover, H-PGDS acts as a mediator of allergies (Yamamoto *et al.*, 2009), suppresses intestinal adenomas in *Apc^{Min/+}* mice (Park *et al.*, 2007), and shows enhanced expression and involvement in the progression of muscle necrosis (Mohri *et al.*, 2009), which is inhibited by HQL-79, a potential inhibitor of H-PGDS (Tanaka *et al.*, 2011). Thus H-PGDS inhibitors are considered to be useful in suppressing several diseases, including neurodegenerative diseases and muscular dystrophy (Mohri *et al.*, 2006, 2009). The H-PGDS mRNA level is increased through the Oct-1 and AP-2 elements by TPA in megakaryocytes (Fujimori *et al.*, 2000). Moreover, H-PGDS is translocated from the cytoplasm to the ER to produce PGD₂ when the intracellular calcium level is increased (Ueno *et al.*, 2001). However, the posttranslational regulation of COX-1 and H-PGDS proteins has never been identified.

In this study, we show the rapid degradation of COX-1 and H-PGDS proteins through the ubiquitin–proteasome system in response to the elevation of the intracellular calcium level in human megakaryocytic MEG-01 cells. This is the first identification of the ubiquitination-dependent degradation of COX-1 and H-PGDS as a terminal PG synthase involved in the arachidonate cascade.

RESULTS

Rapid decrease in COX-1 and H-PGDS protein levels in response to an increase in the intracellular calcium level

COX-1 is localized in the membranes of the ER and nucleus (Smith *et al.*, 2000), while H-PGDS is translocated from the cytosol to the ER membranes by treatment with A23187 (Ueno *et al.*, 2001), indicating that H-PGDS protein is translocated to the ER membranes in response to elevation of intracellular calcium level. We first measured the intracellular calcium level of human megakaryocytic MEG-01 cells after having treated them with ionomycin. We used ionomycin instead of A23187, because A23187 displays autofluorescence and is thus unsuitable for use in fluorescence analysis. MEG-01 cells were cultured with TPA for 16 h and then incubated with ionomycin and Fluo 4-AM, which binds Ca²⁺. Fluorescence microscopy was used to track the time course of Fluo 4-AM-derived fluorescence after the start of treatment with ionomycin. Intracellular calcium concentration quickly increased, and its level was further enhanced 10 min after the start of treatment with ionomycin (Figure 1, A and B). However, when cells were not treated with the ionophore, no apparent increase in the intracellular calcium level was detected (Figure 1, A and B).

To examine the stability of COX-1 and H-PGDS proteins in MEG-01 cells, we cultured cells for 16 h in medium containing TPA and then incubated them for various times with cycloheximide (CHX) in the presence or absence of A23187. The levels of both proteins were determined by immunoprecipitation and Western blot analysis. The COX-1 protein in MEG-01 cells was stable for at least 60 min (Figure 1, C and D, left). However, once the intracellular calcium

level became elevated, the COX-1 protein level rapidly decreased, with a half-life of <30 min (Figure 1, C and D, left).

The H-PGDS protein in the cells was stable for at least 240 min (Figure 1, C and D, right). However, when the intracellular calcium level was increased by treatment with A23187, the level of H-PGDS protein rapidly decreased, with a half-life of <120 min (Figure 1, C and D, right). These results, taken together, indicate that both COX-1 and H-PGDS proteins were stable at the normal intracellular calcium level, but were rapidly degraded once the intracellular calcium level had increased in the MEG-01 cells.

Accumulation and ubiquitination of COX-1 and H-PGDS in response to the increase in the intracellular calcium level

To investigate whether the COX-1 and H-PGDS protein levels were decreased due to proteasomal degradation, we incubated MEG-01 cells with A23187 for 60 or 240 min with or without MG132, a proteasome inhibitor that irreversibly inhibits the 20S proteasome and causes the accumulation of ubiquitinated proteins normally degraded by the proteasome system (Welchman *et al.*, 2005).

Accumulated COX-1 protein was detected at 10 min after initiation of treatment with A23187 and MG132, and its level increased in a time-dependent manner (Figure 2A), suggesting that COX-1 is a likely substrate of the ubiquitin–proteasome complex, whereas COX-1 did not accumulate when cells were cultured in medium containing A23187 alone (Figure 2A); these results are consistent with previous results (Moraitis and Giguere, 2003; Mishra *et al.*, 2009). To further investigate COX-1 degradation through the ubiquitin–proteasome system, we examined the time dependence of the effect of MG132 on the ubiquitination of the COX-1 protein in the cells. When cells were incubated with MG132 and A23187 for 0, 30, or 60 min, a high-molecular-weight smeared mass of the ubiquitinated COX-1 protein was detected at 30 min, and it increased in a time-dependent manner after an increase in the intracellular calcium level (Figure 2B). No high-molecular-weight smeared mass of ubiquitinated COX-1 protein was detected at 15 min after the elevation of the intracellular calcium level (unpublished data).

H-PGDS protein accumulated at 30 min after the intracellular calcium level was increased, and its level was enhanced in an incubation time–dependent manner (Figure 2C), revealing that H-PGDS becomes a substrate of the ubiquitin–proteasome system. In contrast, the H-PGDS level was not changed when the cells were cultured in medium containing A23187 alone (Figure 2C). Moreover, when MG132 was present, a high-molecular-weight smeared mass of the ubiquitinated H-PGDS protein was detected at 60 min after the elevation of the intracellular calcium level, and the level of the ubiquitinated H-PGDS protein increased in a time-dependent manner (Figure 2D). No high-molecular-weight smeared mass of ubiquitinated H-PGDS protein was observed at 30 min after the enhancement of the intracellular calcium level (unpublished data).

Furthermore, ubiquitinated COX-1 and H-PGDS proteins were not detected when the cells were cultured in medium containing either MG132 or A23187, although a high-molecular-weight smeared mass of ubiquitinated COX-1 and H-PGDS was detected in both A23187- and MG132-treated cells (Figure 3, A and B). These results, taken together, reveal that ubiquitin-dependent degradation of COX-1 and H-PGDS proteins was initiated within 10 and 30 min, respectively, after a rise in the intracellular calcium level in MEG-01 cells.

