DOI 10.5012/bkcs.2010.31.02.384

Development of a Coupled Enzyme Assay Method for Microsomal Prostaglandin E Synthase Activity

Kyung-A Choi, Sung-Jun Park, and Yeon Gyu Yu*

*Department of Chemistry, Kookmin University, Seoul 136-702 Korea. *E-mail: ygyu@kookmin.ac.kr*
Received December 14, 2009, Accepted December 30, 2009

Human microsomal prostaglandin E synthase-1 (mPGES-1) catalyzes the conversion of prostaglandin H_2 (PGH_2) into prostaglandin E_2 (PGE_2). To establish a stable and efficient method to assess the activity of mPGES-1, a coupled enzyme assay system using mPGES-1, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and phosphomolybdic acid (PMA) was developed. In this assay system, PGH_2 was converted to PGE_2 by mPGES-1, and then PGE_2 was further transformed to the 15-keto-PGE_2 by 15-PGDH accompanying the production of NADH, which was easily detected by fluorescence spectrometry in a multi-well plate format. During the reaction, spontaneous oxidation of PGH_2 was prevented by PMA. Using this novel assay, the K_m value of mPGES-1 for PGH_2 and the IC_{50} value of the previously characterized inhibitor, MK-886, were determined to be 0.150 mM and 2.8 μM, respectively, which were consistent with the previously reported values. In addition, low backgrounds were observed in the multi-wall plate screening of chemical compounds.

Key Words: mPGES-1, 15-PGDH, Fluorescence, NADH, Coupled enzyme assay

Introduction

Prostaglandins derived from arachidonic acid have been implicated in various physiological processes.[1] Particularly, prostaglandin E_2 (PGE_2) is involved in inflammation,[2-4] reproduction,[5] or tumorigenesis.[6,7] PGE_2 is produced in macrophages and other tissues by inflammatory stimuli[8-11] and mediates fever and pain.[12,13] Hence, the biosynthetic pathway of PGE_2 has been a primary target in the development of anti-inflammatory agents. In human, a cytosolic prostaglandin E_2 synthase (cPGES),[14] and two microsomal membrane-associated prostaglandin E_2 synthases (mPGES-1 and mPGES-2)[2,3,8] have been identified. Among them, mPGES-1 was responsible for the acute increase of PGE_2 induced by proinflammatory stimuli in various tissues.[3,4,7] The knockout of mPGES-1 gene in mouse showed impaired inflammatory and pain responses,[15] indicating that a potent inhibitor of mPGES-1 may be a promising drug for the treatment of chronic inflammatory diseases such as rheumatism.[16]

Human mPGES-1 cleaves the alkylperoxide bond of prostaglandin H_2 (PGH_2), and forms a hydroxide and ketone group. Since the substrate (PGH_2) and product (PGE_2) of mPGES-1 have no distinguishable chromophores, the activity of mPGES-1 cannot be assayed by a spectroscopic method that measures the amount of PGE_2. Instead, the amount of PGE_2 could be measured by anti-PGE_2 antibody.[17] Alternatively, the PGE_2 has been separated by HPLC after the reaction, and its relative amount was directly measured from the elution profile of the reaction products.[8,18,19] A spectrometric method to assay mPGES-1 was previously developed.[20] This method consisted of mPGES-1, COX-2, 15-hydroxyprostaglandin dehydrogenase (PGDH), arachidonic acid and NAD^+. In this assay system, PGH_2 was synthesized by COX-2, and then further converted to PGE_2 and 15-keto-PGE_2 by mPGES-1 and 15-PGDH, respectively. The amount of NADH produced by 15-PGDH during NAD^+-dependent oxidation of the 15-hydroxy group of PGE_2 was measured by absorption at 340 nm or its fluorescence intensity at an excitation and emission wavelength of 340 nm and 468 nm, respectively.

All of the reported assay methods for assessing mPGES-1 activity can only screen simultaneously a small number of chemicals due to the unstable nature of PGH_2. The peroxide bond of PGH_2 is spontaneously cleaved to form PGE_2, PFD_2 or $PGF_{2\alpha}$ in solution with a half life of less than 10 min at 20 °C.[21-23] These products from the spontaneous decomposition of PGH_2 generated high background signal in these assay systems since they could be further oxidized by 15-PGDH accompanying the reduction of NAD^+. Thus, this property of PGH_2 has limited the reaction time to less than a minute in all the reported assay methods.[22] Hence, the stabilization of PGH_2 or the prevention of spontaneous cleavage of the peroxide bond in PGH_2 would enhance the application of the mPGES-1 assay methods for the screening of inhibitors from a large number of chemical compounds.

