CHAPTER 4

RESULTS

Since the title of my PhD thesis is “The Expression and Regulation of MMP-2 and MMP-9 in Human Gingival Fibroblasts and Human Gingival Epithelial Cells”, all of the results in this chapter are mainly divided into three parts. The first part will deal with the expression and activity of MMP-2 and MMP-9 in HGFs and HGECs, respectively. The next two parts are involved with the signaling molecules mediating an up-regulation of MMP-9 expression and activity either via induced PLD activity or via $cPLA_2$in HGECs.This is because it has been shown in this study that MMP-2 mRNA is constitutively expressed in both HGFs and HGECs, and HGFs do not evidently express MMP-9; hence, the MMP-9 activity in HGFs is considered negligible. In addition to constitutive MMP-2 mRNA expression, the MMP-2 protein expression and its activity is neither induced by IL-1β, a pro-inflammatory molecule, nor by *F. nucleatum* cell wall extract, a stimulant that possesses the ability to induce the expression of antimicrobial peptide human β-defensin-2 (Krisanaprakornkit*et al*., 2000) and MMP-9 in HGECs (see below) as well as the expression of IL-8 in HGFs (Krisanaprakornkit*et al*., 2000).

4.1 Expression and activity of MMP -9 and MMP-2 in human gingival epithelial cells

MMP-9 has been found in human gingival keratinocytes *in vitro* (Thomas *et al*., 2001) and an earlier study has shown that this enzyme is expressed during re-epithelialization of palatal wound (Salo*et al*., 1994). In this study, HGECs were grown in culture plates, once the cells are confluent, HGECs were stimulated overnight with various doses of *F. nucleatum* cell wall extract or PMA, a potent MMP-9 activator (Cho *et al*., 2007). Cell-free supernatant were collected for gelatin zymography and Western blot. Total RNA was collected and RT-PCR was performed as described in Material & Methods. PCR products were run on 1.2%agarose gel.

The results showed that MMP-9 mRNA expression was induced byboth *F. nucleatum* cell wall extract and PMA (Figure 4.1), whereasMMP-2 mRNA was constitutively expressed (Figure 4.1). These results were consistent with the findings from a previous study, whichsuggeststhat in the physiological condition,MMP-2 is the major gelatinolytic MMP produced by oral mucosa and cultured skin keratinocytes,while MMP-9 is produced at a low basal level(Mäkelä*et al*., 1999).GAPDH expression was equal among different samples, confirming the quality of all RNA preparations used in RT-PCR(Figure 4.1). No PCR product was detected in the -RT sample, where the reverse transcriptase enzyme was omitted(Figure 4.1).

The real-time PCR showed a significant induction of MMP-9 mRNA expression by 3 and 10 μg/ml ($P<0.05$) and 30 μg/ml ($P<0.01$) of *F.nucleatum*cell wall extract (Figure 4.2). One ng/ml of PMA induced MMP-9 mRNA almost 100 fold, and the induction reached the maximal level at 10 ng/ml of PMA (Figure 4.2). After HGEC stimulation, cell-free

supernatants were collected for protein expression by western blot. MMP-9 protein expression was up-regulated in*F.nucleatum*- and PMA-stimulated samples (Figure 4.3). Furthermore, two major bands of gelatinolytic activity, including the one at 92 kDa for MMP-9 and the other one at 72 kDafor MMP-2, were detected by gelatin zymography.Up-regulation of MMP-9 activity was detected in cell-free culture supernatants collected from HGECs stimulated with both stimulants (Figure 4.4).

Ten μg/ml of *F.nucleatum* cell wall extract and 10 ng/ml of PMA were chosen for the kinetics study. HGECs were stimulated with either *F.nucleatum* cell wall extract or PMA for 0-24 hours. The time-course study demonstrated an early induction of MMP-9 mRNA after stimulation with both stimulants for three hours (Figure 4.5), while MMP-2 mRNA expression was constitutively expressed. Cell-free culture supernatants from Figure 4.5 were collected for MMP-9 protein expression and activity. It was shown that MMP-9 secretion and activity were noticeably detected after 12 hours of stimulation(Figures 4.6 and 4.7, respectively). All of the results shown in this section are representative of three independent experiments with similar findings.

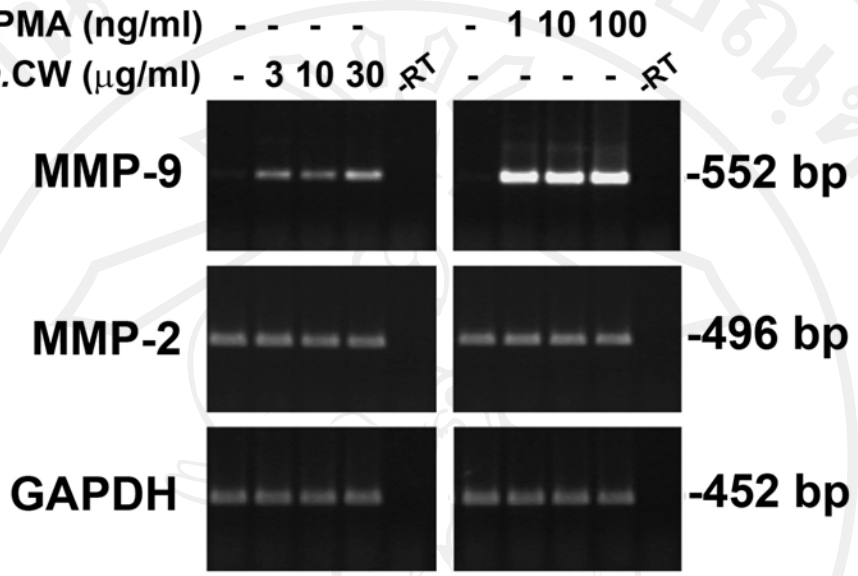

Figure 4.1 Up-regulation of MMP-9 mRNA in human gingival epithelial cells. HGCEs were stimulated with indicated doses of either *F.nucleatum* cell wall extract (*F.n.*CW) or PMA overnight. RT-PCR was performed as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The sizes of PCR products were as predicted.Data are representative of three independent experiments.

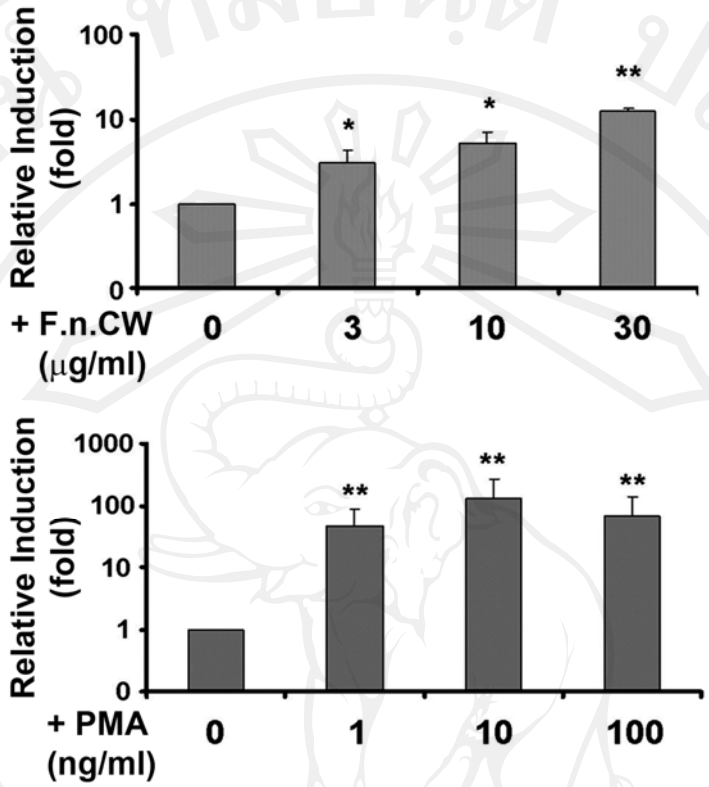

Figure 4.2Real-time PCR assay of MMP-9 mRNA expression.cDNA samples from Figure 4.1 were used to quantify the relative induction (fold) of MMP-9 to GAPDH mRNA expression. Data in bar graphs are presented as mean ± SD, and N for each cell datum = 3 (*, $P<0.05$; **, $P<0.01$).

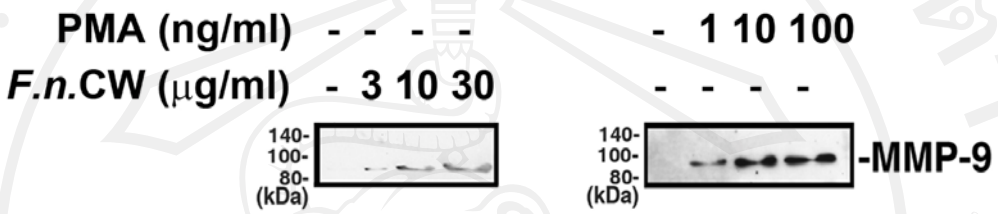

Figure 4.3MMP-9 protein induction in HGECs. Cell-free culture supernatants from Figure 4.1 were resolved on 10% SDS-PAGE along with standard biotinylated protein markers (Cell Signaling Technology) to analyze MMP-9 protein expression by Western Blot as described in Materials and Methods. Data are representative of three independent experiments.

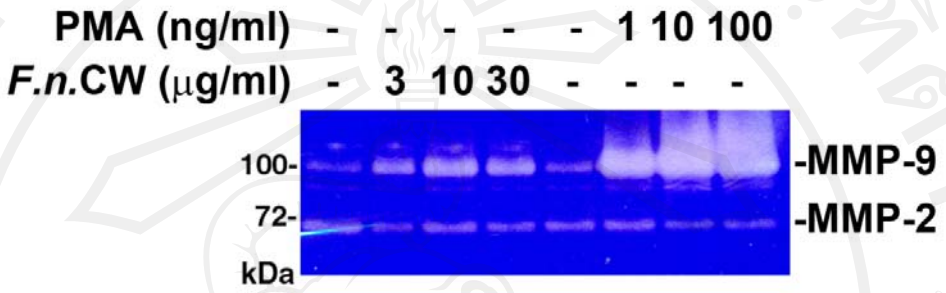

Figure 4.4Induction of MMP-9 activity in HGECs. Cell-free culture supernatants from Figure 4.1 were assayed by Gelatin Zymography as described in Materials and Methods. Note the constitutive and low MMP-2 activity (lower bands at 72 kDa) in HGECs. Data are representative of three independent experiments.

