Cyclic AMP Suppresses Matrix Metalloproteinase-1 Expression through Inhibition of MAPK and GSK-3β

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Expression of matrix metalloproteinase-1 (MMP-1) is stimulated by diverse stimuli and is likely to be regulated by many signaling pathways. cAMP is known to act as a second messenger for various extracellular stimuli and to be involved in the regulation of cell proliferation, apoptosis, and inflammation. Here, we investigated the effect of cAMP on tumor necrosis factor (TNF)-α-induced MMP-1 expression and the molecular events involved in the processes in human skin fibroblasts. We showed that cAMP suppresses TNF-α-induced MMP-1 expression via protein kinase A (PKA) pathway. cAMP inhibited TNF-α-stimulated ERK and JNK activation, which was shown to have an important role in MMP-1 expression. However, MMP-1 expression could also be inhibited by cAMP even when ERK and JNK activities were unaffected, indicating that there might be other target(s) that mediate cAMP-mediated suppression of MMP-1 expression. Further studies revealed that glycogen synthase kinase (GSK)-3β can be inactivated by cAMP/PKA pathway and has important roles in MMP-1 expression, and showed that inactivation of GSK-3β is critical for suppression of MMP-1 expression by cAMP elevation after TNF-α treatment. Taken together, our results suggest that cAMP/PKA pathway can suppress MMP-1 expression through inhibition of multiple signaling pathways, including MAPK and GSK-3β.

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INTRODUCTION

Matrix metalloproteinases (MMPs) constitute a family of structurally related zinc-dependent endopeptidases that are capable of degrading a wide variety of extracellular matrix components (Kahari and Saarialho-Kere, 1997; Woessner, 1998; Nagase *et al.*, 2006). MMPs are known to have important roles in tissue remodeling during physiological processes, including developmental morphogenesis, angiogenesis, and tissue repair, and in tissue destruction during pathological processes, such as arthritis, skin aging, tumor invasion, and metastasis. To date, 23 human MMP gene family members have been identified (Nagase *et al.*, 2006; Lopez-Otin *et al.*, 2009). These can be classified into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs, and other MMPs, according to their structure and substrate specificities (Nagase and Woessner, 1999; Sternlicht and Werb, 2001; Nagase *et al.*, 2006). Human fibroblasts have the capacity to produce several MMPs, including interstitial collagenase (collagenase-1, MMP-1), stromelysin-1 (MMP-3), and 72-kDa gelatinase (MMP-2). The expression of MMPs is usually low in non-stimulated cells, but some are induced by various extracellular stimuli including growth factors, proinflammatory cytokines, tumor promoters, and ultraviolet (UV) and infrared (IR) radiation (Johansson *et al.*, 2000; Sternlicht and Werb, 2001; Rittie and Fisher, 2002; Cho *et al.*, 2008; Schroeder *et al.*, 2008).

The second messenger cAMP has been reported to regulate a variety of cellular activities through cell-type-specific responses (Houslay and Milligan, 1997). cAMP is generated from ATP by adenylate cyclases and converted to 5′-AMP by phosphodiesterases (Sunahara and Taussig, 2002; Houslay and Adams, 2003). Intracellular cAMP levels can be increased by various extracellular stimuli including hormones, growth factors, and neurotransmitters through G-protein (Gs)-coupled membrane receptors (Sunahara and Taussig, 2002). In addition, the cellular cAMP level can be stimulated by a variety of pharmacological agents, such as forskolin, which is a direct activator of adenylate cyclases (Seamon *et al.*, 1981), isobutylmethylxanthine (IBMX), which is an inhibitor of phosphodiesterases (Beavo *et al.*, 1970), and dibutyryl cAMP, a cell-permeable cAMP (Schmidt-Ott *et al.*, 2001). Elevated cAMP activates its downstream effectors, including protein kinase A (PKA) and exchange proteins directly activated by cAMP (Epac), also known as cAMP-regulated guanine nucleotide exchange factors (cAMP–GEFs), which mediate various biological functions of cAMP in a cell-type-specific manner (Doskeland *et al.*, 1993; de Rooij *et al.*, 1998; Feliciello *et al.*, 2005).

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Abbreviations: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; IBMX, isobutylmethylxanthine; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinases; PKA, protein kinase A; TNF, tumor necrosis factor

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Although numerous studies have reported that cAMP signaling pathways have important roles in regulating a variety of biological processes such as cell proliferation and growth, apoptosis, carcinogenesis, inflammation, and immune response (Rosenberg *et al.*, 2002; Schmitt and Stork, 2002; Stork and Schmitt, 2002; Peters-Golden, 2009), the role of cAMP signaling on expression of MMPs remains poorly understood. In this study, we investigated the effect of cAMP on the expression of MMP-1 induced by diverse stimuli, including tumor necrosis factor (TNF)-α, and the signal transduction pathways involved in this process in human skin cells. We found that elevation of cAMP inhibited MMP-1 expression induced by TNF-α, interleukin (IL)-1β, and epidermal growth factor (EGF), and that the suppression of MMP-1 by cAMP was mediated via PKA but not Epac. Further studies indicated that elevation of cAMP before or after TNF-α treatment might attenuate MMP-1 expression through the suppression of TNF-α-induced activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) and/or the inactivation of glycogen synthase kinase (GSK)-3β, which, in this study, was shown to have an important role in MMP-1 expression. Therefore, our results show that cAMP/PKA pathway can suppress MMP-1 expression through the regulation of multiple signaling pathways, including mitogen-activated protein kinase (MAPK) and GSK-3β.

