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Spike protein of SARS-CoV stimulates cyclooxygenase-2 expression *via* both calcium-dependent and calcium-independent protein kinase C pathways

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ABSTRACT **We have previously shown that the nucleocapsid protein of SARS-associated coronavirus (SARS-CoV) activated cyclooxygenase-2 (COX-2) expression (1). In this study, we identified another viral protein, the spike of SARS-CoV, which played an important role in virus-stimulated COX-2 expression after screening all genes from the SARS-CoV genome. We found that an upstream calcium-dependent PKC isozyme PKCα that modulates the downstream ERK/NF-κB pathway through an influx of extracellular Ca^{2+} is induced by the spike protein of SARS-CoV. The ERK/NF-κB was identified to be involved in the activation of COX-2 promoter and production of COX-2 protein in HEK293T cells. We also demonstrated that another unusual pathway, the calcium-independent PI3K/PKCε/JNK/CREB pathway, functioned in cooperation with the calcium-dependent pathway to induce COX-2 expression upon stimulation by spike protein. This pathway can be blocked by PKCε-specific, small interfering RNA, PI3K/JNK kinase-specific inhibitors as well as dominant negative JNK. PKCε-specific siRNA also attenuated the phosphorylation of JNK. Our results provide evidence that helps us understand the function of SRAS-CoV spike protein in SARS pathogenesis.—Liu, M., Yang, Y., Gu, C., Yue, Y., Wu, K. K., Wu, J., Zhu, Y. Spike protein of SARS-CoV stimulates cyclooxygenase-2 expression *via* both calcium-dependent and calcium-independent protein kinase C pathways. *FASEB J.* 21, 1586–1596 (2007)**

Key Words: COX · SARS-CoV · spike protein · transcriptional activation

SEVERE ACUTE RESPIRATORY SYNDROME-associated coronavirus (SARS-CoV) is the etiological agent for the outbreak of severe acute respiratory syndrome (SARS) (2). SARS-CoV is a member of the coronaviridae family of enveloped, positive-stranded RNA viruses containing genes encoding polymerase (polymerase-related proteins), spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins (3). Phylogenetic analysis indicated that the new virus is not related to known groups 1, 2, and 3 of coronaviruses, but represents a fourth group, group 4 in the genus coronavirus (4). Based on available information about other coronaviruses, the spike glycoprotein is the main virulence factor of SARS-CoV, with multiple functions involved in specific receptor binding, cell membrane fusion, and protease susceptibility (5). SARS clinically consists of an incubation phase and respiratory phase. In these two phases, acute inflammatory reaction is the most obvious symptom (6), characterized by the invasion of activated leukocytes into the injured tissue; its process is critically dependent on the rapid expression of proinflammatory genes (7).

Cyclooxygenase (COX) is a rate-limiting enzyme in the biosynthetic pathway of prostaglandins and thromboxanes from arachidonic acid. Prostaglandins play important roles in many biological processes. Altered prostanoid production is associated with a variety of illnesses, including acute and chronic inflammation, cardiovascular disease, and colon cancer (8). Two isoforms of COX were described: COX-1 and COX-2. COX-1 is constitutively expressed in almost all tissues (9); COX-2 is the inducible form of the enzyme, which is expressed in response to inflammatory and other physiological stimuli and growth factors and is involved in the production of those prostaglandins that mediate pain and support the inflammatory process (10). As an immediate early response gene, the promoter region of COX-2 contains a classical TATA box, an E-box, and a series of binding sites for transcription factors such as nuclear factor κB (NF-κB), NFAT/AP-1, nuclear factor IL-6/CCAAT enhancer binding protein (C/EBP), and cyclic AMP response element binding proteins (CREB), which regulate COX-2 gene expression in transcriptional and post-transcriptional levels (11). It has been shown that several types of viral proteins such as LMP-1 of Epstein-Barr virus (EBV), gp120 of HIV, and HBx of

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human hepatitis virus (HBV) are capable of stimulating COX-2 through a wide range of cellular signal transduction cascades, including Ras-Raf-mitogen-activated protein kinase, c-Jun N-terminal kinase, NF-κB, and JAK1-STAT signaling pathways (12–13).

In our previous study, we identified two viral proteins—the nucleoprotein and spike from SARS-CoV—whose expression was involved in up-regulating COX-2 after screening all genes from SARS-CoV genome. In this study, we focused on the spike and delineated the molecular mechanism by which the spike activated COX-2 promoter and protein production. We postulated that spike protein (S protein) of SARS-CoV might be subjected to signaling effects in human cells in addition to cell infection and viral replication. There is a precedent for such "innocent bystander" effects, whereby viral envelope proteins can induce cell dysfunction and death by triggering specific signaling pathways (14). Because COX-2 is normally induced during inflammatory processes and SARS-CoV induces fever, edema, and diffuse alveolar damage in severely affected individuals, we investigated the roles of SARS-CoV S protein in regulating the expression of COX-2 and the signaling pathways through which it induces COX-2 transcriptional activation.

MATERIALS AND METHODS

Materials

Antiflag antibody, SB203580, PD98059, LY294002, SP600125, GF109203, EGTA, DTT, fura-2/acetoxymethyl ester (AM), and pluronic-F127 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the protein kinase inhibitors were dissolved in Me2SO and used at a final concentration of 50 μM for PD98059, 10 μM for SB203580, 50 μM for GF109203, 30 μM for SP600125, and 25 μM for LY294002. Antibody against COX-2 was from Cayman Chemical Company (Ann Arbor, MI, USA). Antibodies specific for ERK, phospho-ERK, JNK, and phospho-JNK were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

Human embryo kidney cell line (HEK293T), African green monkey kidney cell line (COS-7), and human airway epithelial cell line (A549) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator.

Plasmid construct

The luciferase reporter vector (pGL3) containing a COX-2 promoter region (−891/+9), its truncation mutants, or site-specific mutants was reported earlier (15). A spike construct was created by RT-polymerase chain reaction (RT-PCR) amplification of the spike open reading frame from SARS-CoV (WHU), a viral strain isolated from a SARS patient in China in 2003 (16). To create S-encoding vectors, the spike gene was amplified using the following primers: 5′-AGCTGGATCCACATGTTTATTTTCTTATTATTTCTTAC-3′(sense) 5′-AGCTGAATTCACTGGCTGTGCAGTAATTGATCC-3′(antisense), in which *Eco*RI and *Bam*HI sites were introduced, respectively. The polymerase chain reaction (PCR) product was cloned into *Eco*RI and *Bam*HI sites of pCMV-tag2B to generate plasmid pCMV-S, in which S was tagged by FLAG. The resulting construct was confirmed by DNA sequencing. Mutants of ERK1 and ERK2 were gifts from Dr. Cobb (University of Texas Southwestern Medical Center) and mutants of JNK were gifts from Dr. Karin (University of California at San Diego, San Diego, CA, USA). PKC RNAi vectors were constructed by ligating the corresponding pairs of oligonucleotide to pSilencer 2.0 (Ambion, Inc., Augstin, TX, USA) based on the sequences described by Storz *et al.* (17).

Luciferase assay

HEK293T cells were cotransfected with reporter plasmids and each protein expression plasmid. Cells were lysed with luciferase cell culture lysis reagent (Promega, Madison, WI, USA). Thirty microliters of the cell lysates and 100 μl of luciferase assay substrate (Promega) were mixed and light intensity was detected by the luminometer (Turner T20/20). Assays were performed in triplicate and expressed as means ± SD relative to vector control as 100%.

Western blot analysis

Whole-cell lysates were prepared by lysing HEK293T with PBS pH7.4 containing 0.01% Triton-100, 0.01% EDTA, and 10% cocktail protease inhibitor (Roche). The cytosolic and nuclear protein fractions were separated as follows. Cells were washed with ice-cold PBS, collected by centrifugation, and pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM DTT, 10% protease cocktail inhibitor) for 15 min on ice and vortexed for 10 s. Nuclei were pelleted by centrifugation at 13,000 rpm for 1 min. Supernatants containing cytosolic proteins were collected. Lysates were centrifuged at 13,000 rpm for 10 min. The supernatants were boiled for 5 min with equal volumes of 2 × gel loading buffer. One hundred micrograms of cultured cell lysates were electrophoresed in 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. Nonspecific IgGs were blocked with 5% nonfat dried milk before being incubated with an antibody used in this study. Protein bands were detected using SuperSignal Chemiluminescent (Pierce, Rockford, IL, USA).