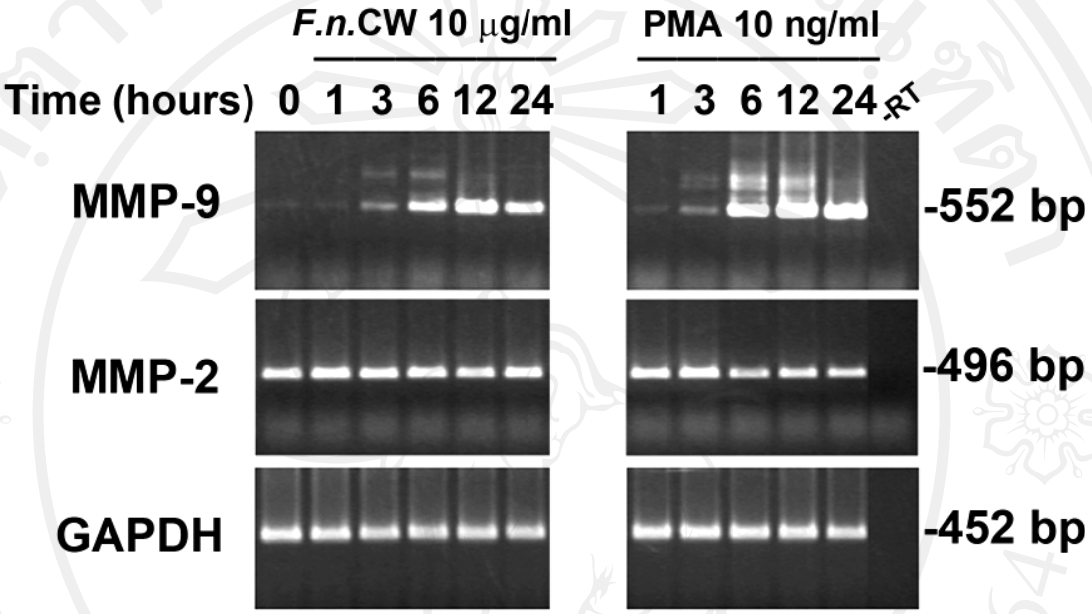

Figure 4.5The time-course study shows an early MMP-9 mRNA induction.HGECs were stimulated with either *F.nucleatum* cell wall extract (*F.n.*CW) or PMA for indicated times. RT-PCR was performed as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. Data are representative of three independent experiments.

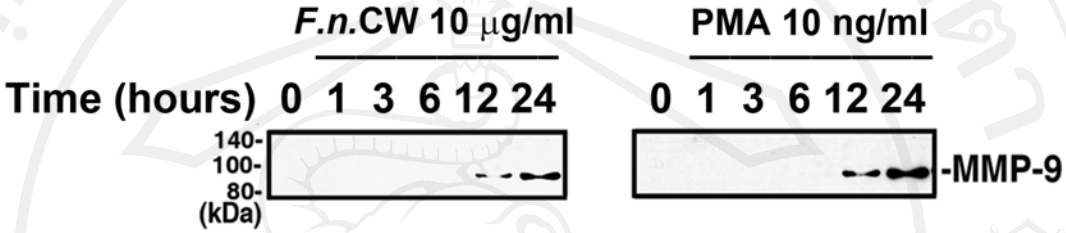

Figure 4.6 Late MMP-9 protein secretion in HGECs. Cell-free culture supernatants from Figure 4.5 were analyzed for MMP-9 protein expression by Western Blot. Data are representative of three independent experiments.

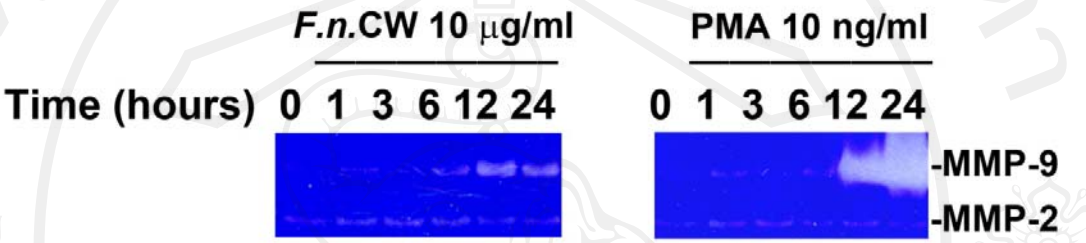

Figure 4.7 Late MMP-9 activity in HGECs. Cell-free culture supernatants from Figure 4.5 were assayed by Gelatin Zymography. Data are representative of three independent experiments.Note the constitutive and low MMP-2 activity (lower bands) in HGECs.

4.2 Constitutive expression of MMP-2 in human gingival fibroblasts

Interleukin-1β (IL-1β), a multi-functional pro-inflammatory cytokine, has been demonstrated to play an important role in the regulation of expression for many genes involved in the inflammatory process. Therefore, in this study, IL-1β was used to stimulate HGFs*in vitro*, since previous studies haveshown that skin fibroblasts and HGFs do express only MMP-2 (Sawicki*et al*., 2005; Zhou and Windsor, 2006, respectively). Thus, MMP-2 expression upon stimulation with either IL-1β or *F. nucleatum* cell wall extract in HGFs will be investigated in this study.

HGFs were treated with various doses (0.01-10 ng/ml) of IL-1β for 24 hours or with 1 ng/ml of IL-1βor 10 μg/ml of *F. nucleatum* cell wall extract for indicated times (0-24 hours).It was found that MMP-2 mRNA was constitutively expressed in HGFs(Figure 4.8). Similarly, MMP-2 mRNA expression was not induced by treatment with 1 ng/ml of IL-1β in any time points of stimulation (Figure 4.9), suggesting that MMP-2 mRNA expression in HGFs is constitutive similar to MMP-2 expression in HGECs (Figures 4.1 and 4.6).

However, theexpression of both latent and active forms of MMP-2 and their activity in cell-free culture supernatants were up-regulated by treatment with 1 ng/ml of IL-1β, implying a possible post-transcriptional mechanism in the regulation of MMP-2 activity (Figure 4.10). Unexpectedly, when cell-free culture supernatants were collected from untreated HGFs for various times (from 0 to 24 hours), it was demonstrated that the MMP-2 activity was already induced in untreated HGFs in a pattern that was similar to IL-1β-treatedor *F. nucleatum*-treated HGFs (Figures 4.11A or 4.11B, respectively). This

indicates that the levels of MMP-2 protein in HGFs increasingly accumulate, reflecting the stability of MMP-2 protein in cell-free culture supernatants.

Moreover, the activity of MMP-2 was already induced even in the absence of cell stimulation, proposing a mechanism for auto-activation of MMP-2 in HGFs irrespective of the presence of stimulants. This self-activation was evident because both latent and active forms of MMP-2 were detected in the cell-free culture supernatants (Figure 4.11). It is possible that MT1-MMP, demonstrated to play a role in the activation of MMP-2 enzymatic activity in other cell types (Sato and Takino, 2010),can contribute to the process of auto-activation of MMP-2 observed in this study.Furthermore, it is interesting to note that MMP-9 expression and activity were not noticeably detected in HGFs, consistent with the result from a previous study that shows expression of only MMP-2, but not MMP-9, in gingival fibroblasts (Mäkelä*et al*., 1994).All of the results shown in this section are representative of three independent experiments with similar findings.

Taken together, all of the experiments from Figure 4.1 to 4.11 show an up-regulation of MMP-9 mRNA and protein expression and of its activity in HGECs upon treatment with either the cell wall extract of *F. nucleatum*, a periodontopathogenicbacteria, or PMA, whereas MMP-2 expression and its activity remain low and unchanged upon stimulation with any of these two stimulants. With respect to the expression and activity of MMP-2 and MMP-9 in HGFs, it is demonstrated that only MMP-2 mRNA, but not MMP-9 mRNA, is expressed. The accumulative induction of MMP-2 protein expression and activity even in the absence of stimulation suggests the stability and a possible auto-activation mechanism of MMP-2 protein in HGFs, respectively. Consequently, all of the

studies in the following sections will be solely conducted to investigate the signaling mechanisms of MMP-9 up-regulation only in HGECs.

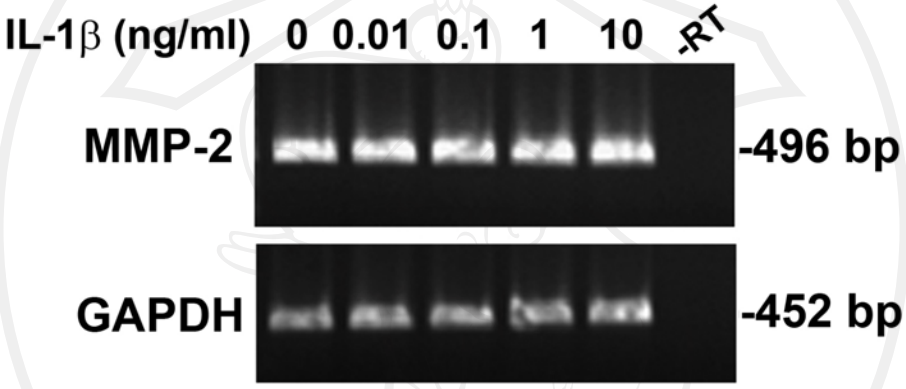

Figure 4.8Constitutive expression of MMP-2 in human gingival fibroblasts. HGFs were stimulated with 0.01, 0.1, 1, 10 ng/ml of IL-1βfor 24 hours. RT-PCR was performed as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

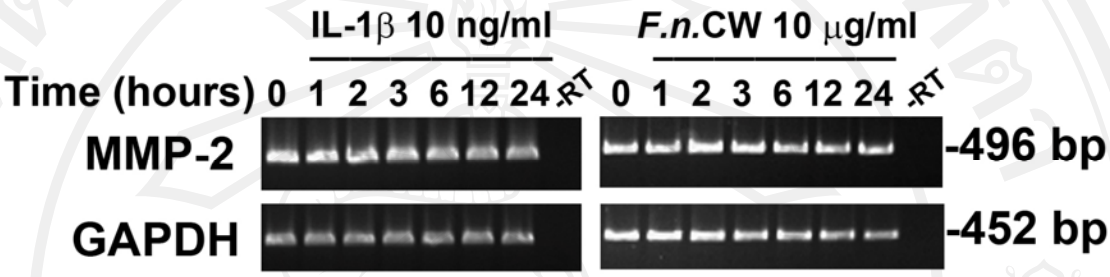

Figure 4.9The time-course study demonstrates constitutive MMP-2 mRNA expression.HGFs were stimulated with 1 ng/ml of IL-1β or 10 μg/ml of *F. nucleatum* cell wall extract for indicated times. RT-PCR was performed as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