RESULTS

Elevation of cAMP inhibits TNF-α-induced MMP-1 expression in human skin fibroblasts

Previous studies have shown that cAMP signaling may have an important role in inhibiting MMP-1 expression in several cell lines (Salvatori *et al.*, 1992). However, the molecular mechanisms involved in the process remain poorly understood. To investigate the effect of cAMP elevation on the expression of MMP-1 in more detail, cultured human skin fibroblasts were pretreated with a cAMP-elevating agent, forskolin, for 45 minutes at the indicated concentrations, followed by treatment with or without TNF-α for 24 hours. The mRNA levels of MMP-1 were measured by quantitative real-time PCR and the protein levels of MMP-1 released into culture media were analyzed by western blot analysis. Forskolin suppressed TNF-α-induced expression of MMP-1 at both the mRNA and the protein levels in a dose-dependent manner (Figure 1a and b). Furthermore, pretreatment of cells with two other cAMP-elevating agents, IBMX and dibutyryl cAMP for 60 minutes also attenuated TNF-α-induced MMP-1 protein expression in a dose-dependent manner (See online Supplementary Figure S1a and b). Taken together, these results indicate that elevation of cAMP suppresses TNF-α-induced MMP-1 expression in primary human skin fibroblasts.

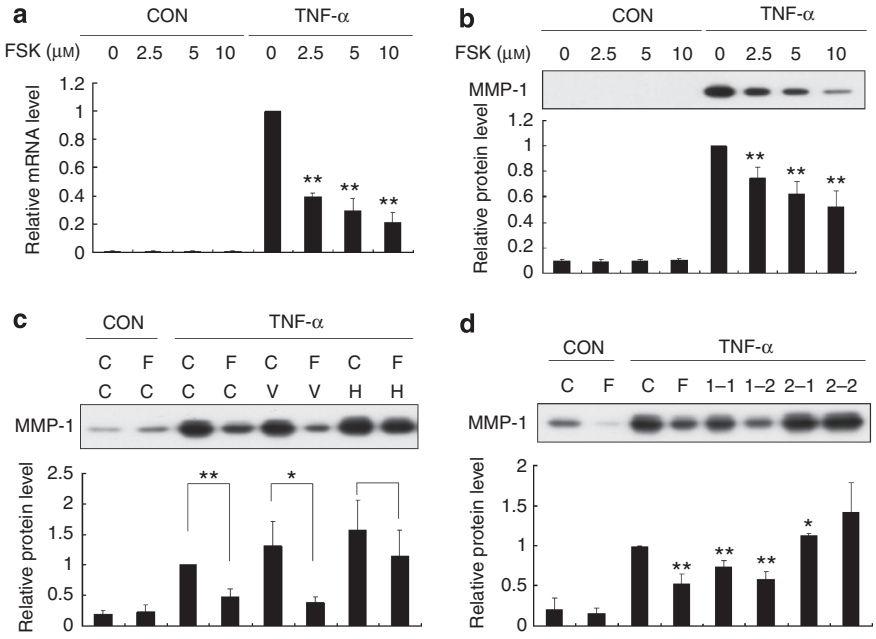

Figure 1. Elevation of cAMP inhibits TNF-α-induced MMP-1 expression via PKA but not Epac in human skin fibroblasts. (**a**, **b**) Serum-starved primary human skin fibroblasts were pretreated with forskolin (FSK) at the indicated concentrations for 45 minutes and then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 24 hours. (**c**) Serum-starved primary human skin fibroblasts were pretreated first with DMSO only (C), the Akt inhibitor, Akt Inhibitor VIII (10 μM; V), or the PKA inhibitor, H-89 (10 μM; H), for 1 hour and then with DMSO (C) or forskolin (10 μM; F) for 45 minutes. Cells were then treated with vehicle only (CON) or TNF-α (5 ng/ml; TNF-α) for 24 hours. (**d**) Serum-starved primary human skin fibroblasts were pretreated with DMSO only (C), forskolin (10 μM; F), the specific PKA agonist, 6-Bnz-cAMP (0.2 mM; 1–1, 1.0 mM; 1–2), or the specific Epac agonist, 8-pCPT-2-*O*-Me-cAMP (0.2 mM; 2–1, 1.0 mM; 2–2), for 45 minutes. Cells were then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 24 hours. The MMP-1 mRNA levels in the cells (**a**) were analyzed by real-time PCR. Each level of MMP-1 mRNA was normalized versus that of the corresponding GAPDH mRNA. The amounts of MMP-1 protein released into culture media (**b**–**d**) were analyzed by western blotting. Data shown are representative of three independent experiments. Values represent the mean ± SD of data from three independent experiments. (**a**, **b**, **d**) *P<0.05, **P<0.01 versus TNF-α only, (**c**) *P<0.05, **P<0.01.