Electrophoretic mobility shift assay (EMSA)

Cell nuclear extracts were prepared from HEK293T cells according to the method described previously (15). NF-κB oligonucleotides and CRE oligonucleotides were synthesized by Takara (Takara Biotech Co. Ltd., Shiga, Japan) based on the cognate human COX-2 promoter sequence: wild-type NF-κB: 5′-CAGTCATTT*CGTCA*CATG-3′, mutant type NF-κB: 5′-CAGTCATTT*GAGCT*CATG-3′, wild-type CRE: 5′-GAGGTGA*GGGG*ATTCCCTTAG-3′, mutant type CRE: 5′-GAGGTGA*ATTC*ATTCCCTTAG-3′.

The complementary oligonucleotides were annealed and purified according to the manufacturer's protocol. Each probe was end-labeled with [γ^{32}P] ATP using T4 polynucleotide kinase (Takara Biotech). EMSA was performed by incubating 2 μg of nuclear extract with a labeled probe (15,000 cpm; ~10 fmol) in binding buffer (4 mM Tris-HCl, 12 mM HEPES-KOH, pH7.9, 60 mM KCl, 12% glycerol, 0.5 mM EDTA, and 1 mM DTT) containing 1 μg of poly(dI-dC) for 25 min at room temperature. To assure the specific binding of transcription factors to the probe, the probe was chased by 100-fold molar excess of cold wild-type or mutant

oligonucleotide. Samples were electrophoresed on 5% nondenaturing polyacrylamide gel with running buffer (×0.25 TBE containing 22.5 mM Tris borate, 0.5 mM EDTA), and the gels were dried and subjected to autoradiography.

Transient transfection

Cells were seeded onto 24-well plates at a density of 1.0×10^5 or 4.0×10^5 cells per 24-well plate or 6-well plate and grown to the confluence reaching ~80% at the time of transfection. Cells were transfected with 0.1 μg or 0.4 μg of plasmid pCMV-S together with 0.45 μg or 1.2 μg plasmid containing luciferase reporter, which was under the control of COX-2 promoter (COX-2-Luc) using Sofast^{TM} transfection reagent (Xiamen Sunma Biotechnology Co. Ltd., China) according to the protocol provided by the manufacturer. In all cotransfection experiments, appropriate vector DNA was used to ensure similar DNA concentrations in all conditions. After 24 h, cells were serum-starved for 24 h before being harvested.

Semiquantitative RT-PCR analysis

The semiquantitative RT-PCR analysis was performed to determine COX-2 mRNA levels. Total RNA was isolated using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cellular RNA samples were reverse-transcribed with a random primer, then amplified by PCR; β-actin primer set was used as an internal control. The primers and reaction conditions were shown as β-actin, 5′-ATGATATCGCCGCGCTCG-3′ and 5′-CGCTCGGTGAGGATCTTCA-3′, COX-2, 5′-TACAATGCTGACTATGGCTAC-3′ and 5′-ACTGATGCGTGAAGTGCTG-3′. PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide.

Measurement of $[Ca^{2+}]_i$

Intracellular calcium was measured using Fura-2. Cells (2×10^4/dish) were transfected with pCMV-flag2B or pCMV-S plasmids. After 24 h, Cells were loaded with Fura-2 by incubation in a solution of 5 μM Fura-2 AM and Pluronic-F127 (0.05%) in D-Hanks buffer (140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml glucose, 0.44 mM KH_2PO_4, 0.37 mM Na_2HPO_4, 20 mM HEPES, pH7.4) at 37°C for 45 min. Fura-2 fluorescence was excited alternately (2 Hz) at 340 nm and 380 nm using a Cairn monochromator (Faversham, Kent, UK) and measured at 510 ± 20 nm using a photomultiplier tube. $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence intensity (r=F340/380) using the formula described by Grynkiewicz *et al.* (18): $[Ca^{2+}]_i = K_d\,[(R\text{-}R_{min})/(R_{max}\text{-}R)](F_{min}/F_{max})$, where with 0.1% aqueous Triton X-100 and 3 mM EGTA, respectively, F_{max} and F_{min} are values of F380 after treatment with Triton X-100 and EGTA, respectively, and K_d = 244 nM.

Statistical analysis

All experiments were reproducible and were carried out in duplicate or quadruplicate. Each set of experiments was repeated at least three times with similar results, and a representative one is shown. The results are presented at the means ±SD. Student's t test for paired samples was used to determine statistical significance. Differences were considered statistically significant at a value of $P \leq 0.05$.

RESULTS

Spike protein of SARS-CoV activated COX-2 expression in a protein concentration-dependent manner

Vero E6 cells were infected by SARS-CoV (WHU); 48 h after infection, total proteins were isolated and examined by Western blot with antibody to COX-2 (**Fig. 1*A***). Results indicated that COX-2 expression levels were significantly increased by SARS-CoV infection (lane 2) or by PMA, a known COX-2 inducer, as positive control (lane 3). Mammalian cells were cotransfected with plasmid pCMV-S and a reporter plasmid carrying the luciferase reporter gene under the control of the COX-2 gene promoter. Results revealed that S protein stimulated the activity of COX-2 promoter in HEK293T, COS-7, and A549 cell lines (Fig. 1*B*). Activation of the COX-2 promoter was in an S protein concentration-dependent manner, because the level of luciferase activity increased as the concentration of plasmid carrying the S gene increased in all three cell types (Fig. 1*B*).

COX-2 mRNA was analyzed using semiquantitative RT-PCR. Results showed that the level of COX-2 mRNA increased in the presence of S protein or 100 nM PMA (Fig. 1*C*).

The expression of COX-2 protein regulated by S protein was determined by Western blot analysis using antibodies against COX-2 and Flag-tagged S protein. Results from Western blot analysis revealed a basal level of COX-2 protein detected in HEK293T cells (Fig. 1*D*, lane 2); the level of COX-2 increased significantly in the presence of SARS-CoV S protein (Fig. 1*D*, lane 1) or in the presence of PMA (Fig. 1*D*, lane 3). These results demonstrated that the S protein of SARS-CoV activated the expression of COX-2 protein in mammalian cells.

NF-κB and CREB recognition sites were involved in the induction of COX-2 by the S protein

Activation of COX-2 gene relies on many consensus *cis*-elements, including NF-κB and CREB binding sites in the COX-2 promoter (**Fig. 2*A***). As S protein activates the expression of COX-2, we investigated the roles of these *cis*-regulatory elements in regulating COX-2 by S protein. Two binding sites for NF-κB and CRE were identified in the promoter of COX-2 (19).

To define the role of NF-κB and CRE recognition sites in activating COX-2 by S protein, two NF-κB binding site mutants (Mut1 and Mut2) and one CRE binding site mutant (Mut3) were generated by site-specific mutagenesis (Fig. 2*A*). Reporter plasmids were then constructed in which the luciferase gene was under the control of COX-2 WT, Mut1, Mut2, and Mut3 promoters, respectively. 293T cells were cotransfected with pCMV-S and plasmids containing the luciferase reporter gene under the control of different COX-2 promoters. Results from luciferase assays showed that Mut1 and Mut3 reduced COX-2 promoter