Substrate and product-independent ubiquitination of COX-1 and H-PGDS

To study the roles of substrates and products of COX-1 and H-PGDS on the ubiquitin-dependent degradation of both enzymes,

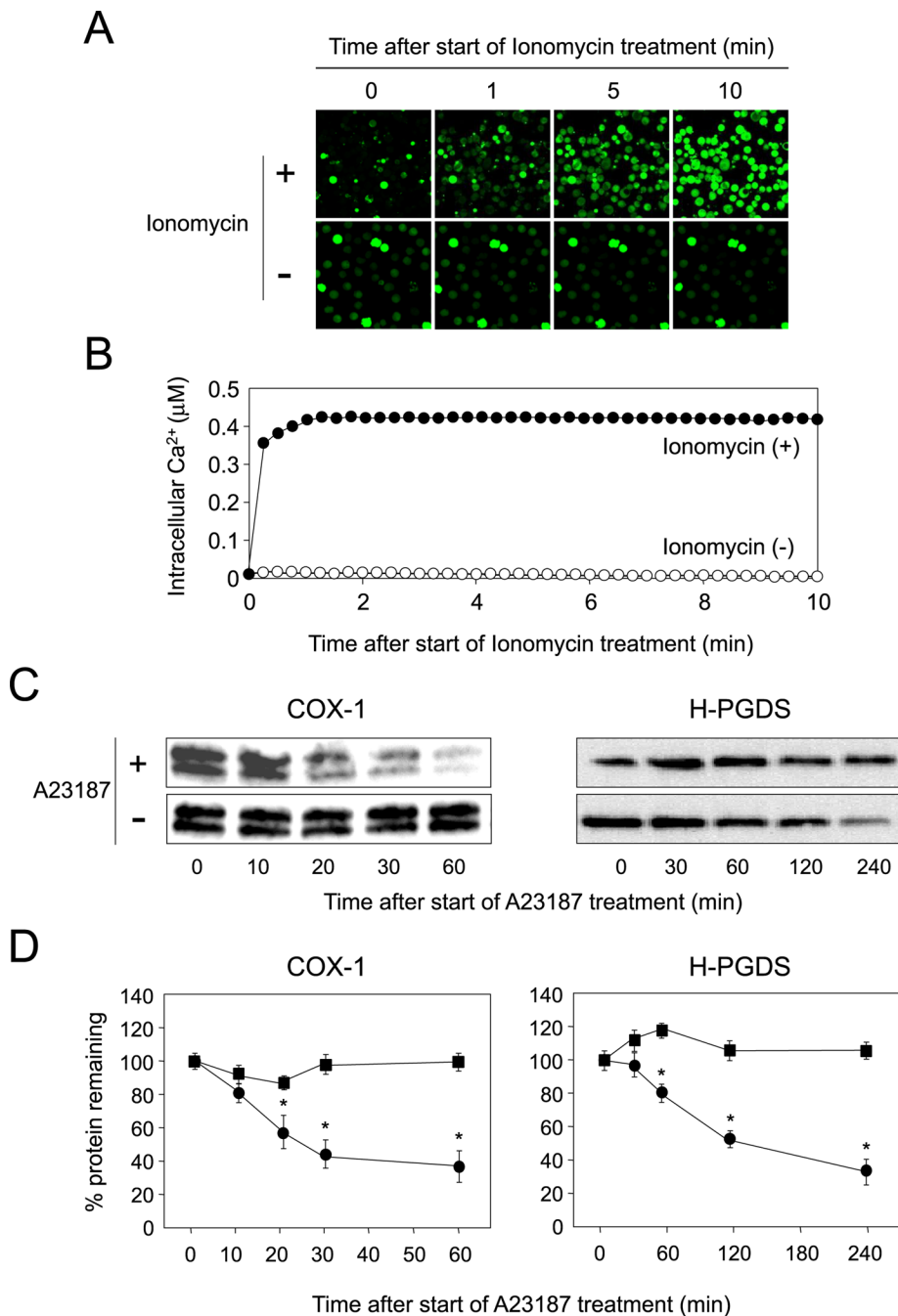


FIGURE 1: Calcium-dependent degradation of COX-1 and H-PGDS proteins. (A) Increasing the intracellular calcium level in MEG-01 cells. Data are representative of three independent experiments. (B) Measurement of intracellular calcium level in MEG-01 cells. Ionomycin: closed circles; vehicle: open circles. Data are representative of three independent experiments. (C) Stability of COX-1 and H-PGDS proteins in MEG-01 cells. Cells were cultured with CHX (10 µM; Sigma) for 15 min prior to addition of A23187 and CHX. Data are representative of three independent experiments. (D) The intensities of immunoreactive bands. The value at 0 min was shown as 100% of protein remaining. Data are representative of three independent experiments and represent the mean ± SD (**p* < 0.01), as compared with A23187(-): closed squares; A23187(+): closed circles.

ubiquitination of both proteins was examined under the influence of inhibitors for cPLA₂, COX, or H-PGDS in MEG-01 cells. PGD₂ production was inhibited with cPLA₂ inhibitor (cPLA₂i); aspirin; indomethacin, COX inhibitors; SC-560 (SC), a COX-1 inhibitor; and HQL-79 (HQL), an H-PGDS inhibitor in MEG-01 cells (Figure 4A). Cells were treated with cPLA₂, COX, or H-PGDS inhibitors and then incubated

~21% of the ionomycin-mediated elevation at 10 s in MEG-01 cells (Figure 5B, inset).

We next examined the ubiquitination-dependent degradation of COX-1 and H-PGDS proteins in the ADP-treated MEG-01 cells. When the cells were cultured in medium containing ADP with or without MG132 for 30 min, ubiquitination of COX-1 protein was

in medium containing A23187 and MG132 for 60 min, and ubiquitination of both proteins was examined. COX-1 was ubiquitinated by treatment with A23187, and this ubiquitination was not inhibited, even in the presence of these four inhibitors (Figure 4B). In addition, COX-1 protein level was decreased to the same extent, even in the presence of each inhibitor in both A23187- and CHX-treated cells, as compared with the vehicle-treated cells (Supplemental Figure S1A, left). Moreover, when cells were cultured under the same conditions without stimulation with ionophore, the COX-1 level was not changed (Figure S1A, right).

Ubiquitination of H-PGDS was detected in A23187-treated MEG-01 cells, and the level of this ubiquitination was not altered by cotreatment with any of the five inhibitors (Figure 4C). Moreover, the protein level of H-PGDS was eliminated by treatment of cells with A23187 and CHX, and its decreased level was not altered, even when the cells were cultured separately in medium containing each inhibitor (Figure S1B, left). Furthermore, the H-PGDS level was not altered when the cells were cultured in the same conditions without A23187 stimulation (Figure S1B, right). These results indicate that the calcium ion-mediated ubiquitination of COX-1 and H-PGDS was independent on the levels of their substrates and their products in MEG-01 cells.

