CAFFEIC ACID PHENETHYL ESTER SUPPRESSES IFN-γ-INDUCED STAT1 ACTIVATION AND GENE EXPRESSION IN ENDOTHELIAL CELLS

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Dysfunction of the endothelium contributes to pathological conditions of the arterial wall including atherosclerosis as a result of immunological and/or inflammatory responses. Caffeic acid phenethyl ester (CAPE) is an anti-inflammatory component of propolis (honeybee resin). CAPE was discussed for its anti-inflammatory and anti-oxidant properties on endothelial cells (ECs). Signal transducer and activator of transcription protein 1 (STAT1), a transcription factor involved in inflammation and the cell cycle, is activated by IFN-γ. In this study, we evaluated whether CAPE can serve as an anti-inflammatory agent during endothelial injuries. Pretreatment of ECs with CAPE dose-dependently inhibited IFN-γ-induced Tyr701 and Ser727 phosphorylation in STAT1 without affecting the phosphorylation of JAK1 and JAK2. Consistently, IFN-γ-induced STAT1 downstream target CXC chemokine, IP-10, was suppressed by CAPE pretreatment. It was also observed that CAPE inhibited promoter activity of IP-10 gene and the secretion of IP-10 protein. Furthermore, the T cell adhesion to IFN-γ-treated ECs was observed to be reduced after CAPE pretreatment. The anti-inflammatory properties of CAPE on IFN-γ-induced JAK-STAT1 activation were approved in the present study, and which provides a molecular basis for further therapeutic usage on vascular disorders.

Key words: CAPE, endothelial cells, JAK, STAT1, IP-10, IFN-γ

Introduction

Dysfunction of the endothelium contributes to pathological conditions of the arterial wall, such as atherosclerosis[1]. Clinical evidence has indicated the expression of cytokines in the human aortic intima during atherogenesis and elevated systemic parameters in patients with coronary atherosclerosis[2,3]. In

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Abbreviations: Caffeic acid phenethyl ester (CAPE), interferon-γ (IFN-γ), IFN-inducible protein of 10 kDa (IP-10), signal transducers and activators of transcription (STATs), Janus kinase (JAK), endothelial cells (ECs).

atherosclerotic lesions from clinical samples as well as in preclinical rodent atherosclerosis models, the expression of interferon-gamma (IFN-γ) has been observed[4,5]. IFN-γ was found to aggravate atherosclerosis in apoE-deficient mice[6]. These results indicate that IFN-γ is a major cytokine that contributes to atherogenesis. Further, it has been reported that IFN-γ enhanced Fas-mediated cell death selectively via augmenting Fas expression and so might be involved in the development of vascular endothelial injury in endotoxic and septic shock[7]. This effect might be one of the damage induced by IFN-γ. Effective blockage of IFN-γ-induced inflammatory responses may offer a remedy for atherosclerosis prevention.

Signal transducers and activators of transcription (STATs) are a family of nuclear proteins mediating the action of a number of cytokines. STAT1 activation cascade is one major signaling pathway converting the IFN-γ signal into gene expression, such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX), vascular cell adhesion molecules (VCAMs), and intercellular cell adhesion molecule-1 (ICAM-1), which is critically involved in different pathologies correlated to the inflammatory process. IFN-γ binds to its receptor, followed by activation of Janus kinase 1/2 (JAK1/2). Subsequently, cytosolic STAT1 is recruited to the receptor complex and is phosphorylated at Tyr701 and Ser727. Upon phosphorylation, STAT1 forms a homodimer and is translocated to the nucleus where it binds conserved DNA sequences in the promoter region of its downstream target genes and activates their transcription. IFN-γ induces the JAK/STAT1 signaling pathway in ECs[8]. IFN-γ-inducible chemokines (CXC) including CXCL10 (IP-10), CXCL9 (Mig), and CXCL11 (I-Tac) are targets of JAK-STAT signaling pathways. Analysis of human atherosclerotic lesions showed that IFN-γ-inducible CXC chemokines were highly expressed in ECs that overly plaque. Increases of chemokines concentration in plasma were shown to be associated with cardiovascular diseases including myocardial infarction and atherosclerosis. These chemokines serve as chemotactic factors for recruiting T cells to inflammation sites[9]. Chronically activated T cells are frequently detected in atherosclerotic lesions[10].