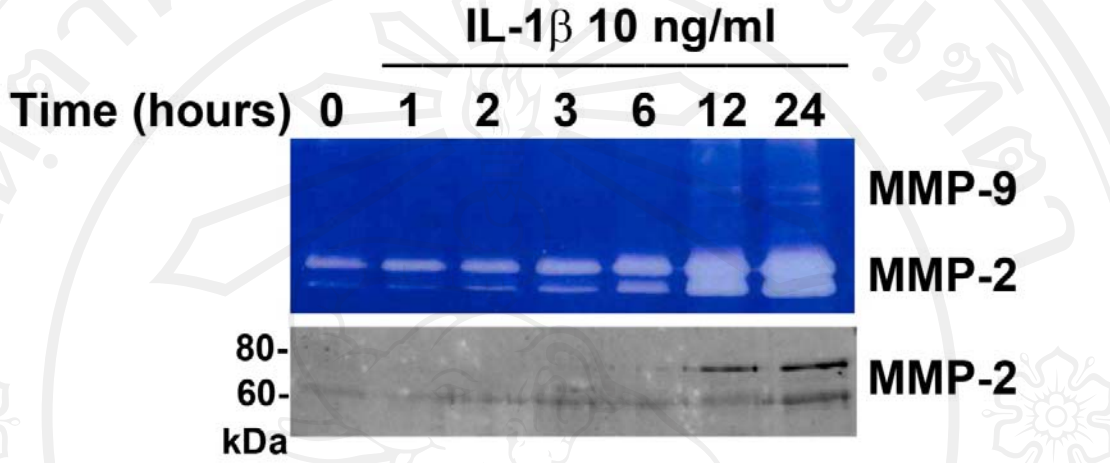

Figure 4.10The expression of MMP-2 protein and its activity in HGFs. Cell-free culture supernatants from Figure4.9 were assayed by Western blot hybridization and Gelatin zymographyto examine the expression and the activity of MMP-2, respectively, as described in Materials and Methods. Note an induction of MMP-2 protein expression and its activity (both pro- and active forms) in cell-free culture supernatants collected from HGFs upon treatment with IL-1β. The results shown are representative of three independent experiments.

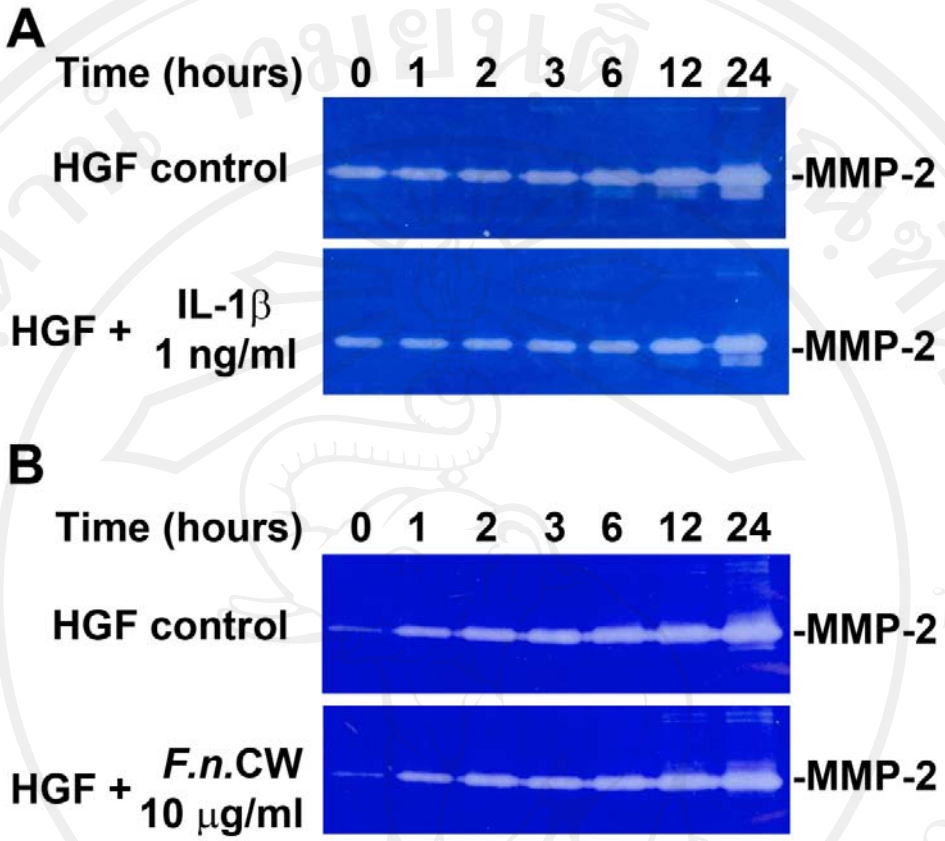

Figure 4.11 Induction of MMP-2 activity in treated and untreatedHGFs. Cell-free culture supernatants collected from both untreated and IL-1β-treated HGFs (A) and both untreated and *F. nucleatum*-treated HGFs (B) for different time points along with the purified peptides of MMP-2 and MMP-9 (R&D Systems, Inc.) were assayed by Gelatin zymography as described in Materials and Methods.Note the accumulative induction of the gelatinolytic activity of both pro- and active forms of MMP-2 even in the absence of IL-1β(A) or *F. nucleatum*(B) stimulation. The findings shown are representative of three independent experiments.

4.3 Expression of PLD1 and PLD2 mRNA and protein and their activity in human gingival epithelial cells

To determine the involvement of PLD1 and PLD2 in MMP-9 up-regulation in HGECs, the expression of PLD1 and PLD2 and their activity in HGECs were first investigated. HGECs were stimulated with 3, 10, 30 μg/ml of *F. nucleatum* cell wall extract or 1, 10, 100 ng/ml of PMA for 24 hours or left untreated as a control. The results showed that both PLD1α and β splice variants were constitutively expressed, while PLD2 mRNA was up-regulated by all doses of PMA tested (Figure 4.12). The intensity of the PLD1αband was less than that of PLD1β(Figures 4.12and 4.13), suggesting thatHGECs preferentially express the latter splice variant. According to the densitometry, both PLD1α and βsplice variants were expressed in much greater levels than was PLD2 by ~10- and 37-fold in control and *F. nucleatum*-stimulated samples and by 9- and 27-fold in PMA-stimulated sample, respectively (Figure 4.13).

Furthermore, the cell lysates were collected for analyzing the PLD1 and PLD2 protein expression. Consistently, expression for PLD2 protein at 106 kDa was detected at the low level in HGECs (Figure 4.14). Consistent with constitutive mRNA expression for two PLD1 splice variants, two immunoreactive bands at 120 and 80 kDa were detected with the PLD1 antibody (Figure 4.14), and there was no alteration in PLD1 protein expression in response to both stimulants.

For further analyzing the functional activity of PLD enzymes in HGECs, HGECs were stimulated with 10 μg/ml of *F. nucleatum* cell wall extract or 100 ng/ml of PMA for indicated times. The functional activity of PLD enzymes in HGECs was monitored indirectly using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent), a sensitive

fluorogenic probe for H_2O_2. The result showed that PLD activity was transiently induced when HGECs were stimulatedwith *F. nucleatum* cell wall extract from 3 to 12 h (Figure 4.15), corresponding with an early MMP-9mRNA induction by *F. nucleatum* cell wall extract (Figure 4.5).

It was interesting to note that the induction of PLD activity by *F. nucleatum*cell wall extract was significantly reduced at 24 hours, whereas the level of MMP-9 mRNA expression at 24 hours was still high. It is possible that PLD enzymes function as intermediate molecules and other downstream signaling molecules in the PLD pathway, such as,phosphatidic acid (PA), may participate in MMP-9 up-regulation and/or MMP-9 mRNA is stable and accumulated during the prolonged stimulation. In addition, the PLD activity was markedly induced after being stimulated with PMA for 12 hours(Figure 4.15), consistent with MMP-9 mRNAinduction by PMA (Figure 4.5).

Moreover, the PLD product, PA mass, was evaluated by TLC. Consistent with the induced PLD activity, PA mass was elevated in the lipid extracts from HGECs stimulated with *F. nucleatum* cell wall extract from 3 to 24 hours(Figure 4.16). In contrast, the changes in PA mass were not detected with the present method in control HGECs (Figure 4.16). Interestingly, PA mass was still detected at 24 hours(Figure 4.16), although the PLD activity was significantly reduced (Figure 4.15). This may be because diacylglycerol (DAG) kinases can generate PA from DAG (Nanjundanand Possmayer, 2003), in addition to PLD enzymes that generate PA from phosphatidylcholine.

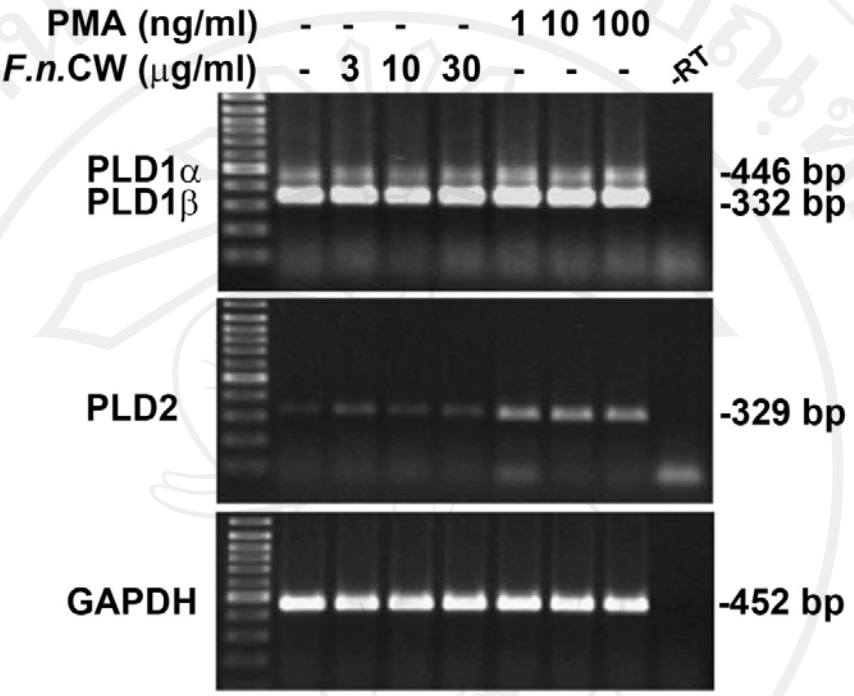

Figure 4.12 Expression of PLD1 and PLD2 mRNA in HGECs.RT–PCR analysis. HGECs were stimulated with various doses of *F.nucleatum* cell wall extract (*F.n.*CW) or PMA for 24 hours or left untreated as a control. Total RNA isolation and RT–PCR was conducted as described in Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