In this study, we developed a novel coupled enzyme assay method that consists of purified recombinant mPGES-1 and 15-PGDH. The activity of mPGES-1 was efficiently assayed by measuring the amount of NADH converted from NAD^+ by 15-PGDH using a fluorescence plate reader. In particular, PGH_2 was stabilized by 0.1 mM phosphomolybdic acid (PMA) and the spontaneous decomposition of PGH_2 to $PGF_{2\alpha}$ was effectively prevented during the reaction period. This method provides a robust assay system that can be applied for screening large chemical libraries.

Materials and Methods

Materials. Prostaglandin H_2 (PGH_2), phosphomolybdic acid (PMA) and MK-886 was obtained from Sigma (USA). A 96-well black non-binding plate, which was used in the mPGES-1 assay, was obtained from Cayman Chemical Company (USA). Glutathione (GSH), nicotinamide adenine dinucleotide (NAD^+), and dithiothreitol (DTT) were purchased from USB Corp.

(USA), Sigma (USA) and Bio Basic Inc. (Canada), respectively.

Preparation of mPGES-1 and 15-PGDH. The coding region of human mPGES-1 containing a His_6-tag at the N-terminus was expressed in *E. coli* Rosetta(DE3) and purified as previously described.[24] Briefly, the expression of recombinant mPGES-1 was induced with 1 mM IPTG for 12 hr at 18 °C, and the recovered cells were lysed by ultrasonication. After the remaining cell debris in the lysate was removed by centrifugation at 5,000 × g for 10 min at 4 °C, and the membrane fraction was precipitated by ultracentrifugation at 100,000 × g for 1 hr at 4 °C. The membrane fraction was solubilized with 20 mL of buffer A (15 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.5 mM EDTA, 1 mM PMSF, 1 mM GSH, and 4% Triton X-100). The solubilized mPGES-1 was further purified by a Ni-NTA and mono-Q column, successively.[24] For the preparation of 15-PGDH, an expression vector of 15-PGDH was prepared by inserting the coding sequence of human15-PGDH (gi: 3248) in p15PGDH (kindly provided from Prof. Cho Hoon at Chosun Univ., Korea) into the *Bam*HI and *Eco*RI sites of pGEX-2T vector. The GST-tag labeled 15-PGDH was expressed in *E. coli* Rosetta(DE3), and purified as previously described.[25] Briefly, the expression of recombinant 15-PGDH was induced with 1 mM IPTG for 12 hr at 25 °C, and the recovered cells were lysed using a microfluidizer. After the remaining cell debris in the lysate was removed by centrifugation at 10,000 × g for 20 min at 4 °C, the lysate was loaded onto GSH-agarose column and the bound 15-PGDH was eluted with 10 mM reduced glutathione in 50 mM Tris-HCl, 1 mM EDTA, 0.1 mM DTT and pH 8.0.

Enzyme assay. To measure the activity of mPGES-1, 0.72 μg of mPGES-1 and 1.1 μg of 15-PGDH were mixed in 200 μL of the reaction buffer (50 mM Tris-HCl, 0.1 mM of PMA, 2 mM reduced form of glutathione, 2 mM NAD^+, 0.1 mM DTT, pH 7.5). The reaction was initiated by adding cold PGH_2 to a final concentration of 6 μM. The amount of NADH in the reaction product was measured by fluorescence reader (DYNEX, USA) using an emission and excitation wavelength of 468 nm and 340 nm, respectively using a plate reader (DYNEX, USA). The inhibitory activity of chemical compounds was measured by incubating 20 μM of the compounds 1 μL in dimethylsolfoxide (DMSO) with mPGES-1 for 30 min prior to the addition of PGH_2. The amount of PGE_2 was measured using anti-PGE_2 antibody and PGE_2-conjugated alkaline phosphatase[17] as described in the instruction manual (Assay Designs, Inc., USA). The protein concentration was measured by the Lowry method using bovine serum albumin as a standard.[26]

Results and Discussion

Preparation mPGES-1 and 15-PGDH. We used pET28a and pGEX-2T expression vectors to express mPGES-1 and 15-PGDH, respectively. The coding sequence of human mPGES-1 containing a N-terminus His_6-tag was cloned in pET28a, and the recombinant protein expressed in *E. coli* Rossetta (DE3) was purified from the crude extract by a NTA-column and mono-Q column, as described previously[24] (Fig. 1A). The coding region of human 15-PGDH was cloned into the pGEX-2T expression vector, which expressed a fusion protein of 15-PGDH linked at the C-terminus to glutathione S-transferase. The recombinant protein was purified from the crude extract by a single step of GSH-agarose column (Fig. 1B). Both mPGES-1 and 15-PDGH were purified to more than 90% purity, and their molecular weights were estimated to be 17 and 52 kDa, respectively.