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant, which is an active anti-inflammatory component of propolis (honeybee resin)[11,12]. Various investigators have demonstrated that CAPE has anti-inflammatory properties both *in vitro* and *in vivo*[11-14]. CAPE was also proposed to be a specific inhibitor of the transcription factor nuclear factor-κB (NF-κB), which may account for its anti-inflammatory actions[15]. These results imply that CAPE has anti-inflammatory properties that may explain its antiatherosclerotic effects. However, the detailed signaling cascades and downstream effects of IFN-γ-related responses in ECs that contribute to CAPE-induced atheroprotection remain to be defined. In this study, we evaluated the protective mechanism of CAPE against IFN-γ-Induced JAK/STAT1 activation in ECs.

Materials and Methods

I. Chemicals

CAPE was obtained from Sigma Chemical Co. (St. Louis, MO). The complementary cDNA probe of IP-10 was supplied from Dr. DL Wang[16]. Recombinant human IFN-γ was purchased from Protech Technology (Taipei, Taiwan). Antibodies to phospho-Tyr701 and phosphor-Ser727 of STAT1, phospho-JAK1, phospho-JAK2, STAT1, and actin were purchased from Santa

Cruz Biotechnology (CA, USA).

II. Cell culture

Bovine aortic endothelial cells (BAECs) derived from the American Type Culture Collection (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technology, Kibbutz, Isreal) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technology) under standard culture conditions.

III. MTT assay

During culturing process, the cell viability and cell number were determined by the trypan blue dye-exclusion method. After cultured onto plates, measurement of cellular 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT) reduction was performed as described previously (16) to quantify ECs' viability. ECs were incubated with CAPE at the indicated concentrations for 24 h, and then the cytotoxic effect was estimated by using MTT assay. MTT level was measured by the absorbance of the MTT-formazan, an indicator of cell viability. The survival ratio of cells is shown as the percentage of untreated control cells.

IV. RNA extraction

For RNA isolation, ECs were washed with cold PBS twice and total RNA was isolated by the TRI reagent (Ambion, TX, USA). Briefly, cells were harvested and treated with 1 ml TRI reagent for 5 min at room temperature and then centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous portion containing RNA was transferred to another RNAase-free microcentrifuge tube, and 0.5 ml isopropanol was added and incubated for 10 min at room temperature, followed by precipitation of total RNA with centrifugation at 12,000 rpm at 4°C for 10 min. The pellet was then washed with 75% ethanol twice and dried at room temperature on ice for 5 min; the total RNA pellet was dissolved in 20 μl H_2O. To remove the contaminated genomic DNA that slightly co-precipitated with RNA, the RNA sample was digested using DNAase with a TURBO DNA-free kit (Ambion, TX, USA). The RNA concentration was measured at A260 nm with a Beckman DU 530 Life Science UV/Vis spectrophotometer.

V. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR was performed using one-step RT-PCR System SuperScript^{TM} Ⅲ (Invitrogen, CA, USA). Forward and reverse (respectively) primer sequences for human IP-10 were: 5`-GACATATTCTGAGCCT-ACAGC-3` and 5`-GCAGAGCATATATCTATCTG-3` (1030 bp). GAPDH served as an internal control. Thermal cycling conditions were as follows: reverse transcription at 55°C for 30 min; denaturation at 94°C for 2 min; 25 cycles of denaturation at 94°C for 0.25 min, annealing at 56°C for 0.5 min, and elongation at 68°C for 1 min; and an additional elongation step of 5 min at 68°C. These amplified DNA were analyzed by electrophoresis in 1% agarose gels.

VI. Transient transfection and promoter activity assay

A full-length IP-10 promoter construct was purified using an EndoFree Plasmid Maxi Kit DNA Purification System (Qiagen, CA, USA). Transfection was performed by the LipofecTAMINE method (Invitrogen), and a pSV-β-galactosidase plasmid was cotransfected to normalize the transfection efficiency.

In brief, ECs were plated onto 6-well plates at a density 1×10^5 cells/well and grown overnight. Cells were co-transfected with 2 μg of each promoter construct and 1 μg of the pSV-β-galactosidase plasmid for 5 h by the LipofecTAMINE method. After transfection, cells were recovered with 10% FBS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the procedures of the manufacturer (Promega, WI, USA). Luciferase activity was normalized to β-galactosidase activity in cell lysates and was expressed as an average of three independent experiments.