PKA but not Epac is critical for cAMP- mediated inhibition of MMP-1 expression

It has been reported that many biological activities of cAMP are mediated through the PKA pathway (Houslay and Milligan, 1997; Mayr *et al.*, 2001; Schmitt and Stork, 2002), even though recent studies have shown that some biological functions of cAMP can also be mediated by Epac (Borland *et al.*, 2009; Woolson *et al.*, 2009). To investigate whether the PKA pathway is specifically involved in cAMP-mediated MMP-1 suppression, cultured human skin fibroblasts were pretreated with a specific Akt inhibitor, Akt Inhibitor VIII, or a PKA inhibitor, H-89, for 1 hour, followed by treatment with forskolin for 45 minutes, and then stimulation with TNF-α for 24 hour. TNF-α-induced MMP-1 expression from the cells pretreated with Akt Inhibitor VIII or H-89 was slightly, but not significantly, increased, compared with that from cells pretreated with vehicle only. However, H-89, but not Akt Inhibitor VIII, antagonized cAMP-mediated suppression of MMP-1 expression, indicating that PKA activity specifically mediates the effect of cAMP on MMP-1 expression (Figure 1c). Furthermore, to confirm these results and to test whether another cAMP effector, Epac, is also involved in the MMP-1 suppression, human skin fibroblasts were pretreated with 6-Bnz-cAMP, a specific PKA agonist, or 8-pCPT-2′-O-Me-cAMP, a specific Epac agonist, for 30 minutes, and then treated with TNF-α for 24 hour. Only 6-Bnz-cAMP, but not 8-pCPT-2′-O-Me-cAMP, suppressed TNF-α-induced MMP-1 expression (Figure 1d). Taken together, these results indicate that the inhibition of MMP-1 expression by cAMP is mediated by PKA but not Epac.

Elevation of cAMP inhibits ERK and JNK activities, which have an important role in TNF-α-induced MMP-1 expression

It has been reported that the cAMP-signaling pathway can regulate MAPK either positively or negatively, depending on the stimulus and/or the cell type (Houslay and Kolch, 2000; Stork and Schmitt, 2002) and that ERK and JNK have a crucial role in the expression of MMP-1 induced by various extracellular stimuli including UV, heat shock, and phorbol 12-myristate 13-acetate (PMA) (Reunanen *et al.*, 1998, 2002; Westermarck *et al.*, 1998; Park *et al.*, 2004; Kim *et al.*, 2005). Therefore, we wondered whether TNF-α-induced ERK and JNK activities would also be affected by cAMP and, if so, whether they would have a role in MMP-1 expression in human skin fibroblasts. To test this possibility, we treated cultured fibroblasts with TNF-α, harvested the cells at various time points, and examined the activation of ERK and JNK by western blot analysis with antibodies against the phosphorylated forms of these MAPK. Our results showed that TNF-α induced the rapid and strong activation of ERK and JNK, as the phosphorylated forms of these two MAPK were detected and peaked at 15 minutes and then returned to their basal levels at 30 minutes (Figure 2a). Therefore, we pretreated cultured fibroblasts for 45 minutes with or without forskolin and then treated the cells with TNF-α for 15 minutes. TNF-α-stimulated ERK and JNK activation at 15 minutes were significantly attenuated by forskolin. However, previous treatment of cells with H-89, a PKA inhibitor, further increased TNF-α-stimulated ERK and JNK activation and