Ubiquitin-dependent degradation of COX-1 and H-PGDS in ADP-treated MEG-01 cells

The entry of calcium ions into cells treated with a calcium ionophore may not represent physiological conditions. As ADP is known to enhance the intracellular calcium level in MEG-01 cells (Nakata *et al.*, 2005), we next measured the intracellular calcium level in MEG-01 cells incubated with ADP or ionomycin. Cells were cultured with TPA for 16 h and then incubated with Fluo 4-AM in the presence or absence of ADP or ionomycin for 5 min, after a 15-min preincubation with Fluo 4-AM. Intracellular calcium level increased slightly at 10 s, then decreased, and became low level by 3 min after initiation of treatment with ADP (Figure 5, A and B; note Figure 5B, inset), indicating that ADP weakly enhanced the intracellular calcium level in MEG-01 cells. The enhancement of the intracellular calcium level by ADP was

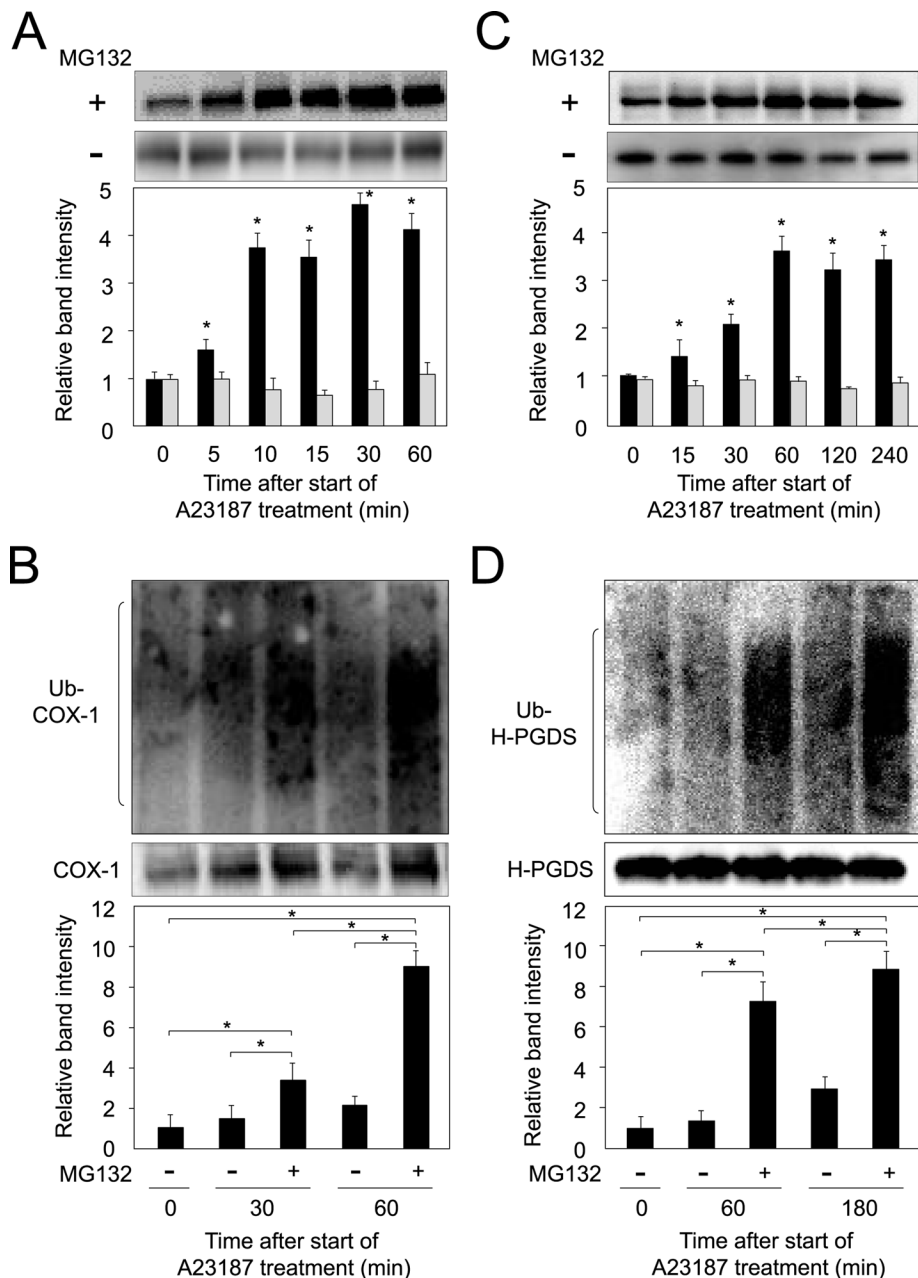


FIGURE 2: Rapid ubiquitination of COX-1 and H-PGDS. (A) Accumulation of COX-1 in response to an increase in the intracellular calcium level was dependent upon the incubation time with MG132. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as compared with before treatment (0 min). MG132(-): gray columns; MG132(+): black columns. (B) Ubiquitin-dependent degradation of COX-1 (Ub-COX-1). High-molecular-weight smeared signals were measured. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as indicated by the brackets. (C) Accumulation of H-PGDS was dependent on the incubation time with MG132. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as compared with before treatment (0 min). MG132(-): gray columns; MG132(+): black columns. (D) Time dependence of the change in the ubiquitinated H-PGDS (Ub-H-PGDS) level in MEG-01 cells. High-molecular-weight smeared signals were measured. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as indicated by the brackets.

observed, as compared with the levels of ubiquitination in the pre-treated cells or only MG132-treated cells (Figure 5C). In addition, COX-1 protein level was decreased in both ADP- and CHX-treated cells, as compared with the vehicle- or CHX-treated cells (Figure

5A). Moreover, ubiquitinated H-PGDS protein was detected at 60 min after initiation of ADP treatment, as compared with the level of ubiquitinated protein in the MG132-treated cells (Figure 5D). Furthermore, the protein level of H-PGDS was reduced by treatment of cells with both ADP and CHX, as compared with the vehicle- or CHX-treated cells (Figure S2B). These results reveal that the calcium-dependent degradation of COX-1 and H-PGDS proteins was also observed in MEG-01 cells treated with ADP, a natural inducer that causes an increase in intracellular calcium level. Thus the degradation of both enzymes was initiated by the treatment with ADP as well as with ionophore.