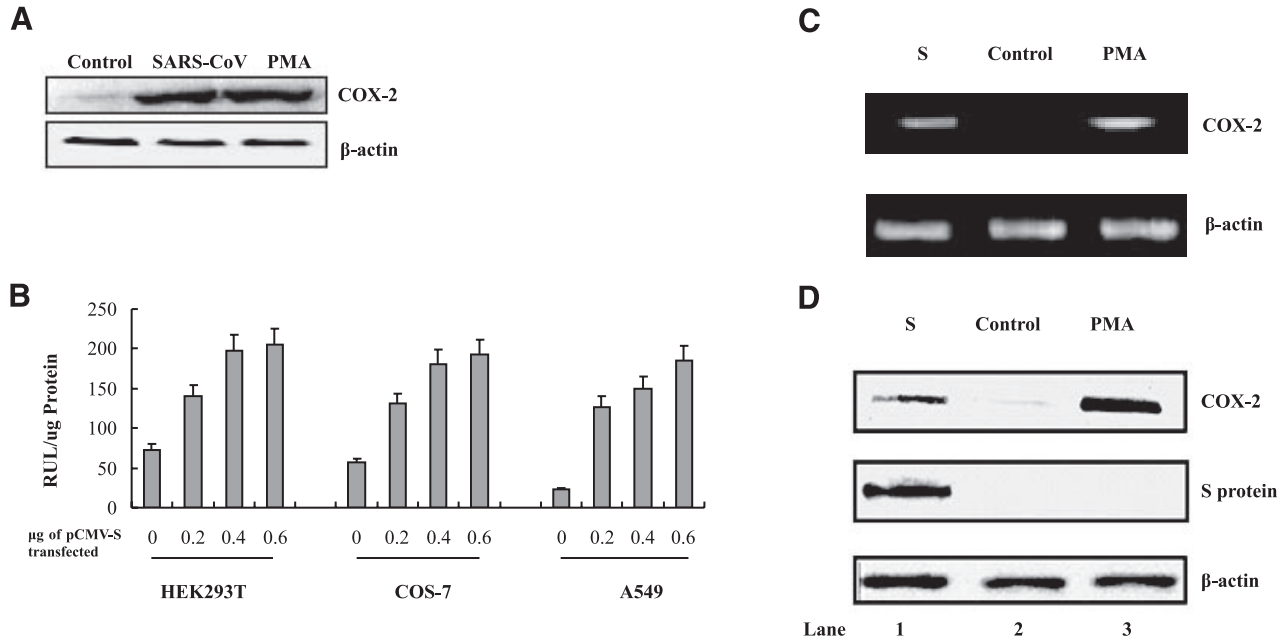

Figure 1. Effects of SARS-CoV S protein on COX-2 promoter and protein production. *A)* SARS-CoV-infected Vero-E6 cells were lysed and COX-2 protein was analyzed by Western blot. The multiplicity of infection (MOI) is 10. *B)* HEK293T, COS-7 or A549 cells were cotransfected with reporter plasmid containing the luciferase under the control of COX-2 promoter (COX-2-Luc) and pCMV-S, the spike-encoding plasmid. Luciferase activity was measured as described in Materials and Methods. Results are expressed as the mean ± SD of three independent experiments performed in triplicate. *C)* Total RNA was extracted from S-transfected or PMA-treated HEK293T cells, COX-2 mRNA was analyzed by RT-PCR. *D)* S-transfected or PMA-treated HEK293T cells were lysed. COX-2 and S proteins were analyzed by Western blot. Similar results were obtained in three independent experiments.

activity to the basal level whereas Mut2 partially reduced COX-2 activity. (Fig. 2*B*). These results indicated that both NF-κB sites, especially the proximal site, and the CRE recognition site were required for the activation of COX-2 expression by S protein.

S protein-induced COX-2 expression required NF-κB and CREB activation

NF-κB is a ubiquitously expressed transcription factor that regulates the induction of genes involved in immune and inflammatory cell function. NF-κB is composed of homo- and heterodimers of Rel family members, typically p65:p50 heterodimers, which are held in the cytosol by the inhibitory IκB proteins. NF-κB activation results from the phosphorylation of IκB, which targets it for ubiquitylation and degradation by the 26S proteasome. After the destruction of IκB, NF-κB can then translocate to the nucleus to activate target genes. We examined the effect of S protein in the translocation of p50, a subunit of NF-κB, from cytosol to nucleus. Results from Western bolt analysis using antip50 antibody revealed that the level of p50 protein was decreased in cytosol and increased in nucleus at 48 h after S protein stimulation (**Fig. 3**).

ERK, JNK, PI3K, and PKC but not P38 MAP kinase were involved in S protein-induced COX-2 activation

It is well known that members of the MAP kinase family regulate several transcription factors that are important in the proinflammatory response (20). Regulation of COX-2 expression was shown to depend on different kinase activators in various cell types (21–24). Based on our findings that S protein activates COX-2 expression and that such activation was dependent on NF-κB and CREB, we investigated the roles of kinases on the activation of COX-2 by S protein and the molecular mechanisms underlying this event.

Cells transfected with pCMV-S and the reporter plasmid COX-2-Luc were treated with PD98059 (ERK-specific inhibitor), SB203580 (P38 MAP kinase-specific inhibitor), SP600125 (JNK-specific inhibitor), LY294002 (PI3K-specific inhibitor), and GF109203 (PKC-specific inhibitor), respectively. Results from luciferase activity assays showed that the level of COX-2 promoter activity was significantly decreased in the presence of PD98059, SP600125, LY294002, GF109203, but was not affected by treatment of SB203580 (**Fig. 4*A***). The kinase inhibitors did not affect the expression of S protein significantly in the inhibitor-treated cells. Western blot analysis revealed a corresponding reduction of COX-2 protein (Fig. 4*B*).

We further determined the role of ERK and JNK in the activation of COX-2 induced by S protein by introducing three dominant kinase-inactive mutants—mERK1, mERK2, and mJNK—whose expression can block kinase activities by competing with endogenous kinases (25–26). Cells were cotransfected with pCMV-S, COX-2-Luc reporter, and each of three kinase mutants, respectively. Results from luciferase activity assays showed that all three mutants reduced COX-2 pro-

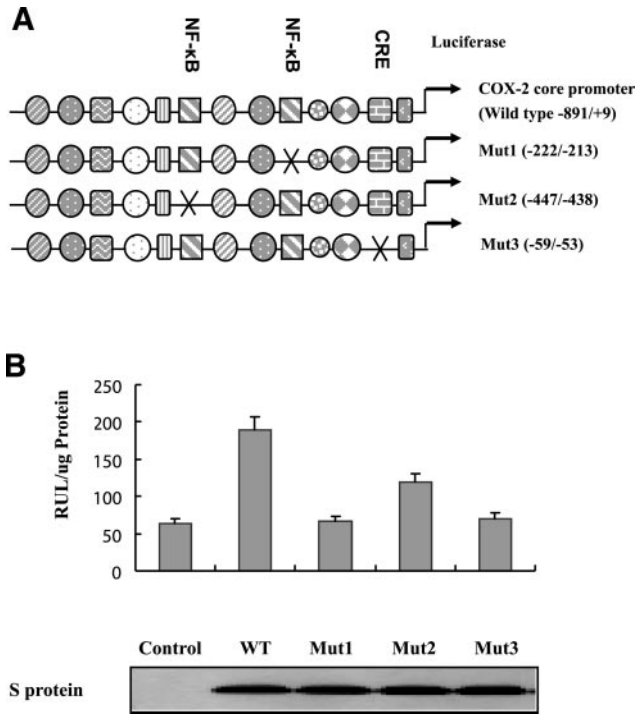

Figure 2. NF-κB and CREB were involved in S-induced COX-2 expression in HEK293T cells *A)* COX-2 core promoter structure including wild-type, two NF-κB mutants, and a CRE mutant. *B)* Cells were transfected with wild-type COX-2 promoter (−891/+9), NF-κB site (−222/−213, −447/−438) mutants Mut1 and Mut2, or CRE (−59/−53) mutant Mut3. The induction level of luciferase activity was compared with that of vector control. Data are expressed as mean ± SD of three independent experiments. The lower panel shows the expression of S protein.

moter activity (Fig. 4*C*). We also used Western blot to detect expression of mERK1, mERK2, and mJNK; results showed that protein expression levels increased slightly in all groups, with the dose increase of plasmids DNA transfected. Protein expression levels did not show a remarkable dose-dependent increase compared with that of DNA-transfected plasmids. The results may provide an explanation for the incomplete inhibition by three kinase mutants. Taken together, these data suggested that ERK, JNK, PI3K, and PKC, but not P38 MAP kinase, were involved in S protein-induced COX-2 activation.

S protein stimulated phosphorylation of ERK1/2 and JNK resulted in the binding of NF-kappaB and CREB to COX-2 promoter

To further investigate the role of S protein in the activation of signal transduction pathways, we determined the phosphorylation status of ERK1/2 and JNK in human cells by Western blot analysis using antibodies specifically recognizing phosphorylated ERK1/2 and JNK. Results showed that the levels of phosphorylated ERK1/2 (P44/42) and phosphorylated JNK increased in a time-dependent fashion in the presence of S protein (**Fig. 5*A***). Phosphorylation of ERK1/2 (P44/42) was blocked by GF109203 but not LY294002 (Fig. 5*B*). By contrast, the level of phosphorylation of JNK was significantly reduced by both GF109203 and LY294002 (Fig. 5*B*). These results suggested that S protein-induced ERK phosphorylation depends on PKC but not PI3K whereas JNK phosphorylation depends on both.