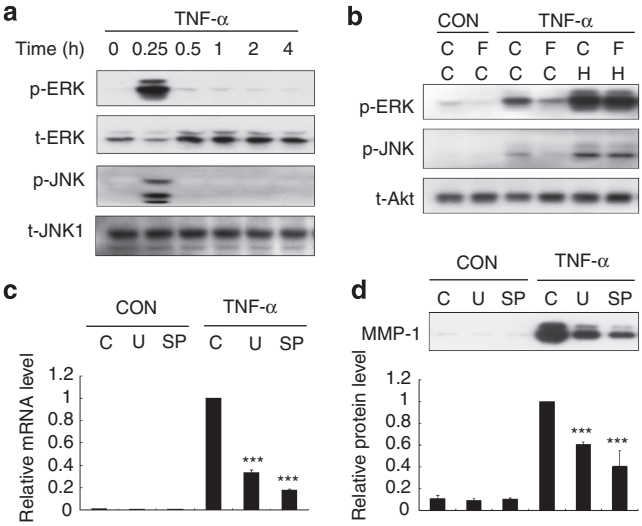

Figure 2. cAMP/PKA pathway inhibits ERK and JNK activities, which have important roles in TNF-α-induced MMP-1 expression. (**a**) Serum-starved primary human skin fibroblasts were treated with TNF-α (5 ng ml^{-1}) for the indicated times. Activation of ERK and JNK were analyzed in total cell lysates by western blotting using antibodies against phosphorylated forms of ERK and JNK, respectively. Total protein levels were analyzed with antibodies against ERK or JNK1. (**b**) Serum-starved primary human skin fibroblasts were pretreated first with DMSO only (C), or the PKA inhibitor, H-89 (10 μM; H), for 1 hour and then with DMSO (C) or forskolin (10 μM; F) for 45 minutes. Cells were then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 24 hours. Activation of ERK and JNK were analyzed in total cell lysates by western blotting using antibodies against phosphorylated forms of ERK and JNK, respectively. Total protein levels were analyzed with antibodies against Akt. (**c**, **d**) Serum-starved fibroblasts were pretreated with DMSO only (C), U0126, a MEK inhibitor (20 μM; U), or SP600125, a JNK inhibitor (20 μM; SP) for 1 hour and then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 24 hours. The MMP-1 mRNA levels in the cells (**c**) were analyzed by real-time PCR. Each level of MMP-1 mRNA was normalized versus that of the corresponding GAPDH mRNA. The amounts of MMP-1 protein released into culture media (**d**) were analyzed by western blotting. Data shown are representative of three independent experiments. Values represent the mean ± SD of data from three independent experiments. ***$P<0.001$ versus TNF-α only.

significantly abrogated the suppression of TNF-α-stimulated ERK and JNK activation by forskolin (Figure 2b), indicating that basal and forskolin-induced PKA activities have a role in suppressing the activation of ERK and JNK in human skin fibroblasts. We also investigated the role of ERK and JNK in TNF-α-induced MMP-1 expression. The inhibition of ERK and JNK by pretreatment with U0126, a MEK inhibitor, and SP600125, a JNK inhibitor, respectively, reduced the MMP-1 expression at both the mRNA and the protein levels (Figure 2c and d), confirming that ERK and JNK have an important role in TNF-α-induced MMP-1 expression in human skin fibroblasts. Taken together, our results suggest that cAMP/PKA pathway may suppress TNF-α-induced MMP-1 expression through the inhibition of ERK and JNK activities.

TNF-α-induced MMP-1 expression can be suppressed by cAMP, even when ERK and JNK activities are unaffected by it

Next, we were interested in whether the ERK and JNK pathways were the only targets through which cAMP

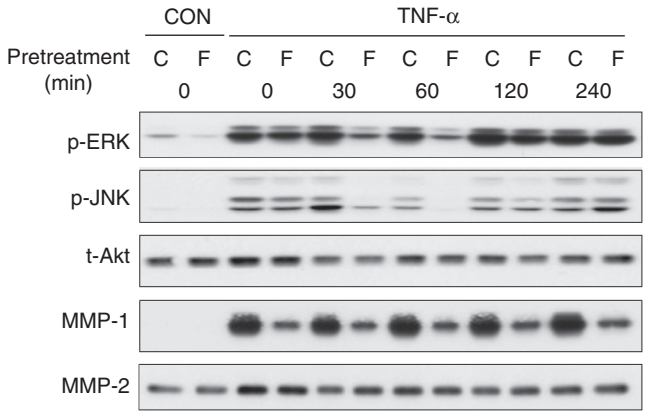

Figure 3. TNF-α-induced MMP-1 expression can be inhibited by cAMP-signaling pathway, even when ERK and JNK activities are unaffected by cAMP elevation. Serum-starved primary human skin fibroblasts were pretreated with DMSO only (C) or forskolin (10 μM; F) at the indicated times and then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 15 minutes or 24 hours. The protein levels of each kinase indicated above were analyzed in total cell lysates at 15 minutes, and the MMP-1 and MMP-2 protein levels in culture media at 24 hours were analyzed by western blotting. Data shown are representative of three independent experiments.