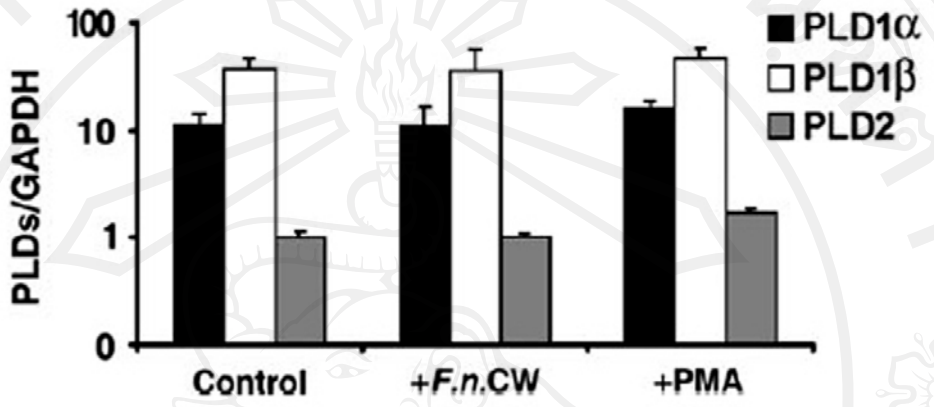

Figure 4.13 Densitometric analyses of mRNA expression for two PLD1 splice variants and PLD2 in control, *F. nucleatum*-stimulated (+*F.n.*CW) and PMA-stimulated (+PMA) samples. The *y*-axis of a bar graph represents the mean ratios of mRNA expression for the α (filled bars) and β (empty bars) splice variants of PLD1 and PLD2 (stippled bars) normalized by the levels of GAPDH expression of three separate experiments. The lowest ratio of PLD2 expression relative to GAPDH was set to one.

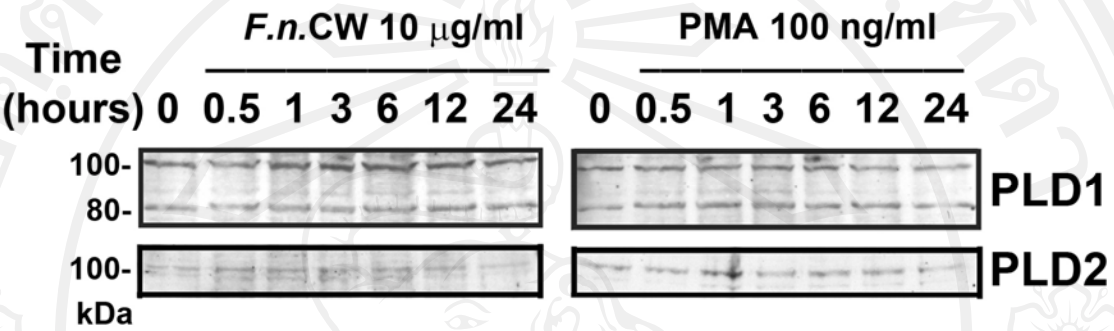

Figure 4.14 Expression of PLD1 and PLD2 protein in HGECs by Western blot analysis.HGECs were stimulated with *F. nucleatum* cell wall extract (*F.n.*CW) or PMA for indicatedtimes (0–24 h). Total protein extraction and western blot analysis using primary antibodies againstPLD1 or PLD2 were conducted as described in Materials and Methods. The data shown are representative of three separate experiments.

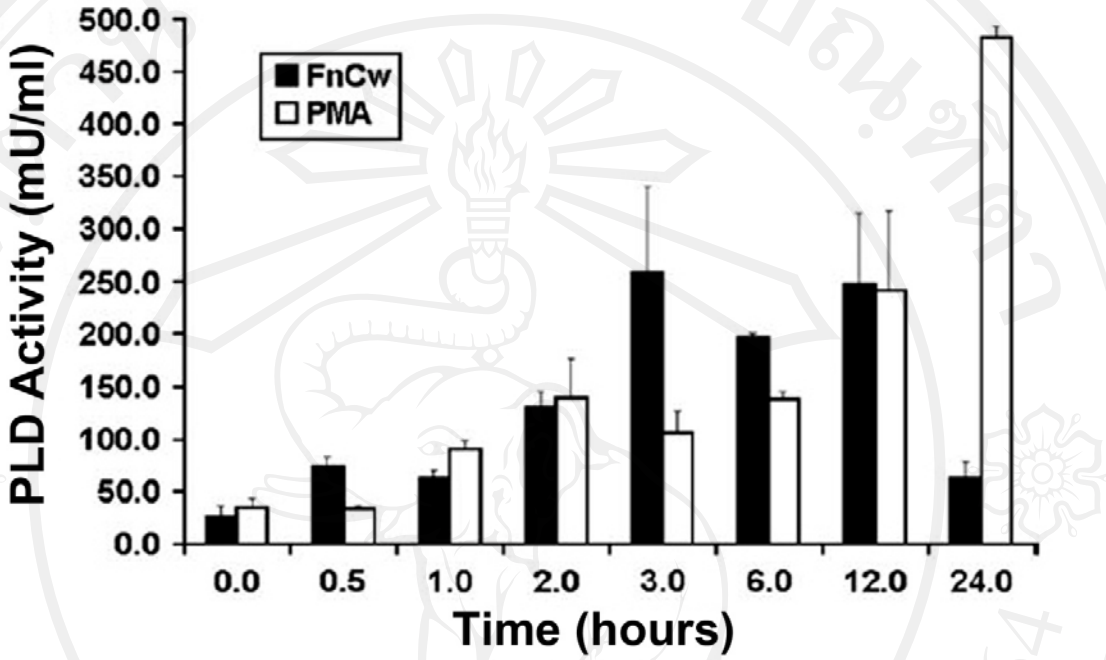

Figure 4.15 An assay for PLD activity in HGECs.HGECs were stimulated with 10 μg/mlof *F. nucleatum* cell wall extract (*F.n.*CW) or 100 ng/ml of PMA, and the assay for PLD activity was conducted as described in Materials and Methods. Data are given as mean PLD activity in mU/ ml ±SD of three independent experiments.

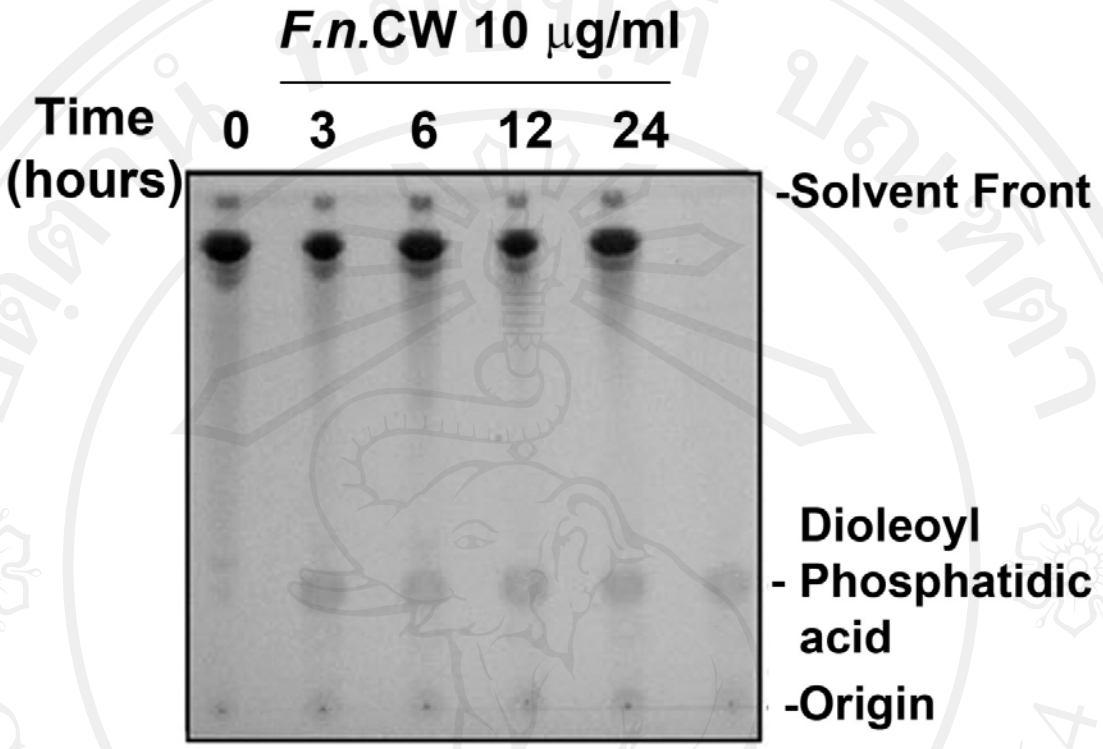

Figure 4.16 Thin-layer chromatogram (TLC) shows the time course study of phosphatidic acid (PA) formation in HGECs.HGECs(5 x 10^6 cells) were incubated with*F. nucleatum* cell wall extract (*F.n.*CW) for various times, and the stimulation was stopped with lipid extraction. Lipid extracts and 0.12 mM of dioleoylphosphatidic acid, as a positive control,were separated by TLC as described in Materials and Methods.

4.4Involvement of PLD enzymes in induction of MMP-9 expression and secretion

PLD is a group of phospholipid esterase enzymes that can catalyze a transphosphatidylation reaction utilizing short-chain primary alcohols as phosphatidyl-group acceptors (Morris *et al*., 1997). Therefore, in the presence of primary alcohol,*i.e.*, methanol, ethanol, 1-butanol or 1-propanol, the PLD catalyzed transphosphatidylation reaction will generate the acidic lipids – phosphatidylmethanol, phosphatidylethanol, phosphatidylbutanol, or phosphatidylpropanol (Ella *et al*., 1997). This reaction is very specific for primary alcohols as secondary and tertiary alcohols, such as, *t*-butanol, are not acceptors of the phosphatidyl-group. There are few studies in human cancer cells that show an inhibitory effect of primary alcohols in laminin-induced MMP-2 secretion (Reich *et al*., 1995; Willige*et al*., 1995).