Optimization of coupled enzyme assay by PMA-dependent stabilization of PGH_2. To establish the coupled enzyme assay, the fluorescence intensity of NADH was measured at 468 nm under various conditions. The specific activity of mPGES-1 (120 μmol/min/mg)[24,27] was 6-fold lower than that of 15-PGDH (830 μmol/min/mg).[28] In the reaction mixture, about 10-fold higher specific activity of 15-PGDH (1.1 μg) than that of mPGES-1 (0.72 μg) was used to efficiently measure the activity of mPGES-1. As shown in Fig. 2A, NADH was produced right after the addition of PGH_2 to the reaction mixture and reached to constant level after 20 min or longer incubation (Fig. 2A, ▲), indicating most of PGH_2 was consumed within 20 min. In comparison, a significant amount of NADH was also produced from the reaction mixture lacking mPGES-1 (Fig. 2A, ■). This result indicates that more than 70% of PGH_2 was decomposed in the absence of mPGES-1 and used as substrates of PGDH. To prevent the spontaneous decomposition of PGH_2, the production of NADH from the reaction mixture containing 15-PGDH, NAD^+ and PGH_2 was measured in the presence of 0.1 mM PMA, which had been identified as stabilizers of peroxidicarbonates.[29] As shown in Fig. 2B, the amount of NADH generated from the reaction mixture containing only 15-PGDH was undetectable (Fig. 2B, ■). Whereas, significant amount of NADH was produced after the addition of PGH_2 in the reaction mixture containing both mPGES-1 and 15-PGDH (Fig. 2B, ▲), and saturated after 10 min. The fluorescence intensity from the reaction mixture

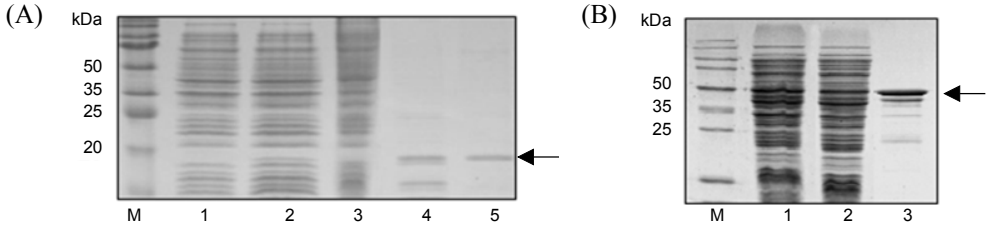

Figure 1. SDS-PAGE analysis of the purified recombinant mPGES-1 (A) and 15-PGDH (B). (A) The recombinant mPGES-1 samples were analyzed by 15% SDS-PAGE. Lane M, molecular weight markers; lane 1, crude extract before IPTG-induction, lane 2, after IPTG-induction; lane 3, membrane fraction; lane 4, after NTA-column; lane 5, and after Q-sepharose column. (B) The recombinant 15-PGDH samples were analyzed by 12% SDS-PAGE. Lane M, molecular weight markers; lane 1, crude extract after IPTG-induction; lane 2. unbound proteins from glutathione column; lane 3 after glutathione column. The purified mPGES-1 and 15-PGDH were indicated as arrows.