VII. Immunoblot analysis and enzyme-linked immunosorbent assay (ELISA)

After treatment, ECs were lysed with buffer containing 0.1% SDS (sodium dodecyl sulfate) and 2-mercaptoethanol, and then subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Antigens were analyzed using monoclonal antibodies. Antigen-antibody complexes were detected using horseradish peroxide-conjugated rabbit anti-mouse immunoglobulin G (IgG), and results were analyzed by enhanced chemiluminescence system (Pierce, IL, USA). Concentrations of secreted IP-10 proteins in the culture supernatant collected from ECs after IFN-γ stimulation with or without CAPE pretreatment were determined. Supernatants of ECs were collected and stored at -20°C prior to ELISA assay. The concentration of secreted IP-10 protein was determined using a sandwich ELISA kit (BioSource, CA, USA) according to the manufacturer's instructions.

VIII. Cell adherence assay

Cell adherence assay was performed using Jurkat T cells. Jurkat T cells were suspended in RPMI 1640 containing 0.1% FBS and labeled with fluorescence solution (3μM LavaCell, Active Motif, CA, USA) for 30 min and incubated at 37°C in dark. After labeling, Jurkat cells were washed twice and resuspended in fresh medium. Labeled Jurkat T cells adjusted to cell number of 1.2×10^7/ml was added to each well containing ECs and incubated for 1 h. Nonadherent Jurkat cells were removed by washing twice with RPMI1640 with gently shaking. Before harvesting, ECs with adherent Jurkat T cells were first photographed under microscopy and removed with lysis buffer, and fluorescence intensity were measured using Spectrophotometer (Spectra Max M5, Molecular Device, CA, USA). The excitation and emission wavelengths of LavaCell solution are 520nm and 610nm, respectively

IX. Statistical analysis

Statistical analyses were performed using Student's *t*-test. Data are presented as the mean±SE. Statistical significance was defined as $p < 0.05$.

Results

In the present study, we first determined the cytotoxicity of CAPE on ECs. CAPE showed no significant toxicity to ECs with concentration up to 250 μM as revealed by MTT assay (Fig. 1).

STAT cascade activation after IFN-γ stimulation is rapid. ECs after incubation with CAPE at indicated concentration for 1 h were subjected to interferon-γ (IFN-γ, 10 ng/ml) treatment for 10 min. The IFN-γ-induced phosphorylations of Tyr701 and Ser 727 in STAT1 were shown to be suppressed by CAPE pretreatment in a dose-dependent manner. However,

phosphorylation of JAK1 and JAK2 showed no significant alternation after CAPE pretreatment (Fig. 2). The above results lead us to speculate that the inhibitory effect of CAPE on IFN-γ-induced JAK/STAT activation might alter the STAT1-downstream targets CXC chemokines gene expression. Effect of CAPE on IP-10 gene expression was evaluated. Indeed, CAPE pretreatment suppressed the IFN-γ-induced CXC chemokine, IP-10, promoter activity (Fig. 3A). Further study showed that IFN-γ-

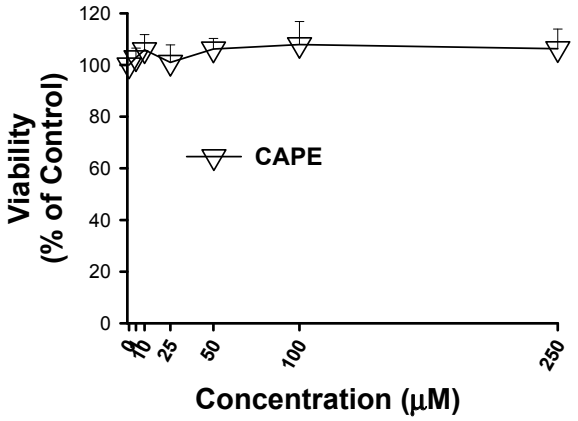

Fig. 1. Effect of CAPE on endothelial cell viability. Caffeic acid phenethyl ester (CAPE) was applied to the cultured ECs for 24 h to evaluate the toxicity of CAPE. Cell viability was measured using the MTT test. All the results were shown as the mean±SE of three independent experiments.