To investigate the roles of ERK1/2 and JNK in S protein-increased NF-κB and CREB binding to COX-2 promoter, we evaluated the effect of kinase inhibitor on NF-κB and CREB binding by EMSA. Results showed that S-induced binding of NF-κB to COX-2 promoter (Fig. 5*C*, lane 3), which was inhibited by a competitive probe (Fig. 5*C*, lane 4), GF109203 (Fig. 5*C*, lane 5), and PD98059 (Fig. 5*C*, lane 6) were not affected by the JNK inhibitor SP600125 (Fig. 5*C*, lane 7). These data suggested S protein-induced NF-κB binding is mediated *via* the PKC/ERK pathway.

Results from EMSA showed that S induced binding of CREB to the COX-2 promoter (Fig. 5*D*, lane 3), which was inhibited by the presence of competitive probe (Fig. 5*D*, lane 8), SP600125 (Fig. 5*D*, lane 4), GF109203 (Fig. 5*C*, lane 5), but not PD98059 (Fig. 5*D*, lane 6). These data suggested that S-induced CREB binding to COX-2 promoter is mediated *via* PI3K/PKC/JNK pathway and is independent of ERK.

PKCα and PKCε were involved in signal transduction pathways induced by S protein

As S-activated ERK and JNK were PKC dependent, we extended our study to identify the specific isozymes of PKC involved in the activation of COX-2 regulated by the S protein using an RNA interference (RNAi) approach. Small interference RNA (siRNA) molecules that specifically knock down genes encoding each PKC isozyme were designed and used to study the function of PKC, based on a previous study (17). Results indicated that PKCα-specific and PKCε-specific siRNA (siPKCα and siPKCε), but not PKCβ1-specific and

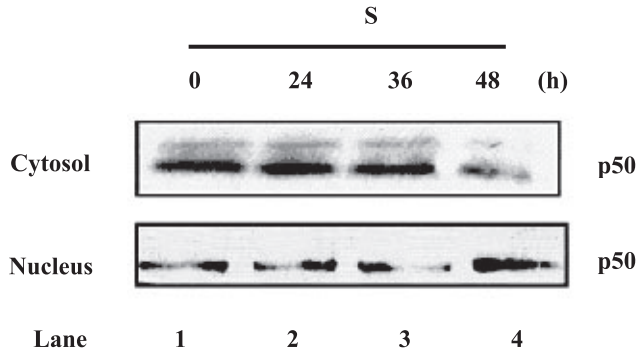

Figure 3. NF-κB was activated by S protein and translocated from cytoplasm to nucleus. Cells were transfected with pCMV-S plasmid; cytosolic and nuclear fractions were then prepared at different time intervals. Levels of cytosolic and nuclear p50 were determined by Western blot with p50-specific antibody. The blot is a representative of three experiments with similar results.

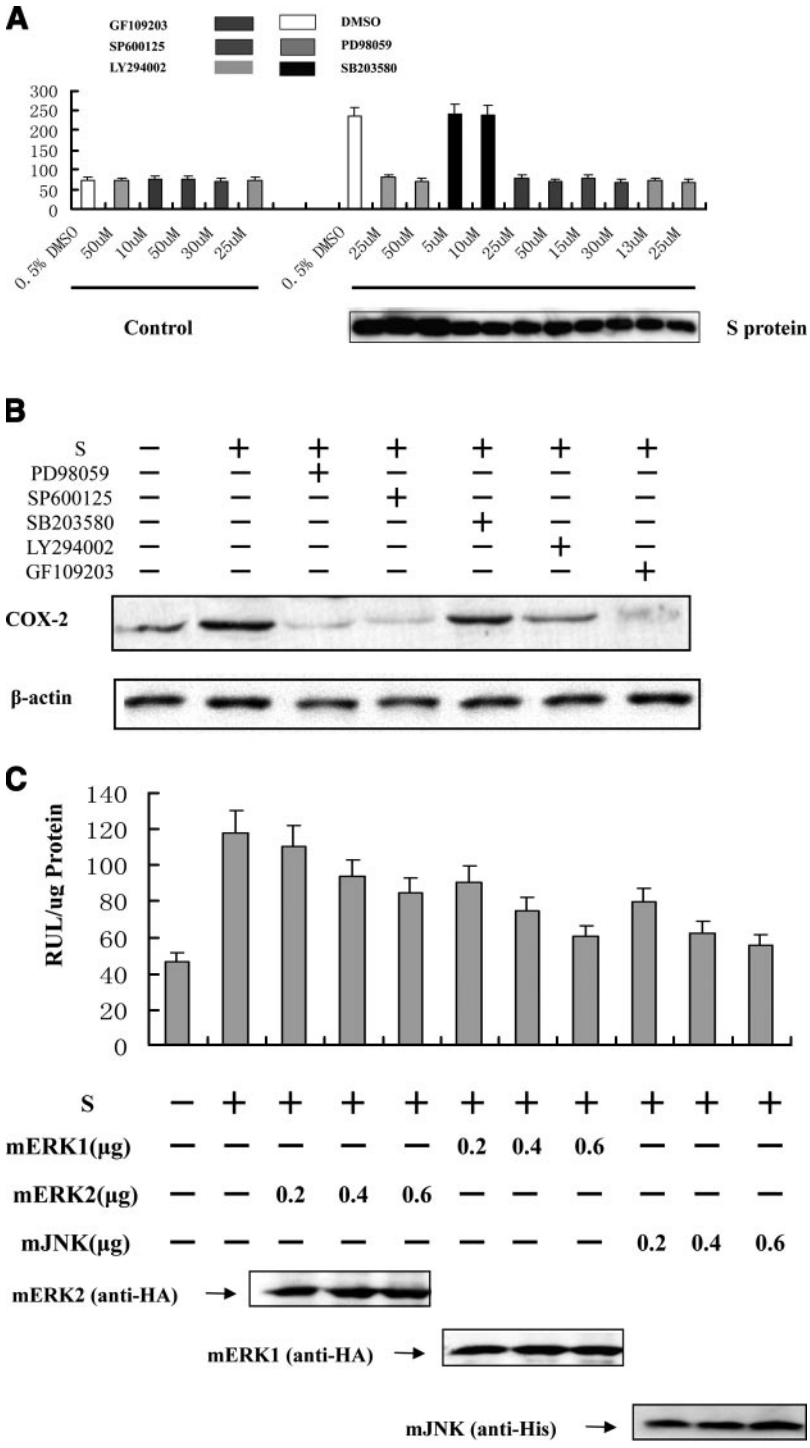

Figure 4. ERK, JNK, PI3K, and PKC were involved in spike-induced COX-2 expression in HEK293T cells. *A)* Cells were cotransfected with pCMV-S and COX-2-Luc for 48 h, then PD98059 (50 μM), SB203580 (10 μM) GF109203 (50 μM), SP600125 (30 μM), and LY294002 (25 μM) were added. The cells were lysed and luciferase activity was measured. Data are expressed as mean ± SD of three independent experiments. *B)* Cells were treated with PD98059 (50 μM), SB203580 (10 μM) GF109203 (50 μM), SP600125 (30 μM), and LY294002 (25 μM) and transfected by pCMV-S plasmid for 48 h. COX-2 protein was analyzed by Western blot. The blot is a representative of three experiments with similar results. *C)* HEK293T cells were cotransfected with COX-2-Luc and dominant negative mutants of ERK1, ERK2, JNK, or control vectors at different concentrations. Luciferase activity was measured. Data are expressed as mean ± SD of three independent experiments. The lower panel showed three kinase mutants expression detected by Western blot using anti-hemagglutinin (HA) antibody for mERK1, mERK2, and anti-His antibody for mJNK.

PKCσ-specific siRNA (siPKCβ1 and siPKCσ) or irrelevant siRNA (siCtrl), were able to significantly inhibit the induction of COX-2 in transiently transfected 293T cells (**Fig. 6*A***). These results suggested that PKCα and PKCε played important roles in the activation of COX-2 by the S protein in human cells.

Expression of PKCα, PKCε, PKCβ1, and PKCσ were determined before and after knockdown by specific siRNA using Western blot in HEK293T cells. Results showed that protein expression of different PKC isoforms were decreased significantly in the presence of PKC isoform-specific siRNAs (siPKCα, siPKCε, siPKCβ1, and siPKCσ). siCtrl, a similar vector (pSilencer 2.0 from Ambion) containing an irrelevant sequence that does not show significant homology to any human gene sequence, was used as a negative control (Fig. 6*B*). The results indicated that various PKC isoform-specific siRNAs worked efficiently in the assays.