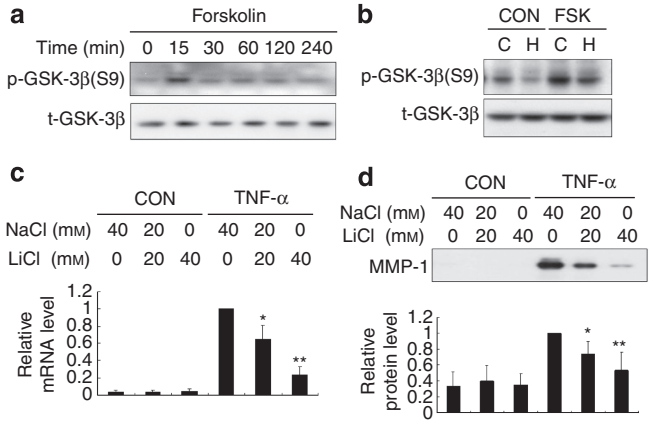

Figure 4. cAMP/PKA pathway induces the phosphorylation of GSK-3β at serine 9, and GSK-3 has an important role in TNF-α-induced MMP-1 expression. (**a**) Serum-starved primary human skin fibroblasts were treated with forskolin (10 μM) at the indicated times. (**b**) Serum-starved fibroblasts were pretreated with DMSO only (C) or H-89 (10 μM; H) for 1 hour and then treated with DMSO only (CON) or forskolin (10 μM; FSK) for 15 minutes. The protein levels of each kinase indicated above in total cell lysates were analyzed by western blotting. (**c**, **d**) Serum-starved primary human skin fibroblasts were pretreated with NaCl or LiCl at the indicated concentrations and then treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) for 24 hours. The MMP-1 mRNA levels in the cells (**c**) were analyzed by real-time PCR. Each level of MMP-1 mRNA was normalized versus that of the corresponding GAPDH mRNA. The amounts of MMP-1 protein released into culture media (**d**) were analyzed by western blotting. Data shown are representative of three independent experiments. Values represent the mean ± SD of data from three independent experiments. *$P<0.05$, **$P<0.01$ versus TNF-α only.

regulated MMP-1 expression. Therefore, we pretreated cultured fibroblasts with forskolin at various time points, treated with TNF-α, and measured the activities of ERK and JNK in total cell lysates at 15 minutes and the MMP-1 protein levels in culture media at 24 hours post-treatment. TNF-α-induced ERK and JNK activation were attenuated when cells were pretreated with forskolin for 30 or 60 minutes but not at 0, 120, or 240 minutes before TNF-α treatment (Figure 3), indicating that the inhibition of TNF-α-stimulated ERK and JNK activation by cAMP elevation requires pretreatment of the cells with forskolin within a specific time frame before TNF-α treatment. In contrast, TNF-α-induced MMP-1 expression was inhibited by cAMP at all time points (Figure 3), even when TNF-α-induced ERK and JNK activation were unaffected by cAMP. These results indicate that there might be other target(s) that mediate the suppression of MMP-1 expression by cAMP.

cAMP/PKA pathway inhibits GSK-3β activity, which has a critical role in MMP-1 expression

GSK-3β has been shown to regulate many transcription factors, including p53, β-catenin, and NF-κB, and the expression of various genes (Beurel and Jope, 2006; Rocques *et al.*, 2007). In addition, studies have reported that PKA and PKC, in addition to PKB/Akt, can phosphorylate GSK-3β at serine 9 in some cell lines, leading to the inactivation of GSK-3β activity (Goode *et al.*, 1992; Fang *et al.*, 2000). Therefore, we were interested in whether cAMP could also phosphorylate GSK-3β at serine 9 in human skin fibroblasts. To test this, we treated cultured cells with forskolin, harvested the cells at various time points (from 0 to 240 minutes), and checked the phosphorylation by western blot analysis. Phosphorylation of GSK-3β at serine 9 was increased by forskolin at 15 minutes and then reduced to the basal level at 30 minutes post-treatment (Figure 4a). However, the phosphorylation of GSK-3β at serine 9 by cAMP was significantly reduced by pretreatment with H-89, indicating that cAMP phosphorylates GSK-3β at serine 9 via PKA in human skin fibroblasts (Figure 4b). We then investigated the role of GSK-3β in MMP-1 expression. First, cultured fibroblasts were pretreated with NaCl (as a control) or LiCl (as a well-known GSK-3 inhibitor) at different concentrations and treated with TNF-α; the levels of MMP-1 mRNA in cells and protein in culture media were measured at 24 hours post-treatment. LiCl but not NaCl reduced TNF-α-induced MMP-1 expression at both the mRNA and the protein levels in a dose-dependent manner (Figure 4c and d). In addition, pretreatment of the cells with two structurally different GSK-3 inhibitors, AR-A014418 and SB415286, also inhibited TNF-α-induced MMP-1 protein expression in a dose-dependent manner (See online Supplementary Figure S2a and b), indicating that GSK-3β has a crucial role in MMP-1 expression in human skin fibroblasts. Taken together, our results indicate that activation of the cAMP/PKA pathway can immediately inactivate GSK-3β, which, in this study is shown to have a critical role in MMP-1 expression in human skin fibroblasts.