Essential roles of the entry of calcium ion into cells upon ubiquitination-dependent degradation of COX-1 and H-PGDS

To investigate the molecular mechanism of the calcium-dependent degradation of COX-1 and H-PGDS proteins, we investigated the roles of intracellular and extracellular calcium ions in the ubiquitination-dependent degradation of COX-1 and H-PGDS proteins. MEG-01 cells were first incubated with TPA for 16 h, and then for an additional 10 min in RPMI1640 containing ionomycin and Fluo 4-AM with ethylene glycol tetraacetic acid (EGTA) and/or CaCl_2 , after preincubation with Fluo 4-AM for 15 min. The intracellular calcium level was increased by treatment with ionomycin (Figure 6, A, top, and B). On the contrary, when cells were cultured in medium with ionomycin and EGTA, the intracellular calcium level was transiently elevated at 1 min after initiation of treatment and then quickly decreased (Figure 6A, middle, and B). Moreover, when cells were incubated in medium containing ionomycin, EGTA, and CaCl_2 , the entry of calcium ions into the cells was greater (Figure 6, A, bottom, and B) than that seen in cells cultured in RPMI1640 containing ionomycin and EGTA (Figure 6, A, top, and B).

We next investigated the ubiquitination of COX-1 and H-PGDS proteins in RPMI1640 with or without EGTA and/or CaCl_2 . When MEG-01 cells were cultured in medium containing A23187, the ubiquitination of COX-1 protein was enhanced, as compared with that in the vehicle control (Figure 6C). Moreover, when cells were incubated in medium containing A23187 and EGTA, the ubiquitination of COX-1 protein was clearly decreased, as compared with that in A23187-treated cells (Figure 6C).

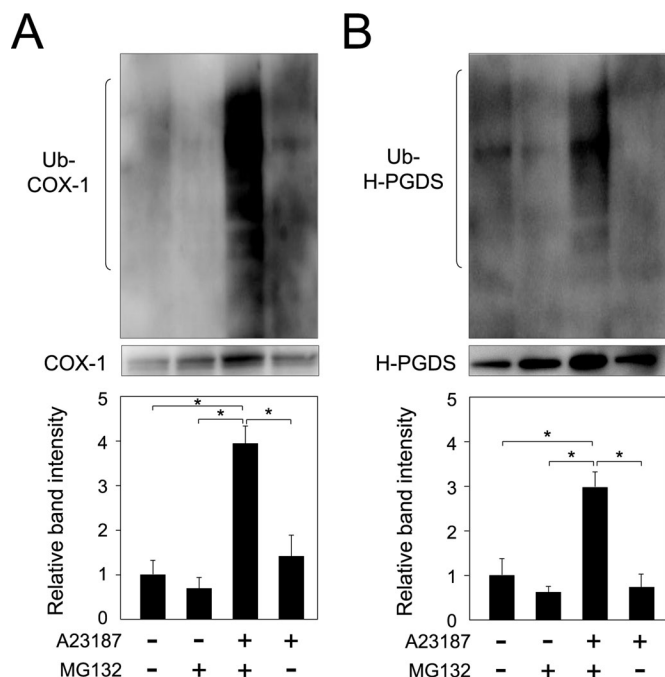


FIGURE 3: Ubiquitination of COX-1 and H-PGDS in the presence of MG132. (A) MG-132-dependent ubiquitination of COX-1 in MEG-01 cells. The cells were incubated in medium with TPA for 16 h and then cultured for 30 min in medium containing A23187 in the presence or absence of MG132. High-molecular-weight smeared signals were measured. Data are representative of three independent experiments and represent the mean \pm SD (* p < 0.01), as indicated by the brackets. (B) Ubiquitination of H-PGDS is dependent on MG132. The cells were cultured for 60 min, as described in the legend of (A). High-molecular-weight smeared signals were measured. Data are representative of three independent experiments and represent the mean \pm SD (* p < 0.01), as indicated by the brackets.

medium with A23187 and EGTA (Figure 6C). In addition, the protein level of COX-1 was reduced by the treatment with A23187 and CHX, and this reduction was restored by cotreatment with A23187, CHX, and EGTA (Figure S3A). Moreover, when cells were cultured in medium containing A23187, CHX, and EGTA together with CaCl_2 , the COX-1 protein level was clearly reduced (Figure S3A).

The ubiquitination level of H-PGDS protein was increased by incubation with A23187, as compared with that in the vehicle control (Figure 6D). Furthermore, when cells were incubated in the presence of A23187 and EGTA, ubiquitination of H-PGDS protein was clearly decreased, as compared with that for cells cultured in medium with A23187 alone (Figure 6D). Conversely, when MEG-01 cells were cultured in medium containing A23187 and EGTA together with CaCl_2 , the ubiquitination level of H-PGDS protein was enhanced, as compared with that for cells cultured in medium containing A23187 and EGTA (Figure 6D). Furthermore, the H-PGDS protein level was decreased by treatment with A23187 and CHX, as compared with that of the vehicle-treated cells (Figure S3B). This decrease was restored by cotreatment with A23187, CHX, and EGTA (Figure S3B). Moreover, when cells were cultured in medium containing A23187, CHX, and EGTA together with CaCl_2 , the H-PGDS protein level was reduced (Figure S3B).

To confirm the requirement of the entry of calcium ion for the ubiquitination of the COX-1 and H-PGDS proteins, we cultured MEG-01 cells in Hank's balanced salt solution (HBSS; containing 1.3 mM CaCl_2) or Ca^{2+} -free HBSS and detected the ubiquitinated