The effects of PKCα and PKCε on the phosphorylation of ERK and JNK were also evaluated by a similar RNAi approach. Results from Western blot analysis showed that phosphorylated ERK1/2 levels stimulated by the S protein were significantly decreased by treatment of PKCα-specific siRNA (siPKCα) (Fig. 6*C*, upper

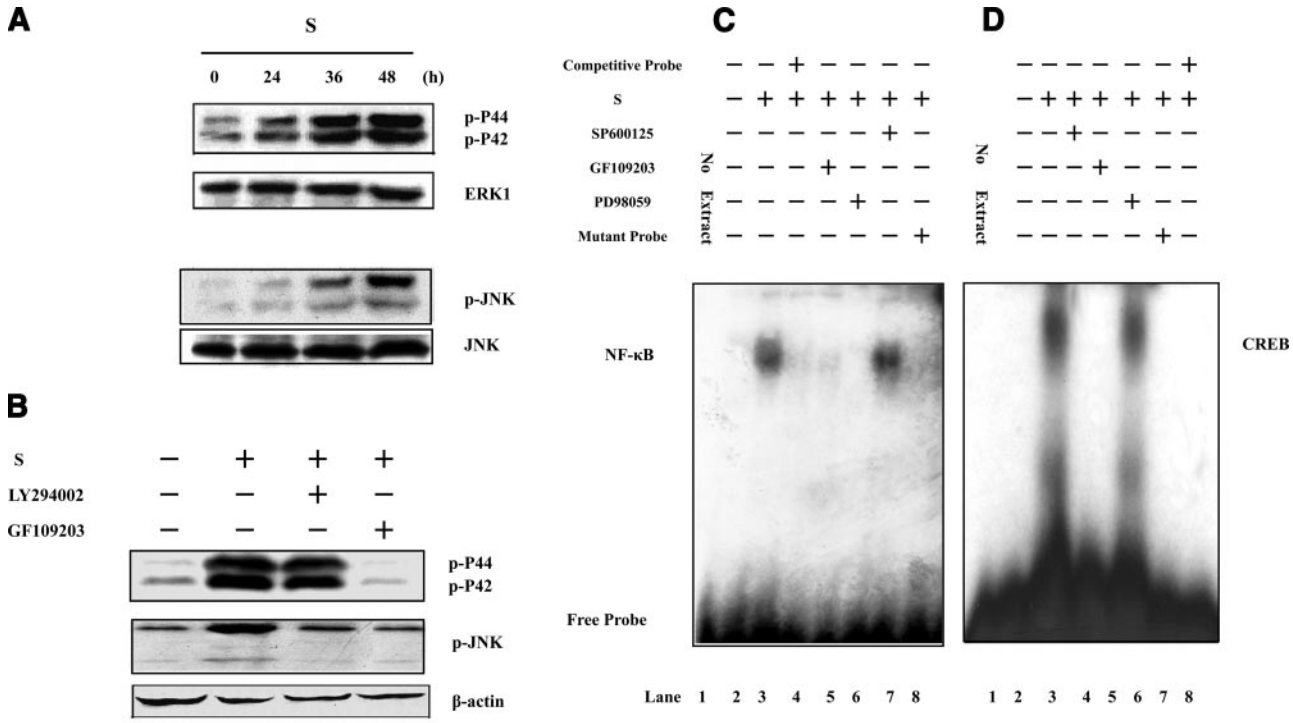

Figure 5. Two major signaling pathways were involved in spike-induced COX-2 activation in HEK293T cells. *A)* Time-dependent ERK and JNK phosphorylation shown by Western blot. The blot is a representative of three experiments with similar results. *B)* Differential inhibition of p-ERK and p-JNK by LY294002 (25 μM) or GF109203 (50 μM). The blot is a representative of three experiments with similar results. *C)* Cells were treated with PD98059 (50 μM), GF109203 (50 μM), and SP600125 (30 μM), then transfected with pCMV-S in HEK293T cells for 48 h. Nuclear extracts were prepared and NF-κB DNA binding activity was determined by EMSA. *D)* Experiments were repeated as in panel *C* except the probe was replaced by CRE.

panel). Similarly, phosphorylated JNK activated by the S protein was inhibited by PKCε-specific siRNA (siPKCε) (Fig. 6*C*, lower panel). These results suggested that PKCα was the upstream kinase for S-induced ERK/NF-κB activation and PKCε was the upstream kinase for JNK/CREB activation.

Induction of COX-2 expression by S protein was associated with extracellular Ca^{2+} entry

PKCα belongs to the conventional subfamily of PKC whose activities are calcium dependent (17). Since PKCα was involved in S-induced COX-2 expression, we investigated the role of Ca^{2+} in the induction of COX-2 expression by measuring intracellular calcium using Fura-2 fluorescence. Results showed that the concentration of cytosolic $[Ca^{2+}]_i$ in 293T cells was ~100 nM in the absence of S protein regardless of the status of EGTA and exogenous Ca^{2+} (**Fig. 7*A***). However, after introducing S protein into the cells, the concentration of cytosolic $[Ca^{2+}]_i$ was elevated to >300 nM, reduced to a basal level when treated with 2 mM EGTA, and increased rapidly after adding 2 mM exogenous Ca^{2+} (Fig. 7*A*).

The effect of Ca^{2+} on S-induced COX-2 expression regulated by the S protein was also studied. Human cells were cotransfected with pCMV-S and reporter followed by treatment with EGTA or BAPTA/AM, a cell-permeable Ca^{2+} chelator widely used as an intracellular calcium sponge. Results from luciferase activity analysis showed that up to 2 mM BATPA/AM had no effect on the regulation of COX-2 promoter activity by S protein (Fig. 7*B*). EGTA had no effect on COX-2 expression at 1 mM, but inhibited COX-2 expression when the concentration reached 2 mM (Fig. 7*C*). In addition, Western blot analysis revealed that S-induced COX-2 protein expression was inhibited by EGTA (Fig. 7*D*). These results suggested that the spike protein elevated intracellular Ca^{2+} by triggering extracellular Ca^{2+} entry to the cytosol, thereby activating PKCα and ERK.

To confirm another requirement of extracellular Ca^{2+} for ERK *vs.* JNK activation, we investigated the role of extracellular Ca^{2+} entry in regulating the MAPK pathway. Results showed that S protein-stimulated phosphorylated ERK was reduced in the presence of EGTA. However, phosphorylated JNK was not altered by EGTA (**Fig. 8**). These results suggested that extracellular Ca^{2+} influx was an upstream signal of the PKCα/ERK/NF-κB pathway.

DISCUSSION

We have identified a second gene from SARS-CoV that stimulates COX-2 expression. This is not an unusual event in the process of virus infection. It has been reported that both Core and NS5A from human hepa-