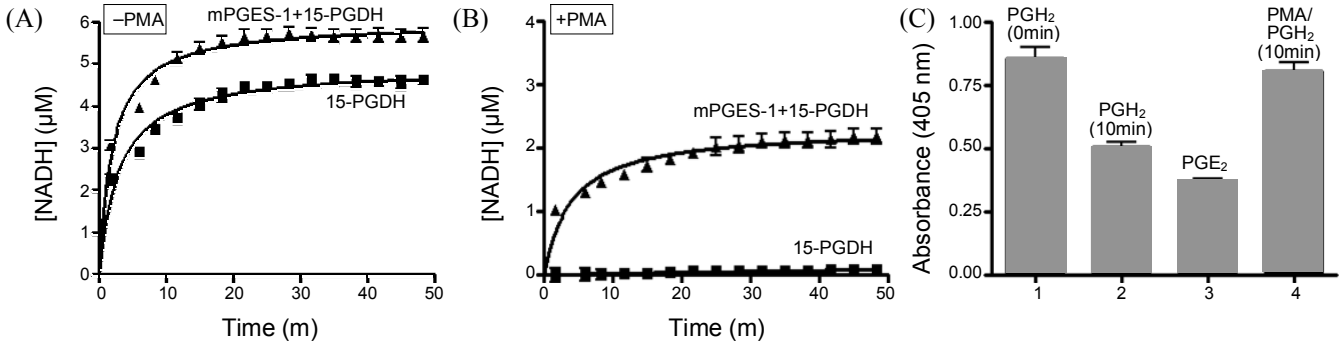

Figure 2. PMA prevents the spontaneous decomposition of PGH_2. (A) The fluorescence intensity of NADH from the reaction mixture containing NAD^+, mPGES-1 and 15-PGDH was measured over 30 min after the addition of PGH_2 at room temperature (▲). Likewise, the fluorescence intensity from the same reaction mixture except lacking mPGES-1 was also measured (■). (B) The fluorescence intensity of NADH from the reaction mixture containing 100 μM PMA, NAD^+, 15-PGDH, with (▲) or without (■) mPGES-1 was measured over 30 min after the addition of PGH_2. (C) One μM of fresh PGH_2 (bar 1), PGH_2 after incubation for 10 min at room temperature (bar 2), PGE_2 (bar 3), or PGH_2 after incubation for 10 min at room temperature with 0.1 mM PMA (bar 4) was incubated in 96-well plate coated with anti-PGE_2 antibody for 10 min at 4 °C, and the amount of PGE_2- labeled alkaline phosphatase bound to the plate was measured.

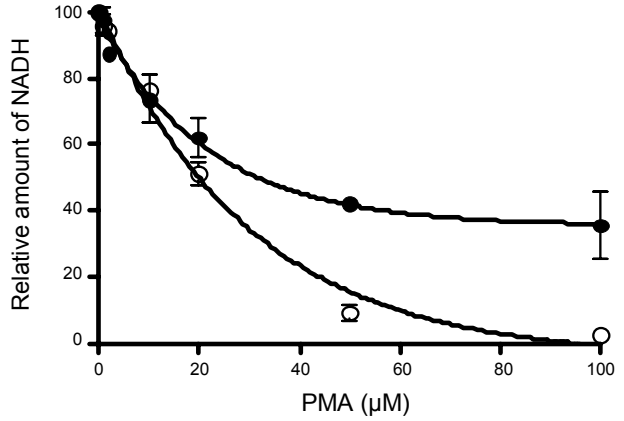

Figure 3. Concentration dependent stabilization of PGH_2 by PMA. The effect of PMA on the stabilization of PGH_2 was examined by measuring the amount of NADH produced from the reaction mixture containing both mPGES-1 and 15-PGDH (●) or the mixture containing only 15-PGDH (○) and different concentrations of PMA after incubation at room temperature for 20 min.

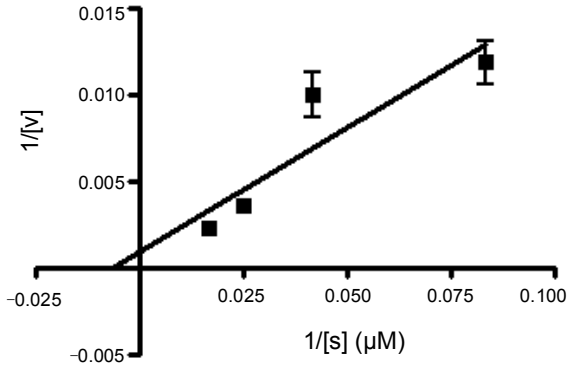

Figure 4. Lineweaver-Burk plot of the coupled-enzyme assay. The initial velocity of the reaction containing 0.2 μM mPGES-1, 1.0 μM 15-PDGH, and 100 μM PMA was measured within 40 sec at different concentrations of PGH_2, and the 1/[v] values were plotted against 1/[s].