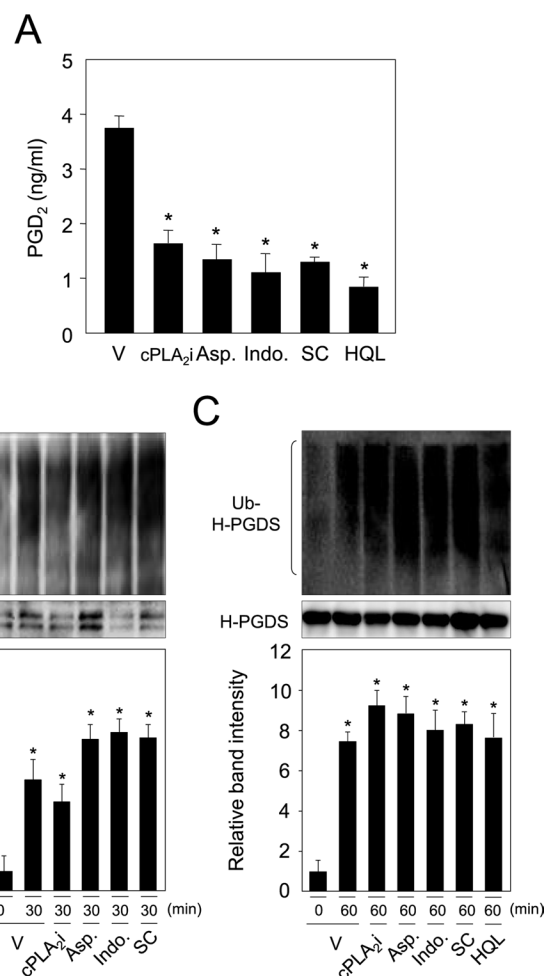


FIGURE 4: Ubiquitination of COX-1 and H-PGDS independent of their substrates and products in MEG-01 cells. (A) PGD_2 production in MEG-01 cells was inhibited by cPLA₂i (10 μM); Aspirin (Asp.; 10 μM); indomethacin (Indo.; 2 μM), COX inhibitors; SC-560 (SC; 5 μM), a COX-1 inhibitor; and HQL-79 (HQL; 3 μM), an H-PGDS inhibitor. MEG-01 cells were incubated in medium containing TPA for 16 h and then incubated with A23187 in the presence or absence of each inhibitor for 30 min. Data represent the mean \pm SD (* p < 0.01) from three independent experiments, as compared with the vehicle. (B) Ubiquitination of COX-1 independent of the inhibitors. Data are representative of three independent experiments and represent the mean \pm SD (* p < 0.01), as compared with the vehicle. (C) H-PGDS ubiquitination under the existence of either inhibitor. MEG-01 cells were incubated for 60 min, as described in the legend of (B), together with A23187 and MG132. Data are representative of three independent experiments and represent the mean \pm SD (* p < 0.01), as compared with the vehicle.

COX-1 and H-PGDS proteins by immunoprecipitation and Western blot analysis. Ionomycin-induced entry of the extracellular calcium ion into the cells was observed with the cells in HBSS (Figure 7, A, top, and B). In contrast, the intracellular calcium level was transiently increased at 1 min in Ca^{2+} -free HBSS, even when the cells were incubated with ionomycin (Figure 7, A, bottom, and B).

Finally, we investigated the requirement of extracellular calcium ions on the ubiquitination of COX-1 and H-PGDS proteins in MEG-01 cells. When the cells were cultured in HBSS containing A23187 and MG132, the ubiquitination level of COX-1 protein was enhanced, as compared with that in the A23187-treated cells

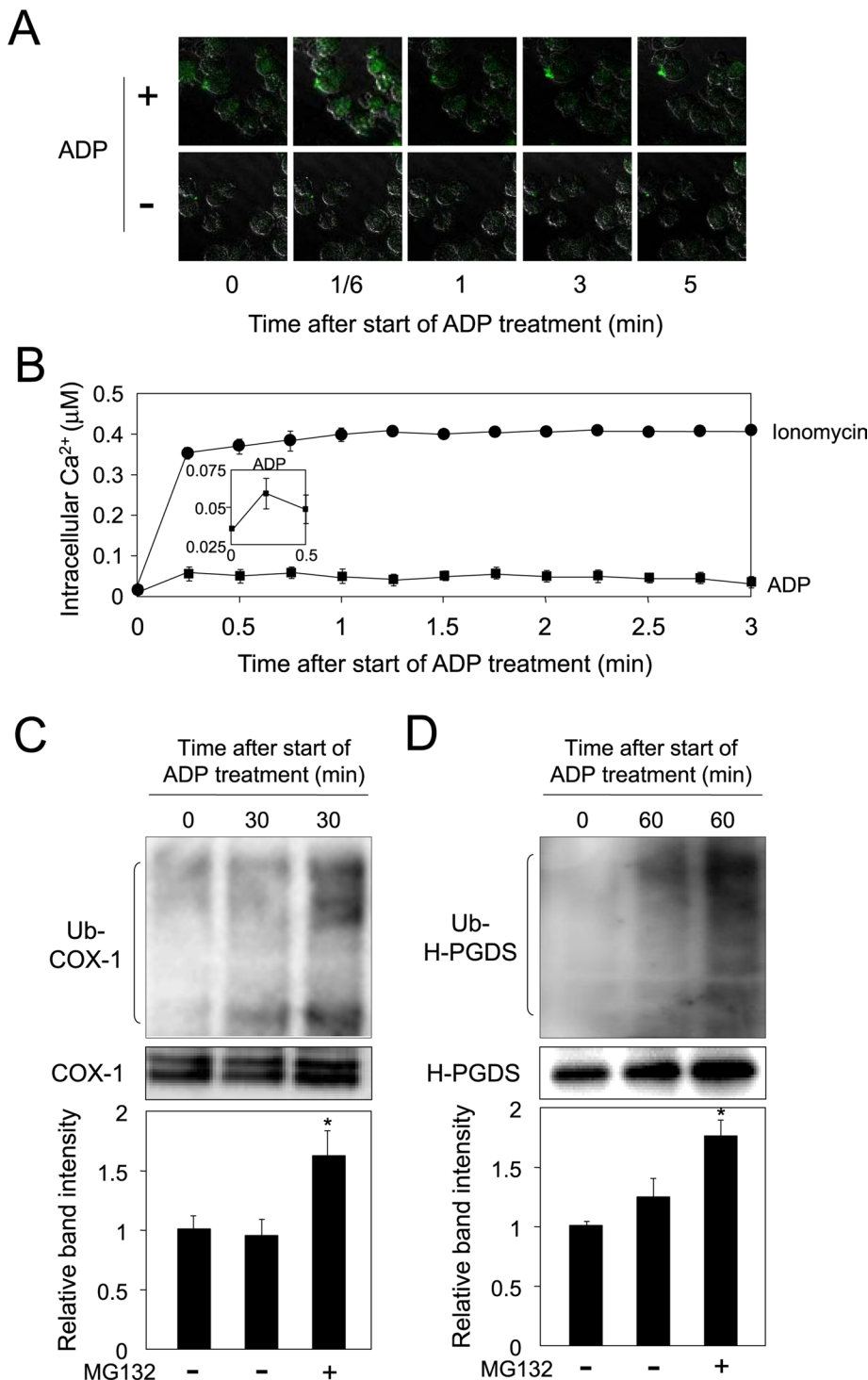


FIGURE 5: ADP enhances intracellular calcium level and concurrently degrades ubiquitinated COX-1 and H-PGDS proteins. (A) Elevation of the intracellular calcium level in ADP-treated MEG-01 cells. ADP (0.5 μM): closed squares; ionomycin (1 μM): closed circles. (B) Measurement of intracellular calcium level in MEG-01 cells. Data are representative of three independent experiments and represent the mean \pm SD. (C) COX-1 protein accumulated in the ADP-treated MEG-01 cells. The cells were incubated in medium containing TPA for 16 h and then incubated with A23187 in the presence or absence of MG132 for 30 min. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as compared with MG132(-) at 30 min. (D) Ubiquitination of H-PGDS protein in the ADP-treated MEG-01 cells. The cells were incubated for 60 min, as described in the legend of (B), together with A23187 and/or MG132. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as compared with MG132(-) at 60 min.