TNF-α-induced MMP-1 expression can be suppressed by activation of cAMP pathway or inhibition of GSK-3 activity several hours after TNF-α treatment

We further investigated the effects of cAMP pathway and GSK-3 activity on MMP-1 expression but focused on their

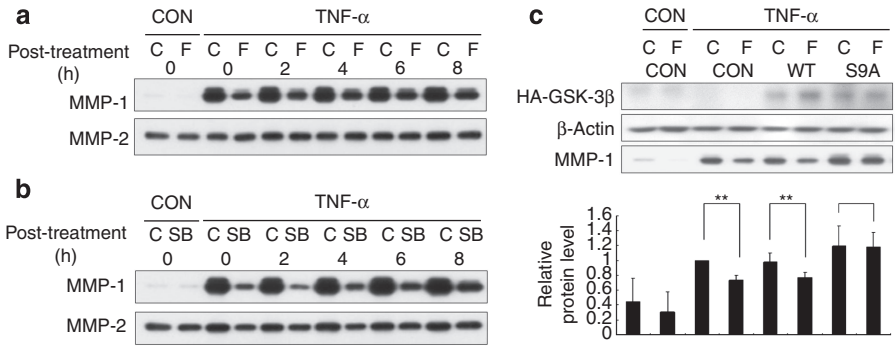

Figure 5. Suppression of MMP-1 expression by elevation of cAMP after TNF-α treatment is mediated through inhibition of GSK-3β. (**a**, **b**) Serum-starved primary human skin fibroblasts were treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α) followed by treatment with DMSO only (C) or forskolin (10 μM; F) or SB415286 (50 μM; SB) at the indicated times. The MMP-1 and MMP-2 protein levels in culture media at 24 hours after TNF-α treatment were analyzed by western blotting. (**c**) Primary skin fibroblasts were transfected with a control plasmid (CON) or the plasmids for expressing the wild-type (WT) or a constitutively active form (S9A) of GSK-3β for 24 hours. Then the transfected cells were serum-starved for 24 hours and treated with vehicle only (CON) or TNF-α (5 ng ml^{-1}; TNF-α), followed by treatment with DMSO only (C) or forskolin (10 μM; F) at 4 hours post-treatment. The protein levels of HA-GSK-3β WT, HA-GSK-3β S9A, or β-actin in total cell lysates and the MMP-1 protein levels in culture media at 24 hours after TNF-α treatment were analyzed by western blotting. Data shown are representative of three independent experiments. Values represent the mean ± SD of data from three independent experiments. $^{**}P<0.01$.

effects on MMP-1 expression after the activation of ERK and JNK by TNF-α had already occurred. To do this, we first pretreated the cells with TNF-α and then treated with or without forskolin (Figure 5a) or SB415286 (Figure 5b) immediately (at 0 hour) and at 2, 4, 6, and 8 hours, respectively. The MMP-1 protein levels in culture media were measured at 24 hours post-TNF-α treatment. Our results showed that TNF-α-induced MMP-1 expression was inhibited by either activation of cAMP pathway or inhibition of GSK-3 activity, even several hours after TNF-α treatment (Figure 5a and b). These results suggest that GSK-3 activity and/or other factors that may be regulated by cAMP/PKA pathway are required at least up to 8 hours post-treatment for efficient induction of MMP-1 by TNF-α in human skin fibroblasts.

cAMP-mediated, but ERK- and JNK-independent, suppression of MMP-1 expression is mediated through inhibition of GSK-3β

To examine whether cAMP-mediated but ERK- and JNK-independent suppression of MMP-1 expression was mainly mediated by inhibition of GSK-3β, we transiently transfected human skin fibroblasts with the control vector only or the vector containing either the wild-type or a constitutively active form of GSK-3β for 24 hours. The transfected cells were serum-starved for 24 hours, treated with forskolin at 4 hours after TNF-α addition, and further incubated for 24 hours. The GSK-3β protein levels in total cell lysates and the MMP-1 protein levels in culture media were measured by western blot analysis. The wild-type and constitutively active form of GSK-3β were equally or similarly expressed (Figure 5c). However, the suppression of TNF-α-induced MMP-1 expression by cAMP was significantly abrogated by overexpression of a constitutive active form, but not the wild-type, of GSK-3β. These results indicate that suppression of TNF-α-induced MMP-1 expression by cAMP elevation after TNF-α treatment is mediated by inhibition of GSK-3β, independently of the regulation of ERK and JNK pathways.

Activation of cAMP pathway also suppresses the expression of MMP-1 induced by IL-1β and EGF in human skin fibroblasts

Next, we investigated whether the expression of MMP-1 induced by other extracellular stimuli could be affected by cAMP pathway in human skin fibroblasts. We pretreated cultured fibroblasts with forskolin or IBMX for 1 hour before treatment with IL-1β or EGF, or treated the cells with forskolin at 4 hours after addition of IL-1β or EGF. The amounts of MMP-1 protein in culture media were measured at 24 hour after treatment with IL-1β or EGF by western blot analysis. As shown in Supplementary Figure S3, the expressions of MMP-1 induced by IL-1β or EGF were significantly suppressed by pretreatment or post-treatment with forskolin or IBMX in human skin fibroblasts, indicating that the cAMP pathway can regulate the expression of MMP-1 induced by various stimuli, including TNF-α, in human skin fibroblasts.