containing PMA was about 30 - 40% compared to the intensity from the reaction mixture lacking PMA, indicating that PMA suppressed the spontaneous decomposition of PGH_2. The stabilization of PGH_2 by PMA was further confirmed by immunoassay using anti-PGE_2 antibody.[17] Alkaline phosphatase coupled PGE_2 (PGE_2-AP) effectively bound to the immobilized anti-PGE_2 antibody (Fig. 2C, bar 1) in the presence of fresh PGH_2. On the contrary, the binding of PGE_2-AP to the immobilized anti-PGE_2 antibody was effectively prevented in the presence of PGE_2 (Fig. 2C, bar 3). The amount of bound PGE_2-AP was significantly reduced in the presence of PGH_2, incubated at room temperature for 10 min (Fig. 2C, bar 2), indicating that substantial amount of PGH_2 was decomposed within 10 min incubation and the decomposed product of PGH_2 interfered the binding of PGE_2-AP to anti-PGE_2 antibody. However, the binding of PGE_2-AP to anti-PGE_2 antibody was not interfered by PGH_2 when it was incubated with 100 μM PMA at room temperature for 10 min (Fig. 2C, bar 4). These results demonstrate that PMA effectively prevented the spontaneous cleavage of PGH_2, and the NADH produced from the reaction mixture containing PMA resulted only from the PGE_2-dependent reduction of NAD^+ rather than the oxidation of PGF_2.

The effect of PMA on the stabilization of PGH_2 was further examined by measuring the production of NADH from the reaction mixture containing various concentrations of PMA. When the reaction mixture of PGH_2, 15-PGDH and NAD^+ were incubated with 10 - 100 μM of PMA for 20 min, the amount of NADH produced from the spontaneous decomposition of PGH_2 was dramatically decreased and reached a basal level at a PMA concentration of 50 μM or higher (Fig. 3, ○). Whereas, the amount of NADH produced from the reaction mixture of PGH_2, mPGES-1, 15-PGDH and NAD^+ decreased to 40% at a PMA concentration of 50 μM or higher (Fig. 3, ●). These results suggest that 50 - 100 μM of PMA was enough to stabilize and suppress spontaneous decomposition of PGH_2. Although the protection of PGH_2 by PMA is not clearly understood, PMA may interact with the alkylperoxide group of PGH_2 and prevent further oxidation by oxygen species. Considering the amount of NADH

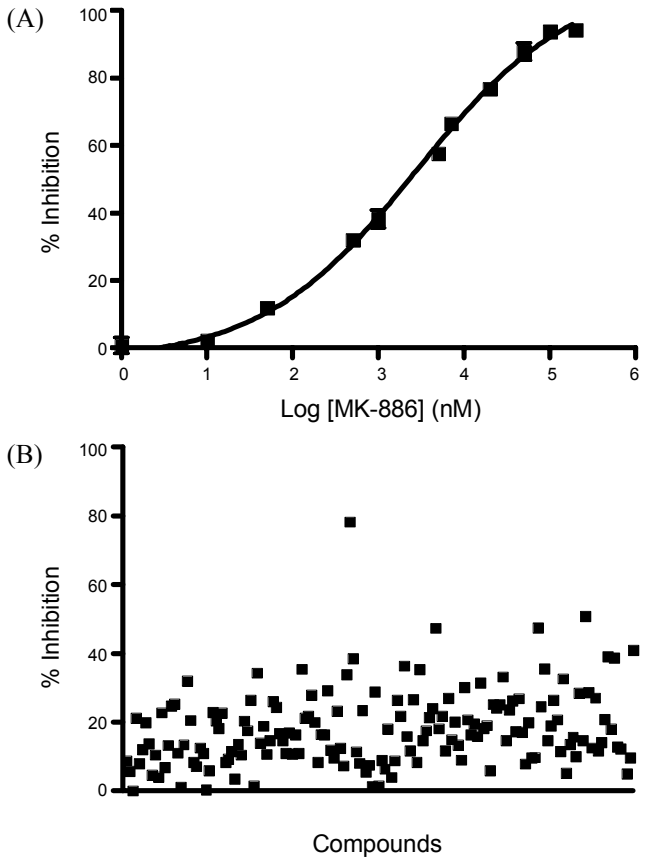

Figure 5. Validation of the coupled enzyme assay method for high-throughput screening. (A) Concentration dependent-inhibitory activity of MK-886 was measured after incubation of different concentrations of MK-886 with the two enzymes for 20 min, and then the amount of NADH produced was measured after 20 min incubation at room temperature. (B) The inhibitory activities of 160-selected compounds measured by the coupled enzyme assay method as described above.

produced from the reaction mixture containing only 15-PGDH as negative control (Fig. 2B and C, ■), the amount of NADH produced from the reaction mixture containing PMA (Fig. 2B, ▲) was similar to the NADH generated from the reaction in the absence of PMA (Fig. 2A, ▲).

