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同半胱胺酸誘發血管平滑肌細胞增生的分子機制

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同半胱胺酸誘發血管平滑肌細胞增生的分子機制

血液中的「同半胱胺酸」(homocysteine)與血管硬化的進行有非常密切的關係，據估計至少有 10%的心臟血管疾病與同半胱胺酸過量有關。細胞培養的實驗結果進一步發現同半胱胺酸有誘發鼠血管平滑肌細胞增生的作用，然而其作用的相關細胞分子機轉，目前尚不是很清楚。內皮素為目前已知具有強力促進血管收縮作用的內生性物質,而且與高血壓及心血管疾病如冠狀動脈硬化的形成有密切關聯，血管平滑肌細胞亦存在著內皮素的接受器。然而有關同半胱胺酸對於內皮素基因的表現作用，在血管細胞上作用的相關機轉，目前相關的文獻仍付之闕如。本研究計劃，釐清內源性的內皮素於同半胱胺酸所誘發血管平滑肌細胞增生的作用中所扮演的角色，並了解同半胱胺酸可以經由增加細胞內活性氧族群，進一步活化內皮素基因表現，據以解釋血液中同半胱胺酸過高易致高血壓及心血管疾病發生的分子機制，並且深入探討同半胱胺酸對內皮素基因表現的作用，其細胞內訊息傳遞的機轉。

關鍵詞：同半胱胺酸; 細胞增生; 內皮素; 訊息傳遞; 血管平滑肌細胞; 活性氧族群

Molecular mechanism of homocysteine-induced proliferation in aortic smooth muscle cells.

Homocysteine is a sulfur-containing amino acid produced from methionine during processing of dietary protein. It has gained considerable attention recently because elevated concentration of total homocysteine are believed to be associated with an increased risk of cardiovascular disease, including coronary artery, cerebrovascular, and peripheral vascular disease. There is evidence that homocysteine can induce vascular smooth muscle cell proliferation in vitro. However, the molecular mechanism(s) of homocysteine in the pathogenesis of cardiovascular diseases remains to be further examined. Endothelin-1 (ET-1) is one of the most potent vasopressors identified to date. It was studied in depth in relation to arterial hypertension and cardiovascular diseases, such as atherosclerosis. Abundant ET-1 receptors present on vascular smooth muscle cells. Recent evidence indicates that reactive oxygen species (ROS) may function as intracellular messengers to modulate signaling pathways. Homocysteine also induces oxidative stress in vascular smooth muscle cells. We previously demonstrated that ROS mediate the induction of ET-1 gene, raised the possibility of its transcriptional regulation by homocysteine in vascular smooth muscle cells. Therefore, it is tempting to hypothesize that endogenous ET-1 mediates the effects of homocysteine and ROS do play a role in homocysteine-induced ET-1 gene expression. However, the direct effect of homocysteine on ET-1 gene expression and the role of ROS in homocysteine-induced cell proliferation and ET-1 gene expression have not been well examined in vascular smooth muscle cells. In this project, we found that endogenous ET-1 mediates homocysteine-induced vascular smooth muscle cell proliferation and explored the detailed intracellular signal transduction pathway of homocysteine-induced ET-1 gene expression in rat aortic smooth muscle cells.

Key Words: homocysteine, proliferation, endothelin-1, signal transduction

報告內容：

前言、

Homocysteine is a sulfur-containing amino acid produced from methionine during processing of dietary protein. It has gained considerable attention recently because elevated concentration of total homocysteine are believed to be associated with an increased risk of cardiovascular disease, including coronary artery, cerebrovascular, and peripheral vascular disease. However, the molecular mechanism(s) of homocysteine in the pathogenesis of cardiovascular disease remains to be clarified. In this project, we examined possible posttranscriptional effects of homocysteine, as well as various signaling pathways and transcription factors known to be important in cell proliferation and endothelin-1 gene regulation in vascular smooth muscle cells.

研究目的、

The goal of this project is to evaluate of the role of ROS in homocysteine-induced cell proliferation and ET-1 gene expression in aortic smooth muscle cells.