(Figure 7C), while the level of accumulated COX-1 protein was not altered when the cells were incubated in Ca^{2+} -free HBSS, even in the presence of A23187 and MG132 (Figure 7C). Protein level of COX-1 was decreased by treatment with A23187 and CHX in the presence of Ca^{2+} , as compared with that cultured in medium containing the various indicated combinations of $CaCl_2$ and A23187 together with CHX (Figure S4A).

H-PGDS protein level was also increased in cells cultured in HBSS containing A23187 and MG132, as compared with the A23187-treated cells (Figure 7D), whereas the level of ubiquitinated H-PGDS protein was not changed when the cells were incubated in Ca^{2+} -free HBSS with A23187 and MG132 (Figure 7D). In addition, the protein level of H-PGDS was decreased in medium containing A23187, CHX, and $CaCl_2$, as compared with the vehicle-treated cells or either A23187 or $CaCl_2$ -treated cells (Figure S4B).

Additionally, the ubiquitination of COX-1 and H-PGDS proteins was dependent upon extracellular Ca^{2+} concentration (Figure S5, A and B), indicating that both COX-1 and H-PGDS protein degraded through the ubiquitination-mediated proteasome system in response to the calcium ions. These results, taken together, reveal that the entry of calcium ions into the cells was essential for the initiation of ubiquitination-mediated degradation of COX-1 and H-PGDS proteins in MEG-01 cells.

DISCUSSION

The ubiquitin-proteasome system is the principal mechanism responsible for the turnover of proteins in eukaryotic cells and is a fundamental mechanism for cellular controls (Clague and Urbe, 2010), such as signal transduction, transcription, and cell cycle progression (Wickliffe *et al.*, 2009). Ubiquitination of a specific substrate is mainly regulated through the modulation of its degradation signal and through the control of the activity of its cognate E3 ubiquitin ligase (Ardley and Robinson, 2005; Welchman *et al.*, 2005). Targeting of proteins to the proteasome generally requires the attachment of a multi-ubiquitin chain to the substrate (Komander, 2009). It is well known that many substrates of the ubiquitin-proteasome system can be ubiquitinated at multiple lysine residues and that multi-ubiquitination occurs with high-molecular-weight proteins, although a number of studies have shown that ubiquitin modification occurs at one or more specific lysine residues in the target protein (Kuhlbrodt *et al.*, 2005; Miller and Gordon, 2005; Hurley *et al.*, 2006).

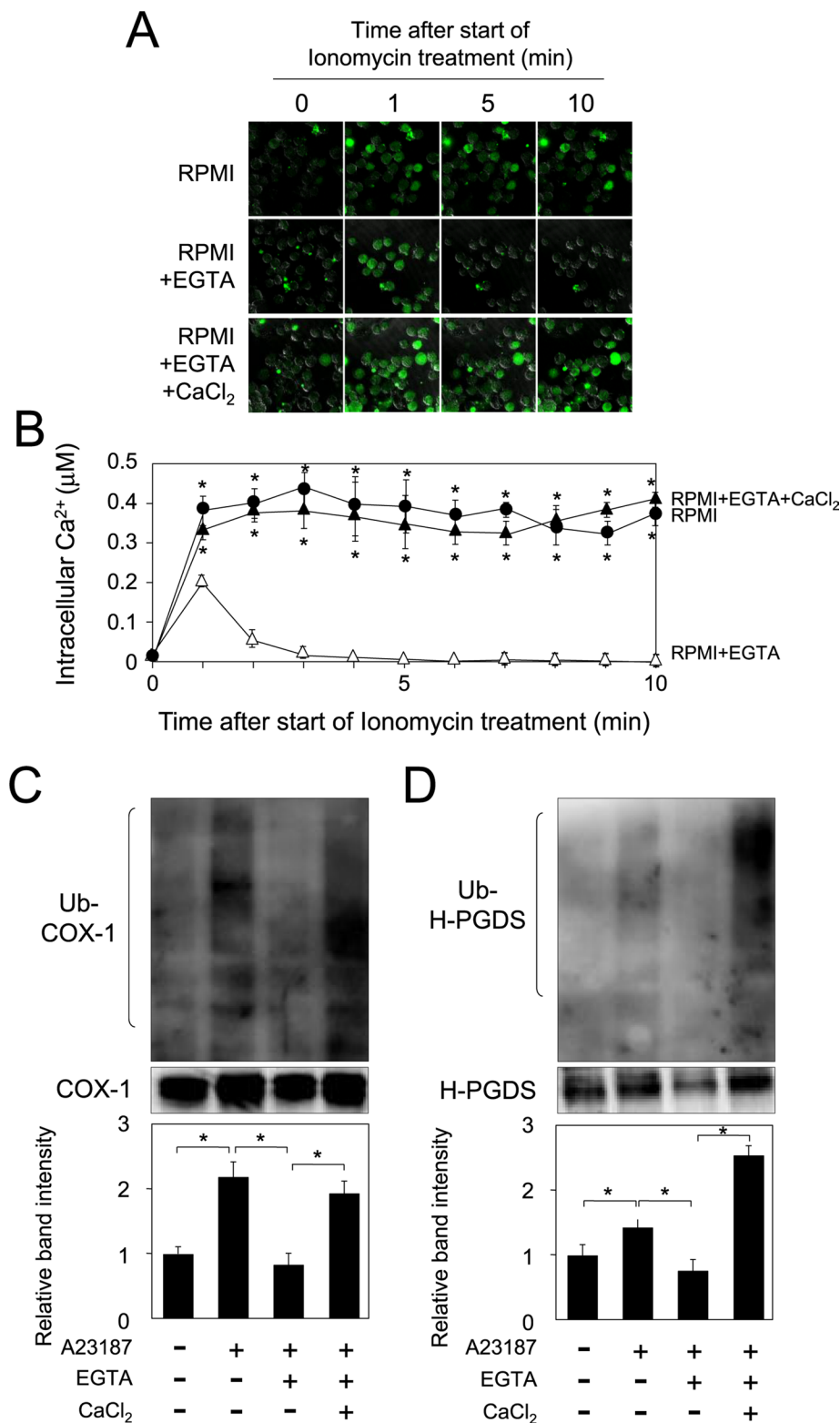


FIGURE 6: Essential roles of calcium entry into cells for ubiquitination of COX-1 and H-PGDS. (A) Suppression of the entry of extracellular calcium ion by EGTA. MEG-01 cells were incubated with TPA for 16 h, washed with PBS, and then pretreated with EGTA (5 mM) for 15 min. They were subsequently incubated for 10 min with Fluo 4-AM (1 μM) and ionomycin (1 μM) in the presence of EGTA (5 mM) with or without CaCl₂ (2 mM). (B) Measurement of intracellular calcium level in MEG-01 cells. Data are representative of three independent experiments and represent the mean ± SD (*p < 0.01), as compared with that for culture in medium containing EGTA. (C) Roles of extracellular calcium ions in the ubiquitination of COX-1 protein in MEG-01 cells. The cells were incubated for 30 min in RPMI1640 containing the various indicated combinations