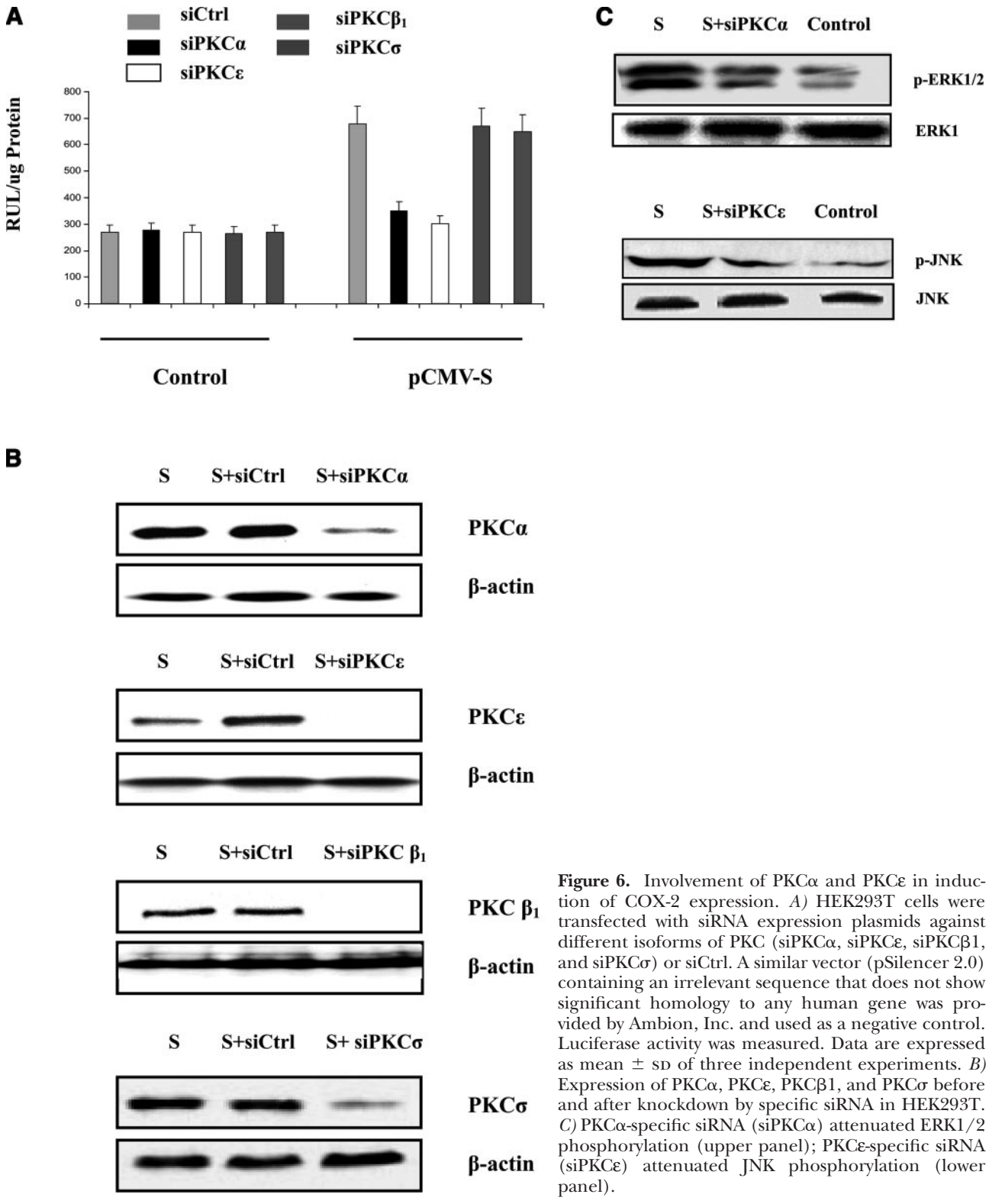

Figure 6. Involvement of PKCα and PKCε in induction of COX-2 expression. *A)* HEK293T cells were transfected with siRNA expression plasmids against different isoforms of PKC (siPKCα, siPKCε, siPKCβ1, and siPKCσ) or siCtrl. A similar vector (pSilencer 2.0) containing an irrelevant sequence that does not show significant homology to any human gene was provided by Ambion, Inc. and used as a negative control. Luciferase activity was measured. Data are expressed as mean ± SD of three independent experiments. *B)* Expression of PKCα, PKCε, PKCβ1, and PKCσ before and after knockdown by specific siRNA in HEK293T. *C)* PKCα-specific siRNA (siPKCα) attenuated ERK1/2 phosphorylation (upper panel); PKCε-specific siRNA (siPKCε) attenuated JNK phosphorylation (lower panel).

titis C virus induce COX-2 expression (27). We postulated that a novel mechanism existed in the cells for spike-activated COX-2 expression based on the fact that localizations of the nucleoprotein and spike are completely different. The nucleoprotein of SARS-CoV is localized in both nucleus and cytoplasm, whereas the spike is localized along the typical secretion pathway from endoplasmic reticulum to plasma membrane (28).

The spike gene of SARS-CoV encodes viral coat protein of 1255 amino acids with a molecular mass of 125 kDa. As previous studies have claimed that the envelope protein of Epstein-Barr virus (latent membrane protein 1) and HIV (Gp120) induce COX-2 expression after introduction into host cells (29–30), we evaluated the effect of S protein on COX-2 expression by transient transfection. Our results show that

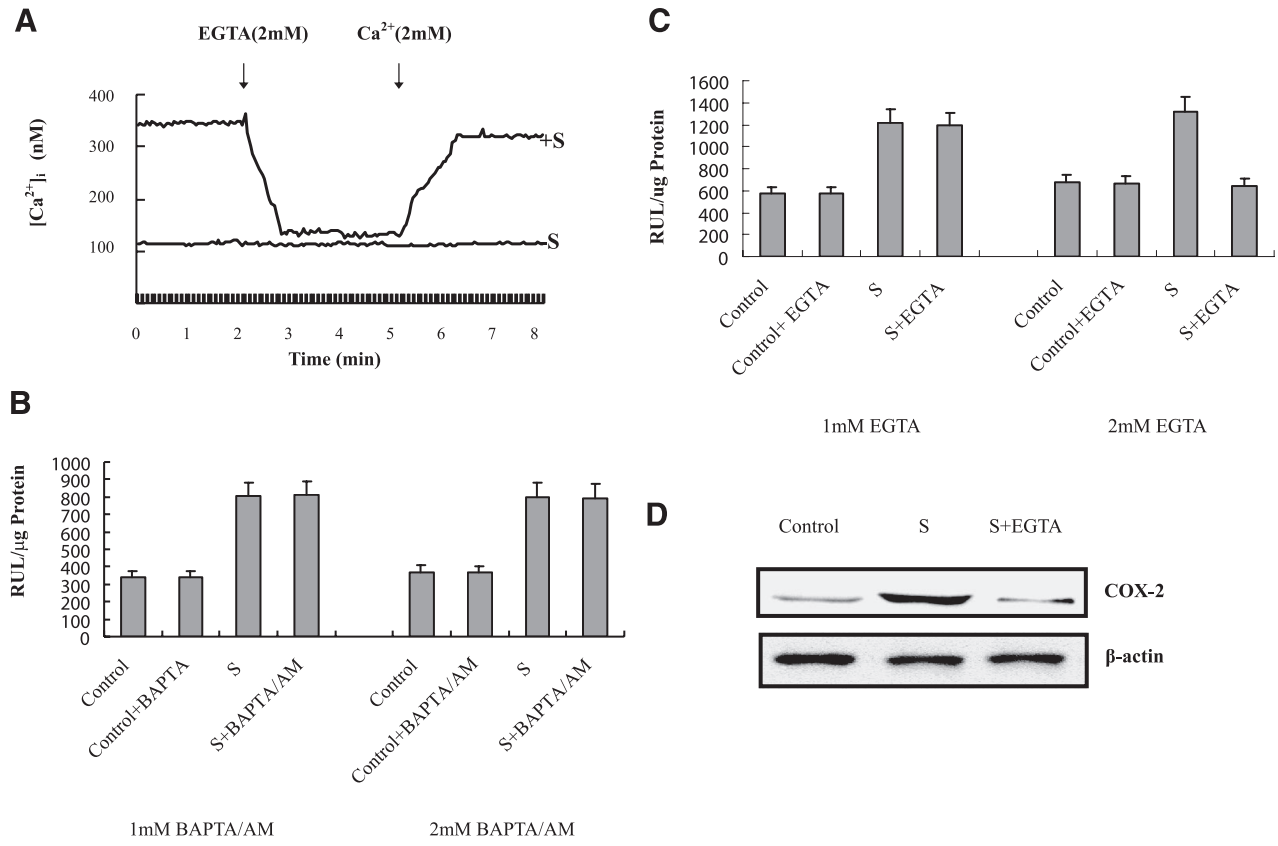

Figure 7. Extracellular Ca^{2+} entry was associated with induction of COX-2 expression. *A)* S overexpression triggered the rapid extracellular Ca^{2+} influx, which was blocked by 2 mM EGTA and restored by 2 mM extracellular Ca^{2+}. *B)* HEK293T cells were cotransfected with pCMV-S and reporter plasmid COX-2-Luc for 24 h, followed by treatment with BAPAT/AM for 8 h. Luciferase activity was measured. Data are expressed as mean ± SD of three independent experiments. *C)* HEK293T cells were cotransfected with pCMV-S and reporter plasmid COX-2-Luc for 24 h, followed by treatment with EGTA for 8 h. Luciferase activity was measured. Data are expressed as mean ± SD of three independent experiments. *D)* HEK293T cells were transfected with pCMV-S plasmid for 24 h, followed by treatment with EGTA for 8 h. COX-2 protein were then analyzed by Western blot. The blot is a representative of three independent experiments.

transfection of S protein resulted in COX-2 promoter activation and protein production.