Thus, 1-butanol and ethanol were used as PLD inhibitors in this study and *t*-butanol was used as a negative control. HGECs were pre-treated with ethanol, 1-butanol, or *t*-butanol at indicated doses prior to *F. nucleatum* cell wall extract or PMA stimulation. Total RNA and cell-free culture supernatants were collected for RT-PCR analysis and gelatin zymography. It was found that MMP-9 mRNA and activity were inhibited by pretreatment with 1-butanol or ethanol in both *F. nucleatum*-stimulated and PMA-stimulated HGECs (Figures 4.17 and 4.18). Interestingly, 1% of 1-butanol almost completely inhibited and 2% of ethanol significantly blocked an up-regulation of MMP-9 activity by 10 μg/ml of *F. nucleatum*cell wall extract or 100 ng/ml of PMA as demonstrated in bar graphs ($P<0.01$) (Figure 4.19). On the contrary, *t*-butanol, had no inhibitory effect on MMP-9 mRNA and activity induced by both stimulants(Figures 4.17, 4.18 and 4.19).

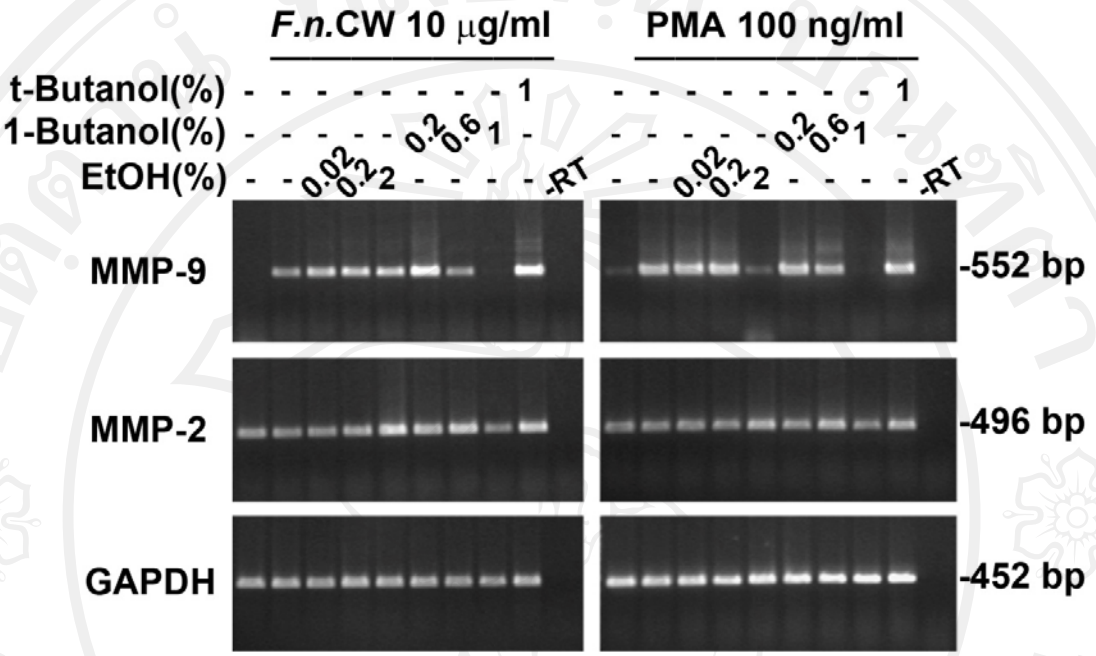

Figure 4.17Dose-dependent inhibition of MMP-9 mRNA induction by the PLD inhibitors, including ethanol and 1-butanol.HGECs were pre-treated with indicated doses of ethanol, 1-butanol, or *t*-butanol for 30 minutes prior to stimulation with 10 μg/ml of*F.nucleatum* cell wall extract (*F.n.*CW)or 100 ng/ml of PMA for 24 hours. GAPDH, serving as an internal control, was equally expressed among samples. The result shown is representative of three independent experiments.

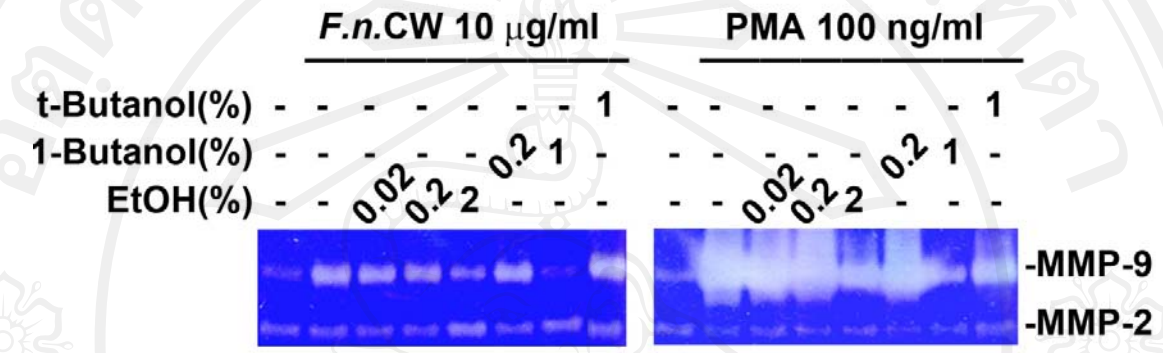

Figure 4.18Dose-dependent inhibition of induced MMP-9 activity by the PLD inhibitors. Cell-free culture supernatants were collected from the experiments in Figure 4.17, and assayed by Gelatin Zymography. Note a little gelatinolytic activity of MMP-2 in HGECs, and no significant change has been observed upon treatment with either the stimulants or the PLD inhibitors.

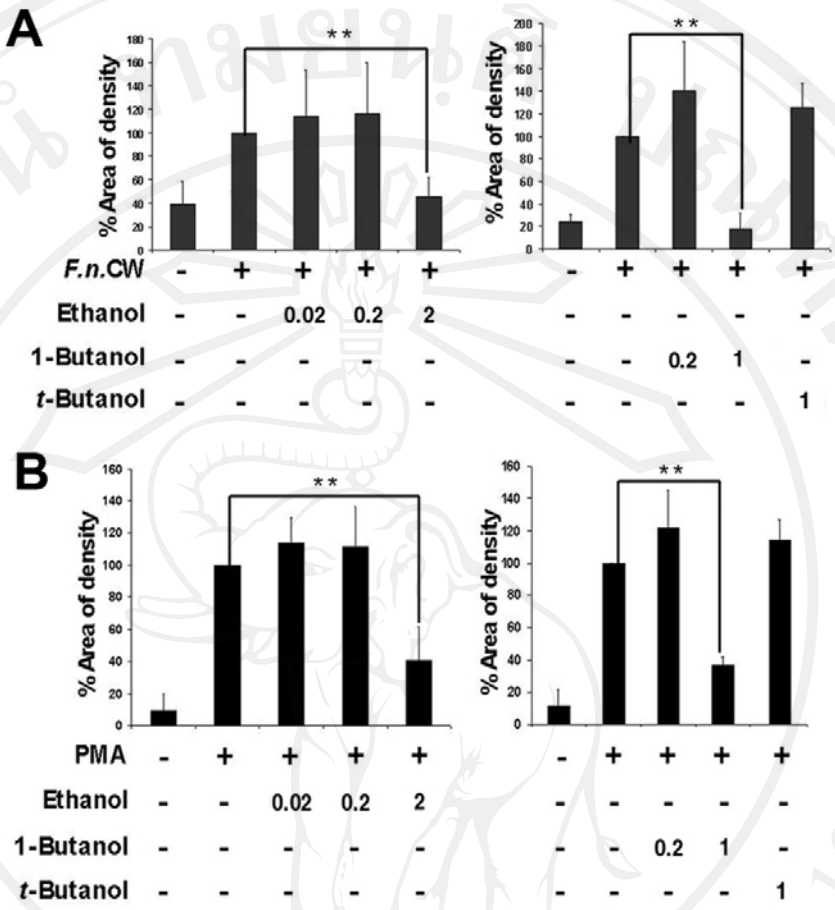

Figure 4.19 Densitometric analyses of the gelatinolytic activities of MMP-9, observed in Figure 4.18. It was found that pretreatment with either 2% of ethanol or 1% of 1-butanol significantly inhibited an up-regulation of MMP-9activity in *F. nucleatum*-stimulated (*F.n.*CW) (A) and PMA-stimulated (PMA) (B) HGECs. The *y*-axis of bar graphs represents the relativepercentage area of density of each sample in comparison with that of either *F.n.*CW-stimulated (A) or of PMA-stimulated (B) sample, which was set to 100. The density of gelatinolytic bands of MMP-9 in each sample was measured from three separate experiments.Data in bar graphs are presented as mean ± SD, and N for each cell datum = 3 (**, P<0.01).

4.5Induction of MMP-9 expression and secretion by dioctanoylphosphatidic acid

Although the experiments in Figures 4.17, 4.18 and 4.19 demonstrated that PLD enzymes are involved in MMP-9 up-regulation by *F. nucleatum* cell wall extract and PMA, they provide no direct evidence that PA, a product derived from the PLD catalytic reaction, is involved in MMP-9 up-regulation. To test this, HGECs were treated with various concentrations of dioctanoylphosphatidic acid (DOPA), which is commonly used for PA to see the direct effect of PA (Müller-Wieprecht*et al*., 1998) on MMP-9 expression and activity. It was found that MMP-9 mRNA was induced by DOPA in a dose-dependent manner (Figure 4.20), confirming a critical role of PLD enzymes and its derived product, *i.e.*, PA, in MMP-9 up-regulation. Consistent with MMP-9 mRNA induction, expression and activity of MMP-9 protein were induced by DOPA in a dose-dependent fashion (Figures 4.21 and 4.22). DOPA induced MMP-9 expression and activity regardless of the stimulation with *F. nucleatum* cell wall extract or PMA, indicating that PA alone can induce MMP-9.

PA can be converted into (diacylglycerol) DAG by the family of enzymes, known as phosphatidic acid phosphohydrolases (PAPs) (Brindley and Waggoner, 1998). Therefore, it is possible that PA is converted into DAG, and DAG is involved in MMP-9 up-regulation in HGECs. Consequently, HGECs were pretreated with various doses of propranolol, a PAP inhibitor,30 minutes prior to stimulation with cell wall extract of *F. nucleatum* or PMA. It was shown that MMP-9 mRNA and activity were inhibited by pretreatment with propranolol in both *F. nucleatum*-stimulated and PMA-stimulated HGECs (Figures 4.23 and 4.34).Interestingly, dioctanoylglycerol (DOG), a chemical reagent commonly used to stimulate cells instead of DAG, could up-regulate MMP-9

mRNA expression (Figure 4.25) and activity (Figure 4.26) even in the absence of stimulants. This confirms the involvement and importance of the PLD- PA-PAP-DAG axis in MMP-9 up-regulation in HGECs.