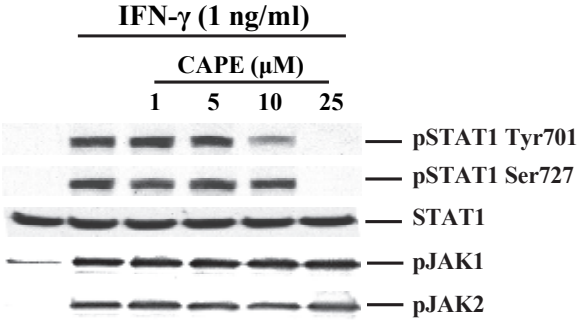

Fig. 2. CAPE inhibits IFN-γ-induced STAT1 phosphorylation in a dose-dependent manner. CAPE inhibits IFN-γ-induced STAT1 phosphorylations on Tyr701 and Ser727 sites in a dose-dependent manner. STAT1 was used to indicate equal amounts of total protein loaded in each lane. Result is a representative of three independent experiments with similar results.

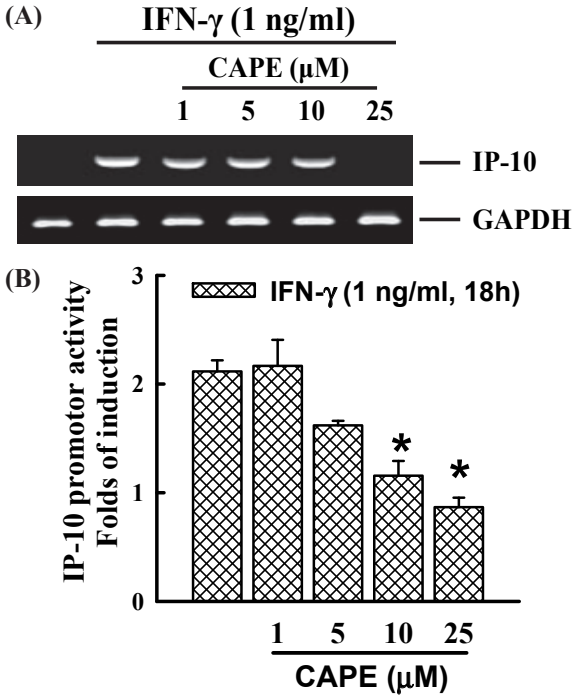

Fig. 3. CAPE inhibits INF-γ-induced IP-10 promoter activity and gene expression. (A) A promoter construct of IP-10 containing luciferase as a reporter gene was cotransfected with the pSV-β-galactosidase plasmid into ECs. The transfected ECs were pretreated with CAPE at the indicated concentrations for 1 h followed by stimulation with IFN-γ (10 ng/ml) for 18 h. Luciferase activities after normalization to β-galactosidase were expressed as folds of induction compared to those of untreated controls. All the results are shown as the mean±SE of three independent experiments. * $p < 0.05$ vs. the control; # $p < 0.05$ vs. IFN-γ-treated ECs. (B) INF-γ-induced expressions of the CXC chemokine gene, IP-10, was investigated by RT-PCR that using GAPDH as an internal control. Result is a representative of three independent experiments with similar results.

induced IP-10 expression was abolished by CAPE pretreatment (Fig. 3B). Consistently, pretreatment of CAPE dose-dependently inhibited IFN-γ-induced IP-10 release. ECs pretreated with CAPE at concentration of 10 μM reduced the IFN-γ-induced IP-10 release to a normal level (Fig. 4). These results indicated that CAPE participated in regulation of IFN-γ-induced IP-10 expression and secretion.

IP-10 was implicated in the recruitment of T cells during inflammation [16]. To address whether the inhibitory effects of CAPE on IP-10 expression and release decreased the T cells recruitment/adhesion to ECs, the adhesiveness of Jurkat T cells to IFN-γ-stimulated ECs with or without CAPE pretreatment was examined. In agreement with the decreased IP-10 release by CAPE, ECs with CAPE pretreatment suppressed IFN-γ-induced Jurkat T cell adhesion (Fig. 5).