DISCUSSION

MMP-1 is known to have an important role in matrix remodeling in many physiological and pathological processes and its expression is regulated by diverse signaling pathways. Even though it has been reported that elevation of cAMP can repress MMP-1 expression (Salvatori *et al.*, 1992), the molecular mechanisms involved in this process have not been elucidated. In this study, we investigated the effect of cAMP elevation on MMP-1 expression in more detail and the underlying mechanisms involved in this process in human skin cells. Our studies showed that the expression of MMP-1 induced by TNF-α is suppressed by several cAMP-elevating agents such as forskolin, IBMX, and dibutyryl cAMP and that the suppression of MMP-1 expression by cAMP is mediated by PKA but not Epac. Many studies have shown that the transcription factor AP-1 has an important role in the expression of MMP-1 induced by diverse stimuli including UV and PMA (Gutman and Wasylyk, 1990; Rittie and Fisher, 2002). The AP-1 transcription factor is composed of homodimeric or heterodimeric complexes containing c-Fos

and c-Jun family members whose activities are regulated by ERK and JNK (Karin, 1995). The ERK pathway increases the expression of these AP-1 components. JNK phosphorylates c-Jun at serine 63 and serine 73, which stabilizes c-Jun protein and increases its transcriptional activity (Karin, 1995; Karin and Hunter, 1995; Davis, 2000). It has been reported that the cAMP/PKA pathway regulates ERK and JNK pathways in some types of cells (Schmitt and Stork, 2002; Zhang *et al.*, 2008). In agreement with these reports, we found that cAMP elevation also suppresses TNF-α-induced ERK and JNK activation in human skin fibroblasts. In addition, our results showed that ERK and JNK also have a critical role in TNF-α-induced MMP-1 expression. Therefore, these results indicate that the inhibition of MMP-1 expression by the cAMP/PKA pathway may be mediated, at least in part, by suppression of ERK and JNK in human skin fibroblasts.

Although ERK and JNK have a critical role in MMP-1 expression, there are obviously other signaling pathways that regulate MMP-1 expression (Jinnin *et al.*, 2005; Faour *et al.*, 2006). Therefore, we wondered whether ERK and JNK were the only targets that mediate the suppression of MMP-1 expression by cAMP/PKA. Our results showed that cAMP elevation could inhibit TNF-α-induced MMP-1 expression, even when TNF-α-induced ERK and JNK activation were not inhibited by cAMP, such as when forskolin was treated immediately before or after TNF-α addition, or even at several hours after TNF-α treatment. This led us to assume that there might be other target(s) that is responsible for suppression of MMP-1 expression by cAMP. It has been shown that the cAMP/PKA pathway can regulate many signaling molecules or transcription factors either directly by phosphorylation or indirectly through synthesis of new proteins (Stork and Schmitt, 2002; Feliciello *et al.*, 2005; Zhang *et al.*, 2005). Among them, we focused on GSK-3β, which had been shown to be phosphorylated at serine 9 and therefore inactivated by PKA (Li *et al.*, 2000; Torii *et al.*, 2008). Indeed, we found that GSK-3β could be phosphorylated at serine 9 via the PKA pathway within 15 minutes after forskolin treatment. In addition, we also found that GSK-3β has a critical role in TNF-α-induced MMP-1 expression in human skin fibroblasts. Then we showed that cAMP-mediated suppression of TNF-α-induced MMP-1 expression is significantly attenuated by overexpression of a constitutively active form, but not the wild-type, of GSK-3β, when forskolin is added at 4 hours after TNF-α treatment. These results indicate that the suppression of MMP-1 expression by cAMP elevation, especially after TNF-α treatment, is mainly mediated by inhibition of GSK-3β activity via the PKA pathway in human skin fibroblasts.