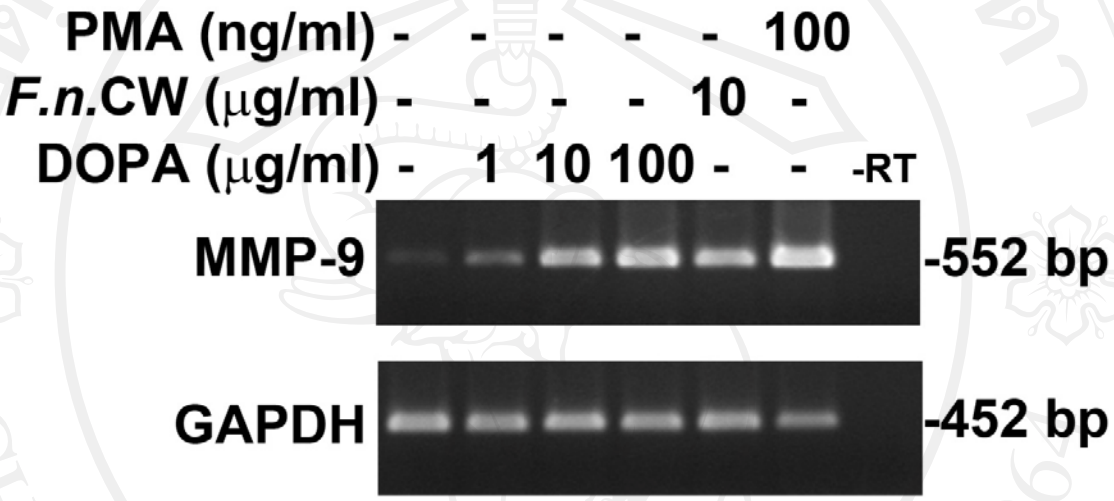

Figure 4.20 Up-regulation of MMP-9 mRNA by DOPA in HGECs.RT–PCR analysis. HGECs were stimulated with 1, 10, 100 μg/ml of DOPA or 10 μg/ml of*Fusobacteriumnucleatum* cell wall extract (*F.n.*CW) or 100ng/ml of PMA for 24 hours or left untreated as a control. Total RNA isolation and RT–PCR was conducted as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

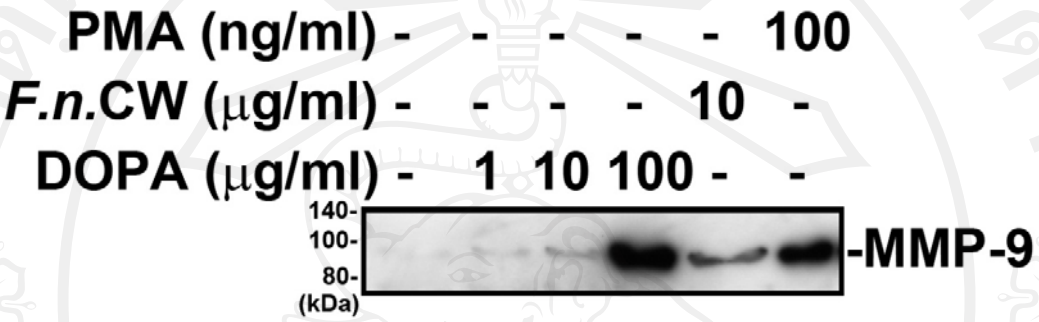

Figure 4.21 Up-regulation of MMP-9 protein by DOPA in HGECs by Western blot analysis.HGECs were stimulated with 1, 10, 100 μg/ml of DOPA or 10 μg/ml of*F. nucleatum* cell wall extract (*F.n.*CW) or 100ng/ml of PMA for 24 hours or left untreated as a control. Cell-free culture supernatants were collected and western blot analysis using the primary antibody against MMP-9 was conducted as described in Materials and Methods. The data shown are representative of three separate experiments.

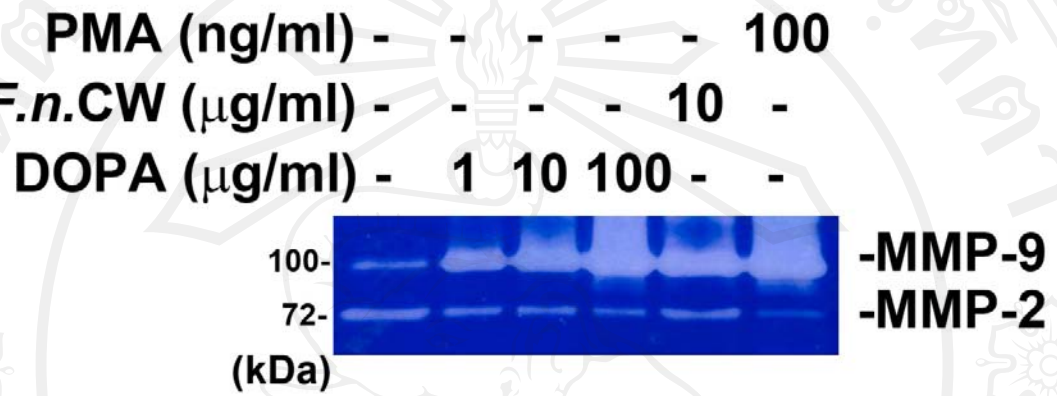

Figure 4.22 Up-regulation of MMP-9 activity by DOPA in HGECs. Cell-free culture supernatants from the experiments in Figure4.20 were assayed by Gelatin Zymography as described in Materials and Methods. Note a dose-dependent induction of MMP-9 activity by DOPA, whereas the MMP-2 activity was not induced by treatment with any of the stimulants.The result shown is representative of three independent experiments.

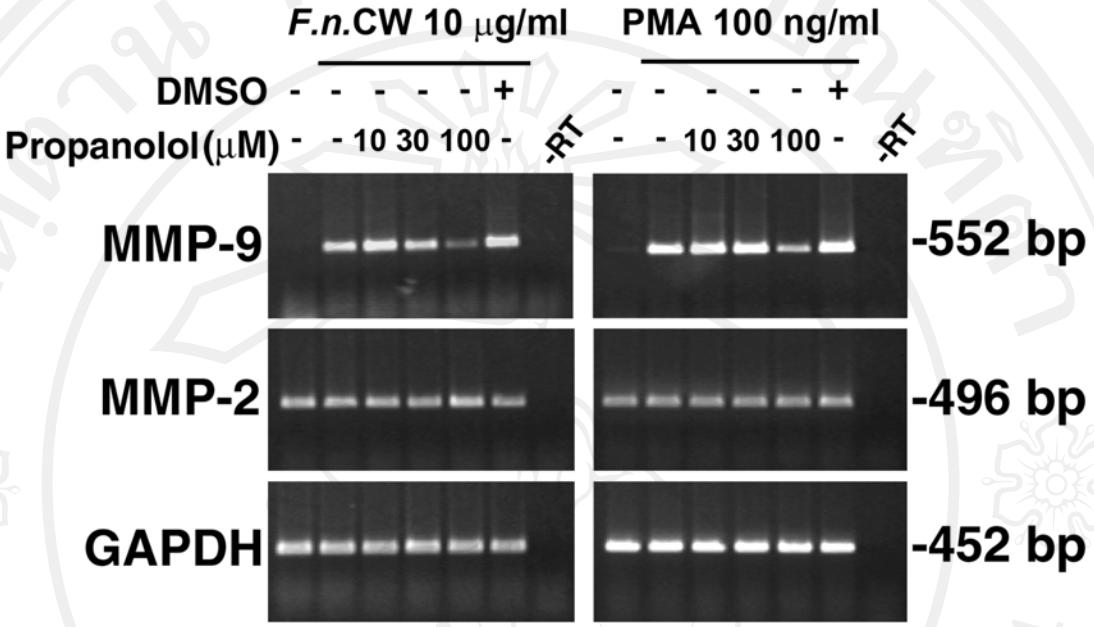

Figure 4.23 Inhibition of MMP-9 mRNA induction by the PAP inhibitor, propanolol.HGECs were pretreated with varying doses of propanolol for 30 minutes prior to stimulation with 10 μg/ml of*F.nucleatum* cell wall extract (*F.n.*CW)or 100 ng/ml of PMA for 24 hours. Note no significant change in MMP-2 expression was observed in any samples. GAPDH, serving as an internal control, was equally expressed among samples. The result shown is representative of three independent experiments.

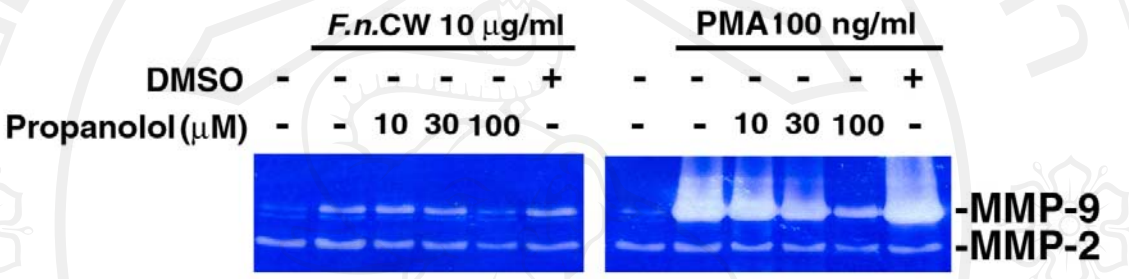

Figure 4.24 Inhibition of induced MMP-9 activity by propranolol. Cell-free culture supernatants were collected from the experiments in Figure 4.23, and assayed by Gelatin Zymography. Note a little gelatinolytic activity of MMP-2 in HGECs, and no significant change has been observed upon treatment with the stimulants or the inhibitor. The data shown are representative of three separate experiments.