These results confirmed that CAPE was able to inhibit STAT-dependent transcriptional activation that leads to suppression of downstream target genes in ECs. Our results suggest the potential for the therapeutic development of CAPE as an anti-inflammatory remedy for suppressing STAT-mediated responses induced by cytokine.

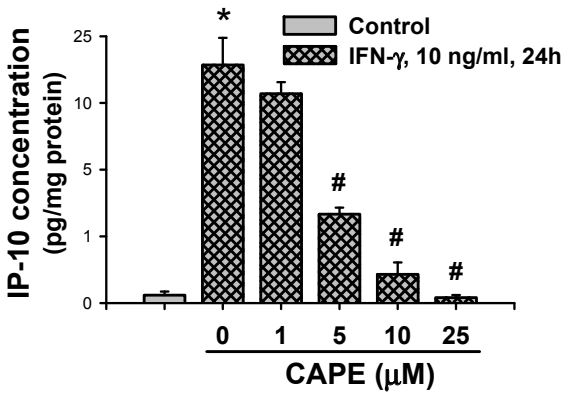

Fig. 4. CAPE inhibits INF-γ-induced IP-10 protein release. After IFN-γ treatment, the cultured medium contains IP-10 was collected for ELISA assay. IP-10 protein release is expressed as pg/mg of total protein. All the results are shown as the mean±SE of three independent experiments. * $p < 0.05$ vs. the control; # $p < 0.05$ vs. IFN-γ-treated ECs.

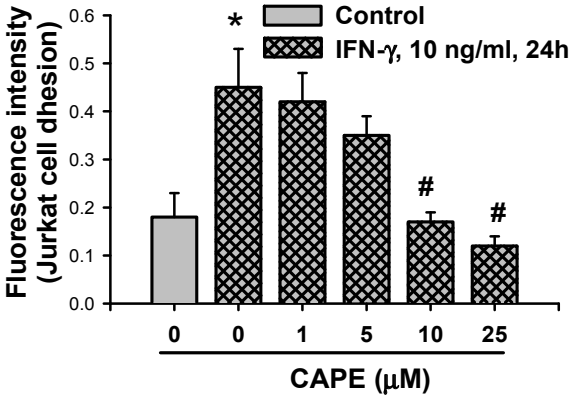

Fig. 5. CAPE suppresses IFN-γ-induced T cell adhesion. ECs were incubated with or without CAPE for 1 h prior to IFN-γ (10 ng ml^{-1}) treatment for 24 h. Treated ECs were then incubated with LavaCell-labeled Jurkat T cells for 1 h. Adherent Jurkat T cells were lysed and the fluorescence intensity was counted. Data are representative for three independent experiments with similar results. Data are shown as mean±SE from 3 separate experiments. * p <0.05 vs. control ECs, # p <0.05 vs. IFN-γ-treated ECs.

Discussion

CAPE is the active component of propolis with anti-inflammatory and antioxidative properties[11]. Anti-inflammatory properties are believed to contribute to its therapeutic effects. In this study, we determined that CAPE inhibited the IFN-γ-induced JAK-STAT1 signaling pathway that results in suppression of downstream inflammation-associated genes. Several lines of evidence support this notion.

First, CAPE inhibited the IFN-γ-induced responses by suppressing STAT1 phosphorylation. Second, it suppressed the IFN-γ-induced IP-10 promoter activity. Third, IFN-γ-induced CXC chemokine gene, IP-10, expression, which contains functional hSIE element in its promoter regions, were suppressed by CAPE pretreatment. Fourth, CAPE inhibited IFN-γ-induced IP-10 protein release. Finally, CAPE pretreatment reduced the adhesion of T cells to IFN-γ-treated ECs. These results provide direct evidence that CAPE exerts atheroprotective effects on ECs by suppressing IFN-γ-induced JAK-STAT1 activation.

The Th1/Th2 is an important switch in the progression of many chronic diseases. It was reported that Th1 cytokines such as IFN-γ and IL-2 are proatherogenic, and Th2 cytokines such as IL-4 and IL-10 are potentially antiatherogenic[17,18]. CAPE-induced down-regulation of Th1-directed CXC chemokines may contribute to the shifting of T-cell responses from Th1 to Th2 in focal areas of atherosclerotic regions. In addition, IP-10, I-Tac, and Mig are antagonists for CCR3[19], a receptor for Th2-specific chemokines. Therefore, inhibition of CCR3 antagonists may promote Th2 cell recruitment/homing to atherosclerotic sites.