Validation of the couple enzyme assay. To determine if this novel coupled-enzyme assay method yielded kinetic parameters that were consistent with previously reported values of mPGES-1, the reaction rate was measured for 40 s at different concentrations of PGH_2 in the presence of 0.1 mM of PMA, 0.2 μM of mPGES-1 and 1.0 μM of 15-PGDH. From the rate of NADH production at different concentration of PGH_2, a Lineweaver-Burk plot was obtained (Fig. 4), and the K_m value of mPGES-1 for PGH_2 was determined to be 0.15 mM with R value of 0.858, which was in good agreement with the reported values of 0.16 mM.[6] This result indicates that the conditions used in this coupled-enzyme assay were suitable to accurately assess mPGES-1 activity.

To test whether the enzyme-coupled assay method was applicable for the screening of inhibitors of mPGES-1, the inhibitory activity of previously known inhibitors of mPGES-1 and randomly selected chemical compounds were measured using this assay method. MK-886, an inhibitory compound of mPGES-1 with an IC_{50} value of 3.2 μM,[3] showed inhibitory activity in the coupled enzyme assay with an IC_{50} value of 2.8 μM (Fig. 5A). To further test the utility of the coupled-enzyme assay for screening chemical libraries, the inhibitory activities of 160-selected compounds in 96-well plate were assayed. Fig. 5B showed the inhibitory activities of the screened compounds at a concentration of 20 μM. The percent inhibition was calculated as follows: $[1-(F-F^0)/(F^{100}-F^0)] \times 100$ (%), where F^{100} was the fluorescence intensity from the positive control not containing the inhibitory compound, F^0 was the fluorescence intensity from the negative control, and F was the fluorescence intensity from the reaction mixture incubated with a particular compound. To assess the quality of the assay, a Z' factor that reflects both the assay signal dynamic range and data variation[30] was determined. The Z' factor for the assay was determined to be 0.86 ± 0.03, indicating that this was reliable high-throughput screening (HTS) assays.

In summary, we have developed an efficient and stable coupled-enzyme assay that consists of mPGES-1 and 15-PGDH. In particular, the spontaneous decomposition of PGH_2 was effectively prevented by PMA, and this stabilizing effect increased the reaction time of the coupled-enzyme assay, which is critical for screening large numbers of chemicals. In addition, the high Z' value clearly indicated that this coupled-enzyme assay system was appropriate for high-throughput screening

Acknowledgments. This work was supported by grants from the 21C Frontier Research Program, Functional Proteomics Research, Ministry of Education, Science and Technology, Korea. We also thank for the support of Kookmin Research Fund.

References

1. Funk, C. D. *Science* **2001**, *294*, 1871-1875.
2. Murakami, M.; Naraba, H.; Tanioka, T.; Semmyo, N.; Natakami, Y.; Kojima, F.; Ikeda, T.; Feuki, M.; Ueno, A.; Oh, S.; Kudo, I. *J. Biol. Chem.* **2000**, *275*, 32783-32792.
3. Mancini, J. A.; Blood, K.; Gordon, J. R.; Claveau, D.; Chan, C. C.; Riendeau, D. *J. Biol. Chem.* **2001**, *276*, 4469-4475.
4. Stichtenoth, D. O.; Thorén, S.; Bian, H.; Peters-Golden, M.; Jakobsson, P. J.; Crofford, L. J. *J. Immunol.* **2001**, *167*, 469-574.
5. Filion, F.; Bouchard, N.; Goff, A. K.; Lussier, J. G.; Sirois, J. *J. Biol. Chem.* **2001**, *276*, 34323-34330.
6. Kamei, D.; Murakami, M.; Nakatani, Y.; Ishikawa, Y.; Ishii, T.; Kudo, I. *J. Biol. Chem.* **2003**, *278*, 19396-19405.
7. Yoshimatsu, K.; Altorki, N. K.; Golijanin, D.; Zhang, F.; Jakobsson, P. J.; Dannenberg, A. J.; Subbaramaiah, K. *Clin. Cancer Res.* **2001**, *7*, 2608-2610.
8. Jakobsson, P. J.; Thorén, S.; Morgenstern, R.; Samuelsson, B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target, *Proc. Natl. Acsd. Aci. U.S.A.* **1999**, *96*, 7220-7225.
9. Catley, M. C.; Chivers, J. E.; Cambridge, L. M.; Holden, N.; Slater, D. M.; Staples, K. J.; Bergmann, M. W.; Loser, P.; Barnes, P. J.; Newton, R. *FEBS Lett.* **2003**, *547*, 75-79.
10. Naraba, H.; Murakami, M.; Matsumoto, H.; Shimbara, S.; Ueno, A.; Kudo, I.; Ohishi, S. *J. Immunol.* **1998**, *160*, 2974-2982.
11. Fournier, T.; Fadok, V.; Henson, P. M. *J. Biol. Chem.* **1997**, *272*, 31065-31072.