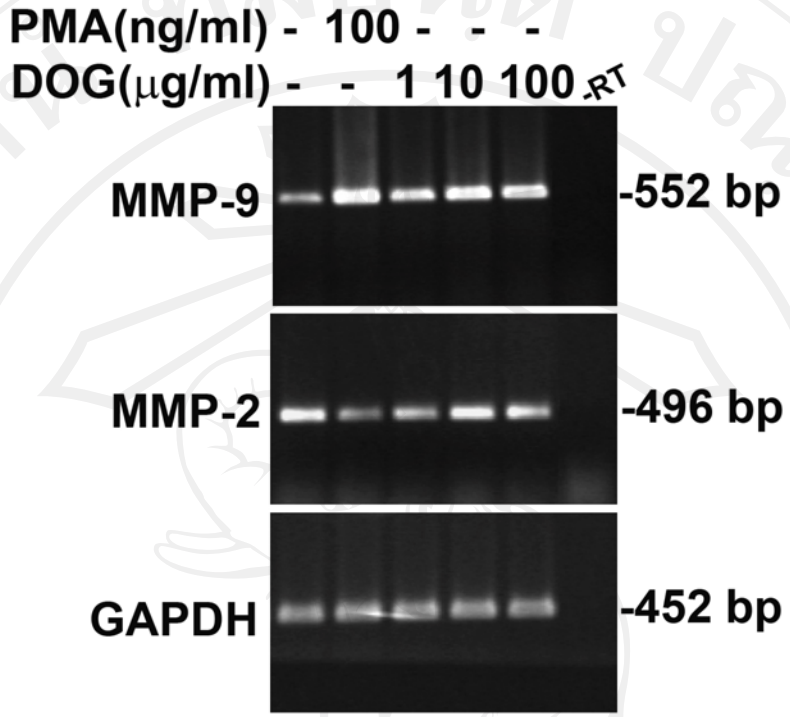

Figure 4.25 Up-regulation of MMP-9 mRNA by DOG in HGECs by RT–PCR analysis. HGECs were stimulated with 1, 10, 100 μg/ml of DOG or 10 ng/ml of PMA for 24 hours or left untreated as a control. Total RNA isolation and RT–PCR was conducted as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

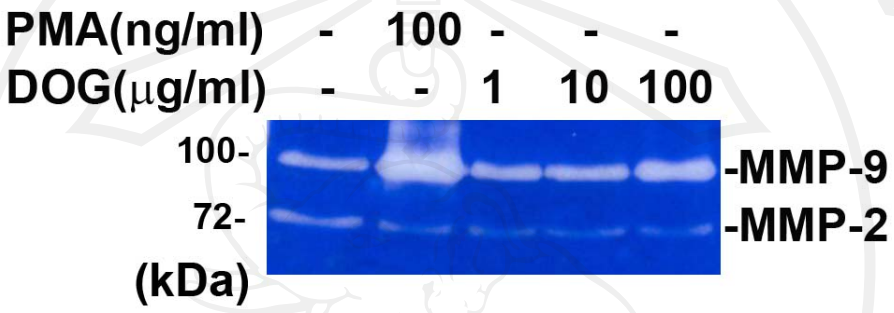

Figure 4.26 Up-regulation of MMP-9 activity by DOG in HGECs. Cell-free culture supernatants from the experiments in Figure4.25were assayed by Gelatin Zymography as described in Materials and Methods. Note a dose-dependent induction of MMP-9 activity by DOG, whereas the MMP-2 activity was not induced by treatment with any of the stimulants. The result shown is representative of three independent experiments.

4.6 Cytosolic phospholipase $A_2\alpha$ is constitutively expressed, but can be transiently activated by phosphorylation.

Among the family of phospholipase A_2 (PLA_2) enzymes, the group IVA phospholipase,also called cytosolic phospholipase $A_2\alpha$($cPLA_2\alpha$), is recognizedas the most important enzyme in exerting the PLA_2activity, and is also known as a key enzyme in theproduction of potent inflammatory mediators, such as, prostaglandins, leukotrienes and plateletactivating factor (Van den Bosch, 1980).To determine the involvement of $cPLA_2\alpha$ in MMP-9 up-regulation in HGECs, $cPLA_2\alpha$ expression in HGECs was first determined. HGECs were stimulated with various doses of either *F.nucleatum* cell wall extract, PMA for the indicated times, or left unstimulated. It was demonstrated that $cPLA_2\alpha$ mRNA and protein were constitutively expressed in HGECs, and its level of expression remained unchanged upon stimulation with the two stimulants(Figures 4.27 and 4.28, respectively).

However, in response to stimulation with *F.nucleatum*cell wall extract and PMA, $cPLA_2$ was transiently activated by phosphorylation (phospho-$cPLA_2$) from 0.5 to 2 hours and from 1 to 12 hours, respectively(Figure 4.28), suggesting distinct signaling pathways used to mediate the phosphorylation of $cPLA_2$between *F.nucleatum*- and PMA-stimulation.Interestingly, the phosphorylated form of $cPLA_2$ (phospho-$cPLA_2$) was localized in the nuclei of stimulated cells (Figure 4.29),suggesting its role as a transcription factor. The immunoreactivity was specific because the signal was not detected in the *F. nucleatum*-treated sample, in which the primary antibody against the phosphorylated form of $cPLA_2$was omitted (no primary antibody) (Figure 4.29).

Furthermore, the method of nuclear extraction was used to confirm the localization of phospho-cPLA$_2$in the nuclei of treated HGECs. It was found that treatment with *F. nucleatum* cell wall extract transiently caused the nuclear localization of phospho-cPLA$_2$ (from 1 to 2 hours), corresponding to the transient phosphorylation of cPLA$_2$ (from 0.5 to 2 hours) in the whole cell lysates of HGECs in Figure 4.28, whereas the expression of cPLA$_2$ and phospho-cPLA$_2$ in the cytoplasmic extract remained constant (Figure 4.30). There was no expression of cPLA$_2$ in the nuclear extract (Figure 4.30).

Collectively, it is demonstrated by this study that cPLA$_2\alpha$ mRNA and protein are constitutively expressed in HGECs. Nevertheless, cPLA$_2$ can be activated by phosphorylation upon cellular treatment with either *F. nucleatum* cell wall extract or PMA. Interestingly, a distinct pattern of cPLA$_2$ activation by these two stimulants is observed, suggesting different signaling pathway used to mediate the inducible effect of these two stimulants. In addition, the phosphorylated form of cPLA$_2$ is transiently localized in the nuclei of HGECs upon stimulation, suggesting its role as a transcription factor.

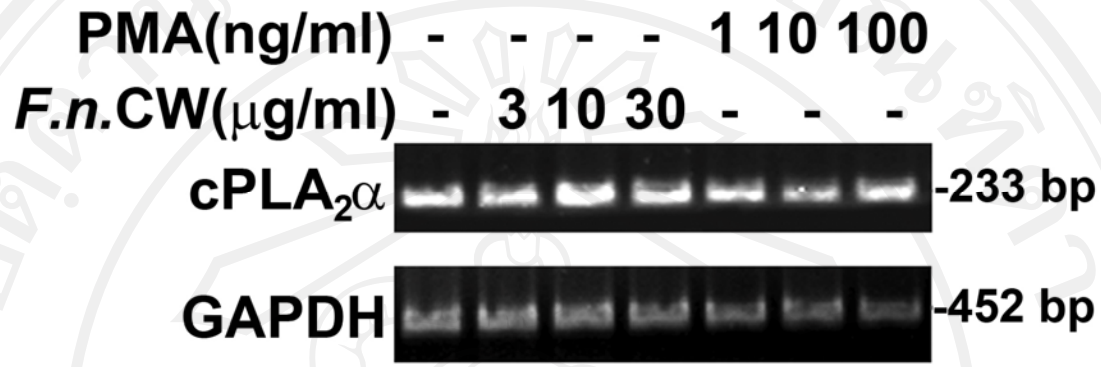

Figure 4.27 Constitutive mRNA expression of cPLA$_2\alpha$.HGECs were stimulated with indicated doses of either *F. nucleatum* cell wall extract (*F.n.*CW) or PMA overnight. RT-PCR was performed as described in Materials and Methods. GAPDH mRNA, serving as an internal control, was equally expressed. The result shown is representative of three independent experiments.

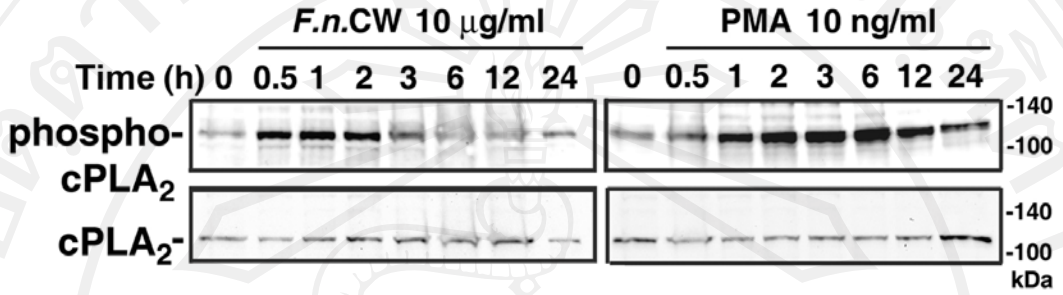

Figure 4.28 Transient activation of $cPLA_2$ by phosphorylation. Forty μg of whole cell lysates were resolved on 10% SDS-PAGE and probed with antibody against $cPLA_2$ or the phosphorylated form of $cPLA_2$ (phospho-$cPLA_2$). Note that the mobility of bands at 110 kDa, greater than the expected molecular weight of $cPLA_2$ at 85 kDa, was consistent with a previous result (Clark *et al*., 1990). The data shown are representative of three independent experiments.

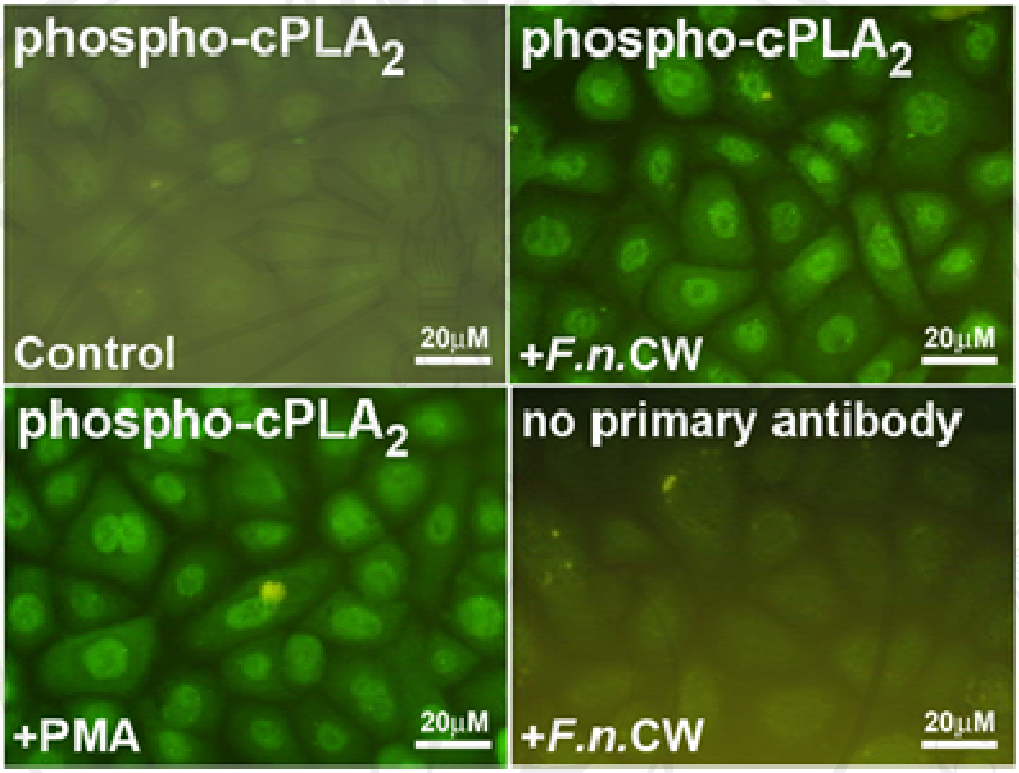

Figure 4.29 Nuclear localization of the phosphorylated form of cPLA$_2$. HGECs, grown on cover slips, were stimulated with 10 μg/ml of*F. nucleatum* cell wall extract (*F.n.*CW)for 1 hour, 10 ng/ml of PMA for 3 hours, or left untreated. Subsequently, cells were processed as described in Materials and Methods. The result shown is representative of three independent experiments.