JAK-STAT signaling is closely associated with inflammation and accounts for various cellular responses to a number of cytokines, growth factors, and hormones. In the present study, the detailed mechanisms underlying the inhibiting mechanism of CAPE on STAT1 activation are not clear. The phosphorylation of STAT1 upstream kinases, JAK1 and JAK2, was not affected by CAPE treatment. Protein tyrosine phosphatase is involved in dephosphorylation of kinases. Further, it is known that cytokine response and the activation of STATs can be negatively regulated. Among the negative regulators are the suppressor of cytokine signaling (SOCS) proteins[20]. While SOCS proteins interact with JAKs and very probably reduce their tyrosine kinase activity, other inhibitors called PIAS (protein inhibitor of activated STAT) bind to activated STAT dimmers and block their DNA binding activity[21]. The possibility that phosphatase, PIAS or SOCSs contributing to the down-regulation of STAT1 activation cannot be ruled out. The molecular mechanisms of this suppressive effect by CAPE on ECs need for further investigation.

ECs under inflammatory stimulation may produce cytokines, chemokines, and cell adhesion molecules, which participate in vascular remodeling and/or injuries such as atherosclerosis[22]. Suppression of the release of these mediators is important for controlling inflammation. IP-10 is constitutively expressed at low levels; however, expression can be highly induced by interferons in leukocytes as well as in nonleukocytes, including ECs and vascular smooth muscle cells (VSMCs)[11,12]. IP-10 was also shown to potentiate leukocyte adhesion to endothelium and to be a potent mitogenic and chemotactic factor for VSMCs[7]. A growing body of evidence suggests that IP-10 may play a role in chronic inflammatory diseases, including coronary artery disease and related manifestations of atherosclerosis[14,20]. In addition, IP-10 can induce the proliferation of smooth muscle cells[23]. Therefore, the inhibition of IP-10 production by CAPE might play a role in suppression of inflammatory damages.

In conclusion, CAPE, an active compound of propolis suppresses IFN-γ-induced STAT signaling pathways and consequently reduces the CXC

chemokine, IP-10, secretion. Suppression of JAK/STAT activation eventually leads to the reduction of T cells recruitment and help to reduce the atherosclerotic progress. Our results provide a molecular mechanism of CAPE-mediated vascular protection and offer a therapeutic basis for the treatment of atherosclerosis.

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蜂膠成分Caffeic Acid Phenethyl Ester抑制干擾素於內皮細胞中所誘導的STAT1活化及其下游基因表現

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內皮細胞功能異常導致免疫失調及發炎反應的發生，在粥狀動脈硬化過程中扮演著決定性的角色。Caffeic Acid Phenethyl Ester (CAPE)為蜂膠中有效的抗發炎成分，曾被討論對於內皮細胞具有抗發炎與抗氧化的功效。訊息傳導與活化轉錄因子1 (STAT1)與發炎反應及細胞週期轉錄過程有關，在內皮細胞中IFN-γ可以活化JAK-STAT之訊息傳遞途徑。本研究中主要是評估在內皮細胞受損時CAPE是否能夠扮演著抗發炎角色的保護作用，首先發現CAPE本身對於內皮細胞之毒性極低，若事先以CAPE處理過後，可以明顯抑制由IFN-γ所誘導的STAT1的Tyr701及Ser727磷酸化現象，且呈現劑量的相關性，但對於JAK1及JAK2的磷酸化則無明顯的抑制作用。同樣的，CAPE會進一步的抑制具有STAT偶合體連接之啟動子基因IP-10的啟動子活化，IP-10基因表現及蛋白質的分泌也都受到明顯的抑制。此外，T細胞沾黏到內皮細胞的多寡會經由干擾素的誘導而增加，此反應也會被CAPE預處理而壓制。總言之，CAPE對於干擾素所誘導的JAK-STAT1活化路徑的抑制所代表的抗發炎特徵在本研究中獲得充分的闡釋，同時這些相關的認知可以提供用於血管動脈硬化之預防與治療劑之開發參考。

關鍵字：CAPE、內皮細胞、JAK、STAT1、IP-10、干擾素-γ

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