Intracellular cAMP levels can be increased by diverse stimuli including hormones, growth factors, and neurotransmitters through G-protein (Gs)-coupled membrane receptors (Sunahara and Taussig, 2002). In addition, prostaglandin E2 (PGE2) has been reported to increase intracellular cAMP when it binds to two subtypes of its receptors, EP2 and EP4 (Sugimoto and Narumiya, 2007). The PGE2 levels can be increased by various extracellular stimuli such as TNF-α, IL-1β, and UV via induction of cyclooxygenase-2 (COX-2), which has an important role in the generation of various PGs including PGE2 from arachidonic acid (Brenneisen *et al.*, 1999; Smith and Langenbach, 2001). Therefore, considering that cAMP elevation can inhibit MMP-1 expression several hours after treatment with extracellular stimuli, it is possible that the increase of intracellular cAMP levels by COX-2 and PGE2, whose induction precedes MMP-1 expression (Chen *et al.*, 2001; Lee *et al.*, 2009), may negatively regulate MMP-1 expression. In fact, it has been recently reported that TNF-α increases PGE2 via COX-2 and that PEG2 inhibits TNF-α-induced MMP-1 expression in a cAMP-dependent manner, in synovial fibroblasts (Kunisch *et al.*, 2009). We have also found that intracellular cAMP levels are increased by TNF-α in human skin fibroblasts (See online Supplementary Figure S4). Further studies are ongoing for understanding the underlying mechanisms by which TNF-α regulates intracellular cAMP levels and the role of the increased cAMP in the expression of various genes regulated by TNF-α, including MMP-1, in human skin fibroblasts.

In this study, we have revealed that GSK-3β activity is required for the MMP-1 expression induced by TNF-α. To our knowledge this is previously unreported. Furthermore, we have shown that GSK-3β activity is critical for MMP-1 induction, even 8 hours after TNF-α treatment. In fact, the steady-state levels of MMP-1 mRNA seem to increase gradually and continuously over 24 hours after TNF-α treatment (data not shown). Therefore, our results suggest that GSK-3β activity is necessary for continuous increase of MMP-1 mRNA levels over several hours upon stimulation with extracellular stimuli such as TNF-α in human skin fibroblasts. Many studies have shown that GSK-3β regulates various transcription factors such as c-Jun and STAT (de Groot *et al.*, 1993; Beurel and Jope, 2008). It has been also reported that NF-κB-dependent gene expressions are differentially regulated by GSK-3β (Steinbrecher *et al.*, 2005). However, at this moment, we do not know how GSK-3β regulates MMP-1 expression. Further work is required to elucidate the molecular mechanisms of GSK-3β-mediated regulation of MMP-1 expression.

In summary, our present studies elucidate the molecular mechanisms by which cAMP regulates the MMP-1 expression induced by extracellular stimuli including TNF-α and show that GSK-3β has an important role in MMP-1 expression in human skin fibroblasts. Increased MMP-1 expression has been shown to be associated with matrix degradation in skin aging and skin carcinogenesis (Airola and Fusenig, 2001; Brenneisen *et al.*, 2002; Rittie and Fisher, 2002). Therefore, our results may help in development of clinical strategies to control skin diseases related with deregulated matrix metabolism.

MATERIALS AND METHODS

Materials

A control plasmid (pDNA3–EGFP) was obtained from Invitrogen (Carlsbad, CA). Expression plasmids for HA-GSK-3β WT and S9A were kindly provided by Dr JW Woodgett.

Cell culture and treatments

Primary human foreskin fibroblasts were established and cultured as described previously (Park *et al.*, 2004). For treatment, primary

human skin fibroblasts were serum-starved for 24–48 hours in DMEM containing 0.25% FBS. The medical ethical committee of Seoul National University approved all described studies, and the participants gave their written informed consent. The study was conducted according to Declaration of Helsinki Principles.

Transfection

Primary human skin fibroblasts were transiently transfected using FuGENE HD (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol.

RNA analysis

Total RNA and the first-strand cDNA were prepared as described previously (Park *et al.*, 2004; Li *et al.*, 2007). Quantitative real-time PCR was performed using an Applied Biosystems 7500 Real-time PCR system (Applied Biosystems, Foster City, CA) and SYBR *Premix Ex Taq* (Perfect Real Time) (Takara Bio, Shiga, Japan) using the following primers for human genes: GAPDH (forward, 5′-CAATGA CCCCTTCATTGACC-3′; reverse, 5′-AAATGAGCCCCAGCCTTCT-3′), MMP-1 (forward, 5′-AAGCGTGTGACAGTAAGCTA-3′; reverse, 5′-AACCGGACTTCATCTCTG-3′).

Western blot analysis

The amounts of MMP-1 and MMP-2 proteins secreted into culture media were analyzed as described previously (Park *et al.*, 2004; Li *et al.*, 2007). In contrast to that of MMP-1, the expression of MMP-2 was not significantly (if any, only very slightly) affected by most of the agents used in this study, including TNF-α, the cAMP-elevating agent, forskolin, and the GSK-3 inhibitor, SB415286, as shown in Figures 3 and 5. Therefore, we used MMP-2 as a loading control for MMP-1 released into culture media (data not shown). The proteins in cells were analyzed using total cell lysates as described previously (Park *et al.*, 2004). Antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and all other antibodies were from Cell Signaling Technology (Beverly, MA).

Statistics

Statistical significance was determined using the Student's *t*-test. Results were presented as mean ± SD. A *P*-value of <0.05 was considered to indicate statistical significance.

Full Materials and Methods and any associated references are available in Supplementary Information.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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