Many viral proteins can induce multiple signaling pathways, which may cross-talk with each other or converge on common downstream effectors (31). In nasopharyngeal carcinoma cells, the latent membrane protein-1 of EBV induced COX-2 expression through MAPK, the JNK/AP-1, and janus-activated kinase (JAK)/STAT signaling pathways and required the transcription factor NF-κB (13).

Our results indicate that S protein activates COX-2 promoter activity by enhancing NF-κB and CREB binding to their specific enhancer elements on COX-2 promoter. Both NF-κB and CREB have been claimed to mediate COX-2 activation induced by diverse stimulating agents. We demonstrated in this study that NF-κB and CREB are essential for S protein-induced COX-2 expression since mutations of binding sites for NF-κB and CREB eliminated S protein-induced activation of COX-2 promoter activity. Based on our finding that S protein activates COX-2 expression, we further investigated the roles of different MAP kinases on the activation of COX-2 by S protein. The results suggested that ERK, JNK, PI3K, and PKC, but not p38 MAP kinase, were involved in the activation of COX-2 regulated by S protein. An active ERK was required for the binding of NF-κB to the COX-2 promoter in response to S protein stimulation; the PI3K/PKC/JNK/CREB pathway is another important signaling in the regulation of COX-2 by S protein.

Although PI3K/JNK/CREB and ERK/NFκB signaling pathways appear to be independent, both were regulated by members of PKC family. Using an RNA interference approach, we demonstrated that the induction of COX-2 expression by S protein of SARS-CoV required the conventional isozyme PKCα and the novel isoform PKCε, since PKCα- and PKCε-specific siRNAs inhibited COX-2-Luc reporter expression activated by the S protein but siRNA specific for PKCβ1 and PKCσ did not influence S protein induced COX-2 promoter activities.

It has been demonstrated that PKCα is calcium dependent and PKCε is calcium independent, suggesting these two PKC isoforms play different roles in transmitting S-induced COX-2 expression. We provided evidence that the S-activated ERK was dependent on PKCα and the S-regulated JNK required PKCε, because PKCα-specific siRNA significantly affected the level of

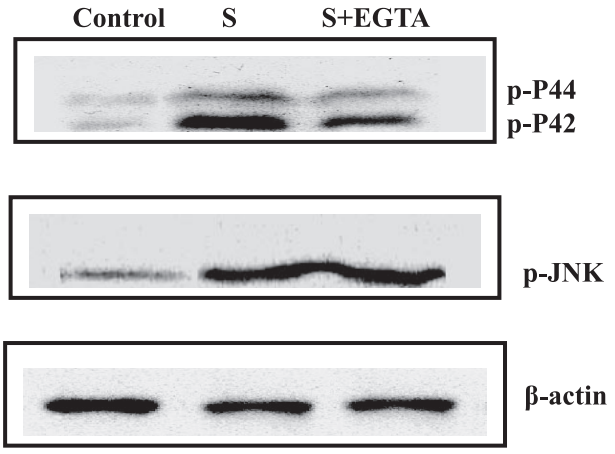

Figure 8. Ca^{2+} signaling triggered the PKCα/ERK/NF-κB pathway. HEK293T cells were transfected with pCMV-S plasmid for 24 h, followed by treatment with EGTA for 8 h. Then levels of p-ERK and p-JNK were determined by Western blot with each specific p-ERK and p-JNK antibody.

ERK1/2 phosphorylation and PKCε-specific siRNA decreased the level of JNK phosphorylation. These results suggested S-activated COX-2 expression was mediated *via* two signaling pathways led by PKCα and PKCε, respectively.

The role of calcium in the regulation of COX-2 by S protein was also investigated in this study. Transient transfection of human cells with S protein induced a rapid increase in the level of intracellular calcium due to the transporting calcium influx from outside the cell. Treatment of cells with 2 mM of EDTA resulted in the chelating of extracellular calcium influx, affected the expression of COX-2, and inhibited the level of ERK phosphorylation. However, the level of JNK phosphorylation was not affected by treatment of the EDTA. These results suggested that an influx of extracellular calcium was required for induction of COX-2.

It has been reported that the elevated Ca^{2+} in cytosol stimulated PKCα through diacylglycerol (DAG), then induced translocation of PKCα to the plasma membrane, where it becomes active and regulates the ERK/NF-κB pathway (32). Although the PKCα pathway is mediated by an influx of extracellular Ca^{2+}, the mechanism of this influx remains unclear. It was reported that endoplasmic reticulum (ER) stress opens up the calcium channel on the plasma membrane to allow extracellular Ca^{2+} entry from outside the cell (33). The ER stress response is suggested to contribute to several types of human disease. ER stress response is induced by overexpression of exogenous membrane or secreted proteins, such as virus proteins (34). Hepatitis C virus and Japanese encephalitis virus infection have been reported to initiate endoplasmic reticulum stress (35). Generally, ER stress is characterized by enhanced expression of ER-resident chaperones, such as glucose-regulated protein (GRP), Bip/GRP78, and CHOP (36). In our study we found that Bip/GRP78 and CHOP mRNA levels were enhanced in the presence of S protein (data not shown), suggesting ER stress was induced in response to SARS-CoV S protein stimulation. The spike protein of SARS-CoV is a glycosylated protein and is localized along the secretion pathway from ER to the plasma membrane (28). It is possible that excessive S proteins pass through the secretion pathway during viral infection and result in ER stress. We speculated that ER stress was the main reason for the influx of intracellular Ca^{2+}, which was required to trigger the PKCα/ERK/NF-κB signaling pathway and to induce COX-2 expression.

In this study, we investigated the role of SARS-CoV S protein in regulating COX-2 gene expression. S protein regulated COX-2 gene expression through two independent signaling pathways: the calcium-dependent PKCα/ERK/NF-κB signaling pathway and the calcium-independent PI3K/PKCε/JNK/CREB pathway. The influx of extracellular Ca^{2+} induced by S protein activates the PKCα kinase and PKCα/ERK/NF-κB pathways. After activation, NF-κB binds to the COX-2 promoter and activates transcription. S protein may also induce kinase PI3K through a mechanism that is unclear at this time, which in turn activates PKCε, a downstream kinase of PI3K (37) and the JNK/CREB signaling pathway. Activated CREB binds to the COX-2 promoter and regulates expression of the COX-2 gene. Our results provide a novel possible explanation for the critical role of the S protein of SARS-CoV in inducing preliminary inflammation and tissue damage leading to devastating SARS. FJ

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REFERENCES

1. Yan, X., Hao, Q., Mu, Y., Timani, K. A., Ye, L., Zhu, Y., and Wu, J. (2006) Nucleocapsid protein of SARS-CoV activates the expression of cyclooxygenase-2 by binding directly to regulatory elements for nuclear factor-kappa B and CCAAT/enhancer binding protein. *Int. J. Biochem. Cell Biol.* **38,** 1417–1428
2. Peiris, J. S., Yuen, K. Y., Osterhaus, A. D., and Stöhr, K. (2003) The severe acute respiratory syndrome. *N. Engl. J. Med.* **349,** 2431–2441
3. Rota, P. A., Oberste, M. S., Monroe, S. S., Nix, W. A., Campagnoli, R., Icenogle, J. P., Penaranda, S., Bankamp, B., Maher, K., Chen, M. H., *et al.* (2003) Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* **300,** 1394–1399
4. Eickmann M., Becker S., Klenk H. D., Doerr H. W., Stadler K., Censini S., Guidotti S., Masignani V., Scarselli M., Mora M., *et al.* (2003) Phylogeny of the SARS coronavirus. *Science* **302,** 1504–1505
5. Simmons, G., Reeves, J. D., Rennekamp, A. J., Amberg, S. M., Piefer, A. J., and Bates, P. (2004) Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 4240–4245
6. Martin, J. T. (2003) Critical care medicine in AJRCCM. *Am. J. Respir. Crit. Care Med.* **169,** 239–253
7. Esteban, V., Ruperez, M., Sánchez-López, E., Rodríguez-Vita, J., Lorenzo, O., Demaegdt, H., Vanderheyden, P., Egido, J., and Ruiz-Ortega, M. (2005) Angiotensin IV activates the nuclear transcription factor-κB and related proinflammatory genes in vascular smooth muscle cells. *Circ. Res.* **96,** 965–973