PGs are known to act as local hormones that are rapidly synthesized in response to various stimuli; this synthesis is followed by quick termination of PG production. The half-lives of PGs are very short, indicating the existence of a critical regulation system for PG production. H-PGDS is localized in the cytosol, and is translocated to the ER membranes in response to elevation of the intracellular calcium level (Ueno *et al.*, 2005). The results of our present study indicated that COX-1 and H-PGDS proteins in human MEG-01 cells were degraded through the ubiquitin-proteasome system in response to a rise in the intracellular calcium level. Treatment of cells with the proteasome inhibitor MG132 markedly increased the steady-state levels of COX-1 and H-PGDS proteins (Figure 2, A and C), demonstrating COX-1 and H-PGDS proteins to be direct substrates of the proteasome. This calcium-mediated ubiquitination of COX-1 and H-PGDS proteins was also observed in human embryonic kidney 293 cells expressing heterologous human COX-1 or H-PGDS protein (Figure S6), suggesting that the intracellular calcium concentration-dependent degradation of COX-1 and H-PGDS proteins is a common event in a variety of cells.

In the arachidonate cascade, COX is known to be a suicidal enzyme, because this enzyme is inactivated during catalysis within several minutes after treatment with endogenous or exogenous arachidonic acid (Smith *et al.*, 2000; Smith and Song, 2002). COX-1 and COX-2 undergo irreversible suicide inactivation during COX catalysis under cell-free conditions (Smith and Song, 2002; Rouzer and Marnett, 2003). In vitro inactivation of COX is accompanied by significant changes in the structure of the enzyme (Mevkh *et al.*, 1993). The inactivated COX-2 is degraded by the ubiquitin-proteasome system (Smith *et al.*, 2000; Mbonye *et al.*, 2006, 2008; Kang *et al.*, 2007; Mbonye and Song, 2009; Wada *et al.*, 2009) acting through the ER-associated degradation system (Hirsch *et al.*, 2009; Mehnert *et al.*,

of EGTA, CaCl₂, and A23187, together with MG132. Data are representative of three independent experiments and represent the mean ± SD (*p < 0.01), as indicated by the brackets. (D) Essential role of the increased intracellular calcium in the ubiquitination of H-PGDS. The cells were cultured for 60 min, as described in the legend of (C). Data are representative of three independent experiments and represent the mean ± SD (*p < 0.01), as indicated by the brackets.

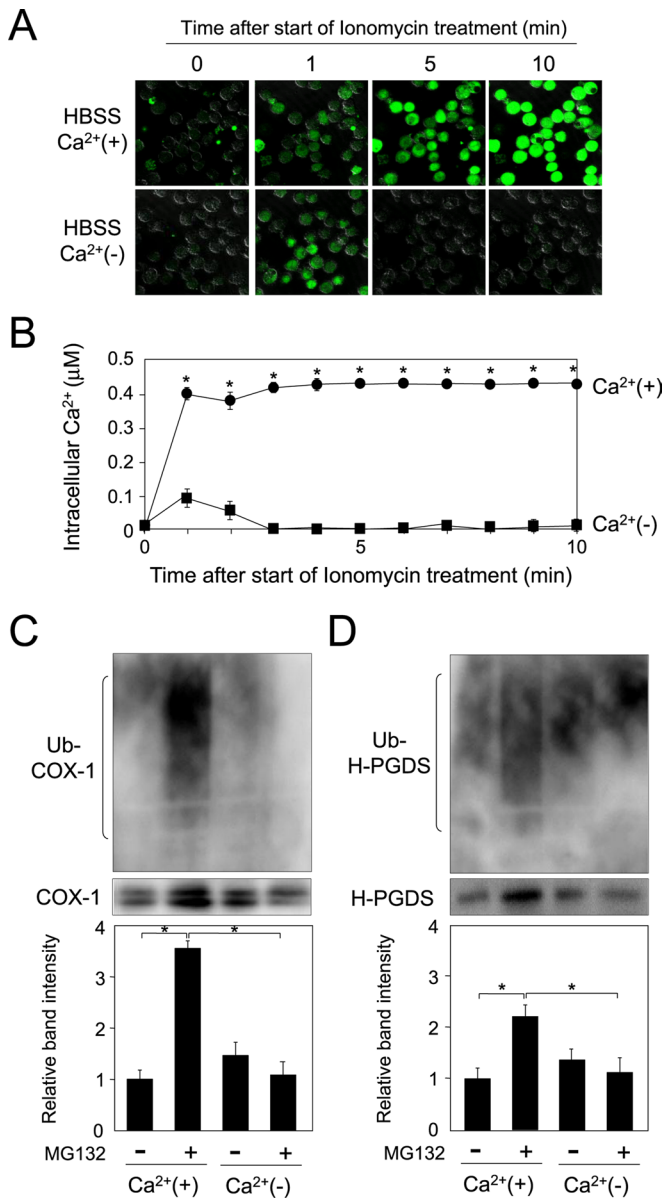


FIGURE 7: Calcium-dependent ubiquitination of COX-1 and H-PGDS. (A) Determination of the intracellular calcium level in the cells cultured in HBSS or Ca²⁺-free HBSS. MEG-01 cells were incubated with TPA for 16 h, and then for 10 min in HBSS or Ca²⁺-free HBSS containing Fluo 4-AM and ionomycin after the preincubation with Fluo 4-AM for 15 min. (B) Measurement of intracellular calcium level in MEG-01 cells. HBSS: closed circles; Ca²⁺-free HBSS: closed squares. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as compared with that for culture in Ca²⁺-free medium. (C) Ubiquitination of COX-1 in MEG-01 cells cultured in HBSS or Ca²⁺-free HBSS with or without MG132. The cells were incubated in RPMI1640 with TPA for 16 h, washed twice with Ca²⁺-free HBSS, and then preincubated in Ca²⁺-free HBSS with 5 mM EGTA for 15 min. The cells were further incubated in HBSS or Ca²⁺-free HBSS with A23187 in the presence or absence of MG132 for 30 min. Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as indicated by the brackets. (D) Ubiquitination of H-PGDS in MEG-01 cells cultured in HBSS or Ca²⁺-free HBSS with or without MG132. The cells were cultured for 60 min, as described in the legend of (C). Data are representative of three independent experiments and represent the mean \pm SD ($*p < 0.01$), as indicated by the brackets.