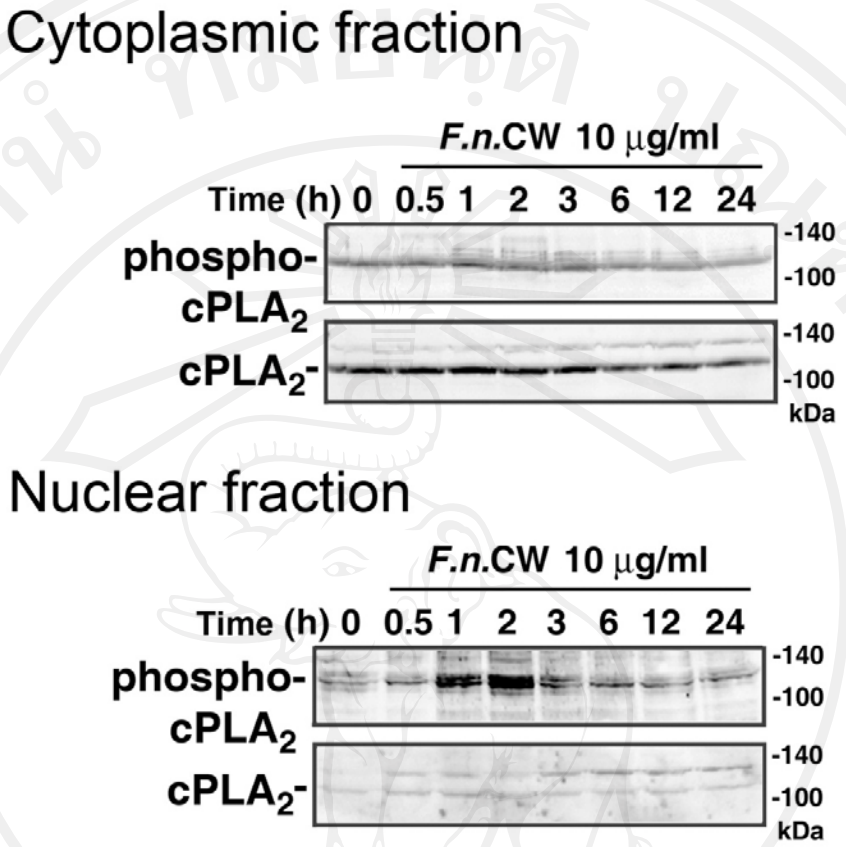

Figure 4.30 Transient activation of $cPLA_2$ by phosphorylation. Cytoplasmic and nuclear extract from control and stimulated-HGECs fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit was analyzed by Western blot. The nuclear or cytoplasmic extracts were resolved on 10% SDS-PAGE and probed with antibody against $cPLA_2$ or the phosphorylated form of $cPLA_2$ (phospho-$cPLA_2$). The data shown are representative of three independent experiments.

4.7 Inductionof MMP-9 expression and activity is controlled by cPLA$_2\alpha$

To test whether cPLA$_2\alpha$ controlled MMP-9 up-regulation, HGECswere pretreated with various doses of the specific cPLA$_2\alpha$inhibitor (pyrrolidine-1, a cell-permeable 1,2,4-trisubstituted pyrrolidine derivative, $C_{49}H_{44}F_2N_4O_5S$)for 30 minutes before stimulation with *F.nucleatum*cell wall extract or PMA for 24 hours. Induction of MMP-9 mRNA expression and activity by both stimulants was almost completely abolished in a dose-dependent manner by pretreatment with 1 μM of the cPLA$_2\alpha$ inhibitor (Figures 4.31 and 4.32, respectively). The real-time PCR showed a significant MMP-9 mRNA inhibition by the cPLA$_2\alpha$ inhibitor ($P<0.01$) (Figure 4.33), suggesting the cPLA$_2\alpha$ involvement in MMP-9 up-regulation in HGECs.

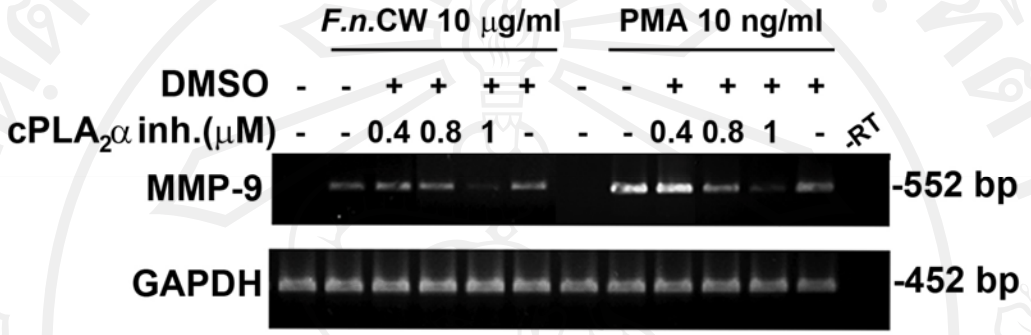

Figure 4.31 Involvement of cPLA$_2\alpha$ in induction of MMP-9 mRNA expression. Dose-dependent inhibition of MMP-9 mRNA induction by the specific cPLA$_2\alpha$ inhibitor (cPLA$_2\alpha$inh.). HGECs were pretreated with indicated doses of the specific cPLA$_2\alpha$ inhibitor for 30 minutes prior to stimulation with *F.nucleatum* cell wall extract (*F.n.*CW)or PMA for 24 hours. GAPDH mRNA, serving as an internal control, was equally expressed. A -RT sample was a negative control where the reverse transcriptase was omitted. The data shown are representative of three independent experiments.

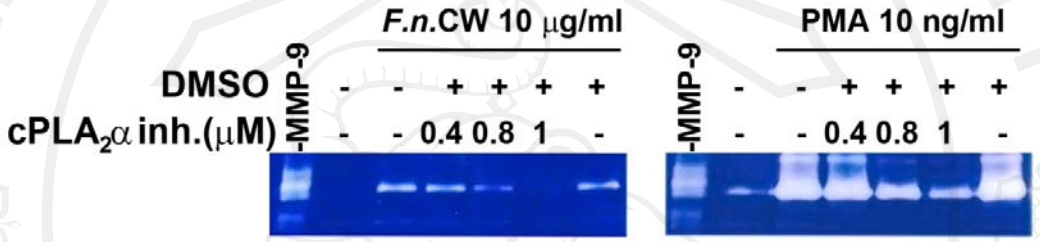

Figure 4.32 Involvement of cPLA$_2\alpha$ in induction of MMP-9 activity. Dose-dependent inhibition of MMP-9 activity in cell-free culture supernatants collected from the experiments in Figure 4.31, as assayed by Gelatin Zymography.

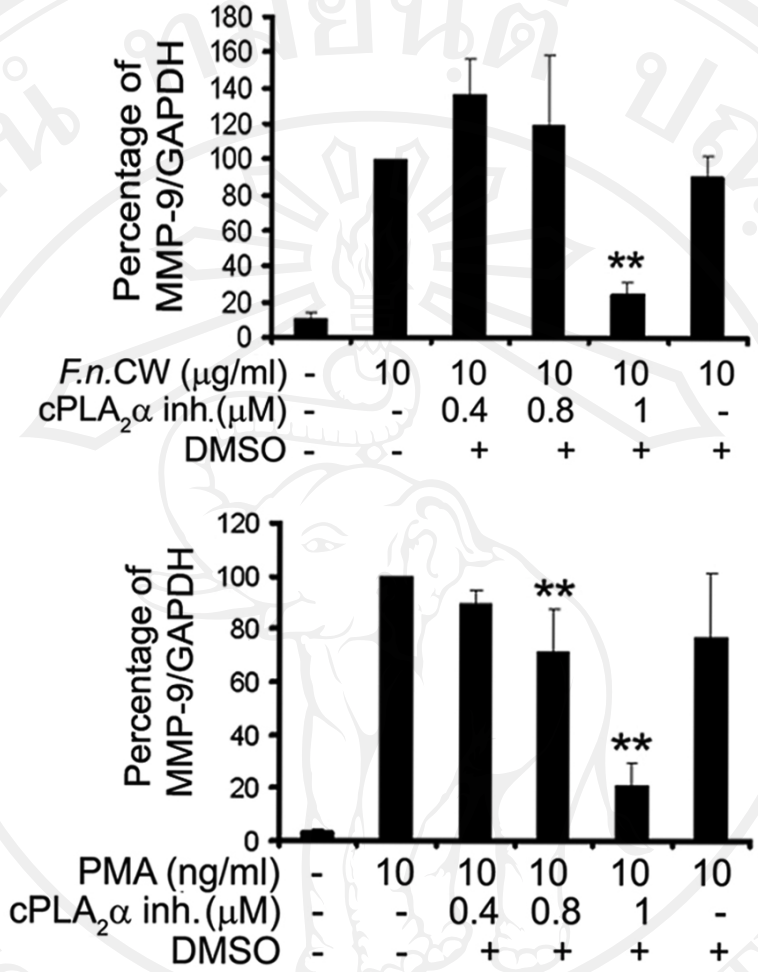

Figure 4.33 Thepercentage of MMP-9 mRNA inhibition as expressed by the expression of MMP-9 relative to GAPDH. Real-time PCR assay.cDNA samples from the experiments in Figure4.31 were usedto quantify the relative expression of MMP-9 to GAPDH mRNA. The level of MMP-9 expression in stimulated sample was set to 100%, and those in other samples were compared with this level. Data in bar graphs are presented as mean ± SD, and N for each cell datum = 3 (**, P<0.01)