8. Hinz, B., and Brune, K. (2002) Cyclooxygenase-2–10 years later. *J. Pharm. Exp. Ther.* **300,** 367–374
9. Lawrence, J. M., Rowlinson, S. W., Goodwin, D. C., Kalgutkar, A. S., and Lanzo, C. A. (1999) Arachidonic acid oxygenation by COX-1 and COX-2. *J. Biol. Chem.* **274,** 22903–22906
10. Iniguez, M. A., Martinez-Martinez, S., Punzon, C., Redondo, J. M., and Fresno, M. (2000) An essential role of the nuclear factor of activated T cells in the regulation of the expression of the cyclooxygenase-2 gene in human T lymphocytes. *J. Biol. Chem.* **275,** 23627–23635
11. Corasaniti, M. T., Bellizzi, C., Russo, R., Colica, C., Amantea, D., and Di, R. G. (2003) Caspase-1 inhibitors abolish deleterious enhancement of COX-2 expression induced by HIV-1 gp120 in human neuroblastoma cells. *Toxicol. Lett.* **139,** 213–219
12. Lara-Pezzi, E., Gomez-Gaviro, M. V., Galvez, B. G., Mira, E., Iniguez, M. A., Fresno, M., Martinez-A C, Arroyo, A. G., and Lopez-Cabrera, M. (2002) The hepatitis B virus X protein promotes tumor cell invasion by inducing membrane-type matrix metalloproteinase-1 and cyclooxygenase-2 expression. *J. Clin. Invest.* **110,** 1831–1838
13. Murono, S., Inoue, H., Tanabe, T., Joab, I., Yoshizaki, T., Furukawa, M., and Pagano, J. S. (2001) Induction of cyclooxygenase-2 by Epstein-Barr virus latent membrane protein 1 is involved in vascular endothelial growth factor production in nasopharyngeal carcinoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6905–6910
14. Dolganiuc, A., Kodys, K., Kopasz, A., Marshall, C., Do, T., Romics L., Jr., Mandrekar, P., Zapp, M., and Szabo, G. (2003) Hepatitis C virus core and nonstructural protein 3 proteins induce pro-and anti-inflammatory cytokines and inhibit dendritic cell differentiation. *J. Immunol.* **170,** 5615–5624
15. Zhu, Y., Saunders, M. A., Yeh, H., Deng, W., and Wu, K. K. (2002) Dynamic regulation of cyclooxygenase-2 promoter activity by isoforms of CCAAT/ehancer binding proteins. *J. Biol. Chem.* **277,** 6923–6928
16. Zhu, Y., Liu, M., Zhao, W. G., Zhang, J. L., Zhang, X., Wang, K., Gu, C. F., Wu, K. L., Li, Y., Zheng, C. Y., *et al.* (2005) Isolation of virus from a SARS patient and genome-wide analysis of genetic mutations related to pathogenesis and epidemiology from 47 SARS-CoV isolates. *Virus Genes* **30,** 93–102
17. Storz, P., Doppler, H., and Toker, A. (2004) Protein kinase cδselectively regulates protein kinase D-dependent activation of NF-κB in oxidative stress signaling. *Mol. Cell Biol.* **24,** 2614–2626
18. Neurath, M. F., Becker, C., and Barbulescu, K. (1998) Role of NF-κB in immune and inflammatory responses in the gut. *Gut* **43,** 856–860
19. Jijon, H. B., Panenka, W. J., Madsen, K. L., and Parsons, H. G. (2002) MAP kinases contribute to IL-8 secretion by intestinal epithelial cells *via* a posttranscriptional mechanism. *Am. J. Physiol.* **283,** C31–C34
20. Zhu, J., Krishnegowda, G., and Gowda, D. C. (2005) Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of plasmodium falciparum: the requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-κB Pathways for the expression of proinflammatory cytokines and nitric oxide. *J. Biol. Chem.* **280,** 8617–8627
21. Syeda F., Grosjean J., Houliston R. A., Keogh R. J., Carter T. D., Paleolog E., and Wheeler-Jones C. P. (2000) Cyclooxygenase-2 induction and prostacyclin release by protease-activated receptors in endothelial cells require cooperation between mitogen-activated protein kinase and NF-κB pathways. *J. Biol. Chem.* **281,** 11792–11804
22. Pratt, P. F., Bokemeyer, D., Foschi, M., Sorokin, A., and Dunn, M. J. (2003) Alterations in subcellular localization of p38 MAPK potentiates endothelin-stimulated COX-2 expression in glomerular mesangial cells. *J. Biol. Chem.* **278,** 51928–51936
23. Yang, T., Huang, Y., Heasley, L. E., Berl, T., Schnermann, J. B., and Briggs, J. P. (2000) MAPK mediation of hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells. *J. Biol. Chem.* **275,** 23281–23286
24. Kim, H., Rhee, S. H., Kokkotou, E., Na, X., Savidge, T., Moyer, M. P., Pothoulakis, C., and LaMont, J. T. (2005) Clostridium difficile toxin A regulates inducible cyclooxygenase-2 and prostaglandin E2 synthesis in colonocytes *via* reactive oxygen species and activation of p38 MAPK. *J. Biol. Chem.* **280,** 21237–21245
25. Khoo, S., Griffen, S. C., Xia, Y., Baer, R. J., German, M. S., and Cobb, M. H. (2003) Regulation of insulin gene transcription by ERK1 and ERK2 in pancreatic beta cells. *J. Biol. Chem.* **278,** 32969–32977
26. Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998) Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes. *EMBO J.* **17,** 3101–3111
27. Nunez, O., Fernandez-Martinez, A., Majano, P. L., Apolinario, A., Gomez-Gonzalo, M., Benedicto, I., Lopez-Cabrera, M., Bosca, L., Clemente, G., Garcia-Monzon, C., and Martin-Sanz, P. (2004) Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins. *Gut* **53,** 1665–1672
28. Nal, B., Chan, C., Kien, F., Siu, L., Tse, J., Chu, K., Kam, J., Staropoli, I., Crescenzo-Chaigne, B., Escriou, N., *et al.* (2005) Differential maturation and subcellular localization of severe acute respiratory syndrome coronavirus surface proteins S, M and E. *J. Gen. Virol.* **86,** 1423–1434
29. Cahir-Mcfarland, E. D., Davidson, D. M., Schauer, S. L., Duong, J., and Kieff, E. (2000) NF-κB inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6055–6060
30. Devergne, O., Hatzivassiliou, E., Kaye, K. M., Kleijner, M., and Kieff, E. (1998) Role of the TRAF binding site and NF-κB activation in Epstein-Barr virus latent membrane protein 1-induced cell gene expression. *J. Viol.***72,** 7900–7908
31. Choi, S. H., and Hwang, S. B. (2006) Modulation of the transforming growth factor-β signal transduction pathway by hepatitis C virus nonstructural 5A protein. *J. Biol. Chem.* **281** 7468
32. Su, H-L., Liao, C-L., and Lin, Y-L. (2002) Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. *J. Virol.* **76,** 4162–4171
33. Bosco, D., Gonelle-Gispert, C., Wollheim, C. B., Halban, P. A., and Rouiller, D. G (2001) Increased intracellular calcium is required for spreading of rat islet β-cells on extracellular matrix. *Diabetes* **50,** 1039–1046
34. Wang, H. C., Wu, H. C., Chen, C. F., Fausto, N., Lei, H. Y., and Su, I. J. (2003) Different types of ground glass hepatocytes in chronic hepatitis b virus infection contain specific pre-S mutants that may induce endoplasmic reticulum stress. *Am. J. Pathol.* **163,** 2441–2449
35. Pavio, N., Romano, P. R., Graczyk, T. M., Feinstone, S. M., and Taylor, D. R. (2003) Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2αkinase PERK. *J. Virol.* **77,** 3578–3585
36. Hung, J. H., Su, I. J., Lei, H. Y., Wang, H. C., Wan, C. L., Chang, W. T., Huang, W. Y., Chang, W. C., Chang, W. S., Chen, C. C., and Lai, M. D. (2004) Endoplasmic reticulum stress stimulates the expression of cyclooxygenase-2 through activation of NF-κB and p38 mitogen-activated protein kinase. *J. Biol. Chem.* **279,** 46384–46392
37. Choudhury, G. G. (2004) A linear signal transduction pathway involving phosphatidylinositol 3-kinase, protein kinase Cε, and MAPK in mesangial cells regulates interferon-γ-induced STAT1α transcriptional activation. *J. Biol. Chem.* **279,** 27399–27409

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