12. Ushikubi, F.; Segi, E.; Sugimoto, Y.; Murata, T.; Matsuoka, T.; Kobayashi, T.; Hizaki, H.; Tuboi, K.; Katsuyama, M.; Ichikawa, A.; Tanaka, T.; Yoshida, N.; Narumiya, S. *Nature* **1998**, *395*, 281-284.
13. Samad, T. A.; Sapirstein, A.; Woolf, C. J. *Trends Mol. Med.* **2002**, *8*, 390-396.
14. Tanioka, T.; Nakatani, Y.; Semmyo, N.; Murakami, M.; Kudo, I. *J. Biol. Chem.* **2000**, *75*, 32775-32782.
15. Trebino, C. E.; Stock, J. L.; Gibbons, C. P.; Naiman, B. M.; Wachtmann, T. S.; Umland, J. P.; Pandher, K.; Lapointe, J. M.; Saha, S.; Roach, M. L.; Carter, D.; Thomas, N. A.; Durtschi, B. A.; McNeish, J. D.; Hambor, J. E.; Jakobsson, P. J.; Carty, T. J.; Perez, J. R.; Audoly, L. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 9044-9049.
16. Kudo, I.; Murakami, M. *J. Biochem. Mol. Biol.* **2005**, *38*, 633-638.
17. Massé, F.; Guiral, S.; Fortin, L. J.; Cauchon, E.; Ethier, D.; Guay, J. *J. Biomol. Screen.* **2005**, *10*, 599-605.
18. Ouellet, M.; Falgueyret, J. P.; Ear, P. H.; Pen, A.; Mancini, J. A.; Riendeau, D. *Protein Expr. Purif.* **2002**, *26*, 489-495.
19. Thorén, S.; Weinander, R.; Saha, S.; Jegerschöld, C.; Pettersson, P. L.; Samuelsson, B. *J. Biol. Chem.* **2003**, *278*, 22199-22209.
20. Percival, M. D. *Anal. Biochem.* **2003**, *313*, 307-310.
21. Smith, W. L. *Am. J. Physiol.* **1992**, *263*, F181-F191.
22. Nugteren, D. H.; Christ-Hazelhof, E. *Adv. Prostaglandin Thromboxane Res.* **1980**, *6*, 129-137.
23. Salomon, R. G.; Miller, D. B.; Zagorski, M. G.; Coughlin, D. J. *J. Am. Chem. Soc.* **1983**, *106*, 6049-6060.
24. Kim, W. I.; Choi, K. A.; Do, H. S. *BMB reports* **2008**, *41*, 808-813.
25. Cho, H.; Oliveira, M. A.; Tai, H.-H. *Arch. Biochem. Biophy.* **2003**, *419*, 139-146.
26. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. E.; Fujimoto, E. K.; Golki, N. M.; Olson, B. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76-85.
27. Thoren, S.; Weinander, R.; Saha, S.; Jegerschold, C; Pettersson, P. L.; Samuelsson, B.; Hebert, H.; Hamberg, M.; Morgenstern, R.; Jakobsson, P.-J. *J. Biol. Chem.* **2003**, *278*, 22199-22209.
28. Cho, H.; Tai, H.-H. *Prostglan. Leukot. Essen. Fatty Acids* **2002**, *66*, 505-509.
29. Frenkel, P.; Abma, C. *United States Patent* **1998**, 5717304.
30. Zhang, J. H.; Chung, T. D.; Oldenburg, K. R. *J. Biomol. Screen.* **1999**, *4*, 67-73.