2010), similar to other ER proteins that have recently been reported (Leichner *et al.*, 2009; Wojcikiewicz *et al.*, 2009; Hoseki *et al.*, 2010). It has been shown that COX inhibitors do not inhibit the proteasome-dependent degradation of COX-2 (Figueiredo-Pereira *et al.*, 2002), similar to our present results, which show that H-PGDS or COX inhibitor did not affect the proteasome-dependent degradation of COX-1 and H-PGDS (Figure 4, B and C). However, when cPLA₂ inhibitor was used, the level of the ubiquitination of COX-1 was lowered, compared with levels seen with other inhibitors (Figure 4B). Moreover, the rate of degradation of COX-1 protein under the influence of cPLA₂ inhibitor was lower than rates seen with other inhibitors (Figure S1A), suggesting the decrease of arachidonic acid levels by cPLA₂ inhibitor might decrease the level of the ubiquitination of COX-1. The initiation of ubiquitination of COX-1 might be required for arachidonic acid-mediated inactivation of COX-1 protein (Mbonye and Song, 2009). Further analysis is needed to verify this hypothesis.

Moreover, when each of COX-1 and H-PGDS proteins was heterologously expressed in 293 cells that expressed negligible levels of COX-1 and H-PGDS, both of these proteins were degraded through the ubiquitin-proteasome system by the increase of the intracellular calcium level (Figure S6), indicating that each of the COX-1 and H-PGDS proteins is independently degraded through the ubiquitin-proteasome system by the increase of the intracellular calcium level.

In summary, the COX-1 and H-PGDS proteins were rapidly degraded through the ubiquitin-proteasome system in response to the increase in the intracellular calcium level. The overall regulation of PG synthesis, including the level of PG synthases, is carried out by the intracellular calcium level. This is the first report that COX-1 and H-PGDS, a terminal PG synthase involved in the arachidonate cascade, are ubiquitinated and concurrently degraded via the ubiquitin-proteasome system. In further study, the physiological meaning of the purpose of rapid degradation of COX-1 and H-PGDS through the ubiquitin-proteasome system should be elucidated. Furthermore, identification of the ubiquitin ligases for both enzymes will be essential for understanding the entire mechanism of the regulation of PGD₂ synthesis in response to an increasing level of intracellular calcium.

MATERIALS AND METHODS

Cell culture

Human megakaryocytic MEG-01 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (vol/vol) fetal calf serum and penicillin (10,000 U/ml) and streptomycin (10,000 U/ml) in a humidified incubator at 37°C and 5% CO₂.

Calcium imaging

MEG-01 cells were seeded on 35-mm glass bottom culture dishes (MatTek, Ashland, MA) and were treated with TPA (20 nM) for 16 h. The cells were then washed with HBSS (Invitrogen, Carlsbad, CA) and preincubated with Fluo 4-AM (1 µM; Dojindo Laboratories, Kumamoto, Japan) for 15 min prior to treatment with ionomycin (1 µM; Calbiochem, Madison, WI) or ADP (0.5 µM; Sigma) in the presence of Fluo 4-AM (1 µM) for 10 min. Fluorescence was monitored with confocal laser scanning fluorescence microscopy (C-LSM510; Carl Zeiss, Jena, Germany) using 488-nm argon laser excitation with a 505- to 530-nm band-pass barrier filter. Fluorescence was recorded every 10–15 s after the initiation of every treatment. Known concentration of calcium solution was used for creation of a standard curve used to measure intracellular calcium levels.

Immunoprecipitation and Western blot analysis

MEG-01 cells were cultured with TPA (20 nM) for 16 h and then washed with phosphate-buffered saline (PBS). They were then incubated for various periods of time in fresh RPMI1640 containing A23187 (5 μ M; Calbiochem) at 37°C in the presence or absence of MG132 (10 μ M; Calbiochem). The cells were then lysed with RIPA buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% (wt/vol) sodium deoxycholate, 0.1% (vol/vol) SDS, 1% (vol/vol) NP-40, 1 mM Na₃VO₄, 1 mM NaF, 50 μ M Na₂MoO₄, and a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). The cell lysates were then centrifuged at 12,000 \times g for 30 min at 4°C to remove cell debris. Supernatants were incubated with anti-human H-PGDS monoclonal antibody (1E6; Cayman Chemicals, Ann Arbor, MI) overnight at 4°C with continuous agitation. Protein G Sepharose (GE Healthcare, Buckinghamshire, UK) was then added to the samples, and incubation was continued for 60 min at 4°C. Immunoprecipitates on the Sepharose resin were washed five times with ice-cold 50 mM Tris-Cl (pH 7.5) buffer containing 150 mM NaCl, 2 mM EDTA, and 0.1% (vol/vol) Triton X-100. Subsequently, the resin was suspended in Laemmli SDS-loading buffer containing 50 mM Tris-Cl (pH 6.8), 10% (vol/vol) glycerol, 2% (wt/vol) SDS, 0.005% (wt/vol) bromophenol blue, and 1% (vol/vol) 2-mercaptoethanol.

Protein samples were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA). Blots were incubated with the specific primary antibodies, that is, anti-ubiquitin (Thermo Fisher Scientific, Rockford, IL), anti-COX-1 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-human H-PGDS polyclonal antibody, washed, and then incubated with the appropriate second antibodies conjugated to horseradish peroxidase (GE Healthcare). Immunoreactive signals were detected by the use of ECL Plus Western blotting detection reagents (GE Healthcare) and a LAS-3000 imaging analyzer (Fujifilm, Tokyo, Japan). The band intensities were measured with Multi Gauge software (Fujifilm). Protein concentrations were measured with Pierce BCA protein assay reagent (Thermo Fisher Scientific).

Calcium-entry study

MEG-01 cells were incubated with TPA (20 nM) for 16 h, washed with PBS, and then pretreated with EGTA (5 mM) for 15 min. The cells were then cultured with 1 μ M Fluo 4-AM in the presence of ionomycin (1 μ M), ADP (0.5 μ M; Sigma), EGTA (5 mM), and/or 2 mM CaCl₂ for the desired times. Intracellular calcium was observed by using confocal laser scanning microscopy (C-LSM510) and measured using calcium solution as the standard.

Statistical analysis

Comparison of two groups was analyzed by Student's *t* test. For comparison of more than two groups with comparable variances, one-way analysis of variance and a Tukey's post hoc test were carried out. *p* < 0.05 was considered significant.

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