文獻探討、

Homocysteine is a thiol-containing amino acid formed as an intermediate metabolite in the conversion of methionine to cysteine. Hyperhomocysteinaemia has recently been recognized as a risk factor of cardiovascular disease (Fanapour et al., 1999). However, the action mechanisms of homocysteine are not well understood. Recent in vitro data has demonstrated the effects of homocysteine on the systemic vasculature. Aortic vascular smooth muscle cells proliferate when exposed to homocysteine (Woo et al., 2000) and increase their production of collagen (Majors et al., 1997). Mitogen-activated protein kinase (MAPK) signaling may also be important in mediating these effects- homocysteine activated extracellular signal-regulated kinase (ERK) in vascular smooth muscle cells (Woo et al., 2000). In endothelial cells, homocysteine induces superoxide anion production which plays a potential role in the early stages of atherosclerosis in the vascular wall (Au-Yeung et al., 2004). However, whether the ROS-sensitive intracellular signaling involves in vascular smooth muscle cell proliferation and the precise mechanisms in response to homocysteine remains to be clarified.

Recent studies in humans (Graf et al., 1997) and animal models (Lapointe et al., 2002) have shown that the expression of endothelin-1 (ET-1) is increased during cardiovascular diseases such as atherosclerosis. It is suggested that ET-1 might contribute to vascular smooth muscle cell proliferation (Hong et al., 2004; Piacentini et al., 2000) resulting in vascular diseases (Ammarguellat et al., 2001). ET-1, a bioactive vasoconstrictor peptide (Yanagisawa and Masaki, 1989), is formed through a specific conversion of its intermediate precursor big ET-1 by an endothelin-converting enzyme. ET-1 works as a paracrine as well as an autocrine. Our previous study revealed that ET-1 mRNA expression was induced by angiotensin II (Ang II) in vascular smooth muscle cells (Hong et al., 2004). Reactive oxygen species (ROS) act as second messengers in receptor-mediated signaling pathways. The increase of ROS in turn regulates various intracellular signal transduction cascades (Tanaka et al., 2001) and the activities of

various transcription factors (Hirotani et al., 2002). Activator protein-1 (AP-1) and GATA2 have been shown to regulate transcription of the ET-1 gene in a cooperative fashion through the GATA and AP1 sites located in the promoter region of ET-1 gene in endothelial cells (Kawana et al., 1995). In addition, Ang II-induced intracellular ROS were involved in ET-1 gene induction through the AP-1 element of ET-1 gene (Hong et al., 2004). Redox-MAPKs have been shown to be important for the post–receptor signaling, through which growth factors stimulate vascular cell proliferation (Pages et al., 1993). However, whether ET-1 expression induced by homocysteine or not and homocysteine acts through this pathway is not known. In this project, we set out to clarify the regulation of ET-1 by homocysteine in vascular smooth muscle cells. We determined the intracellular signal transduction pathways focusing especially on ROS-mediated signaling and transcription factors which involved in this process.

研究方法、

Materials.

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents was from Life Technologies, Inc. ET-1 cDNA is obtained from a human endothelial cell cDNA library as previously described (Wang et al., 1993). PBLCAT2 (containing CAT reporter gene with its promoter) and PBLCAT3 (containing CAT gene only) were constructed as previously described (Cheng et al., 1999). The ECL detection system was from Amersham Pharmacia Biotech. 2',7'-dichlorofluorescin diacetate (DCF-DA) was obtained from Molecular Probes (Eugene, OR, U.S.A.). H2O2 was purchased from Acros Organics (Pittsburgh, PA, U.S.A.). U0126 was obtained from Tocris Cookson Ltd. (Bristol, UK). The plasmid AP-1-Luc containing the firefly luciferase reporter gene driven by a basic promoter element (TATA box) joined to tandem repeats of AP-1 binding element were obtained from Stratagene (La Jolla, CA, U.S.A.). N-acetyl-cysteine (NAC), and all other chemicals of reagent grade, were obtained from Sigma (St. Louis, MO, U.S.A.).

Culture of Aortic Smooth Muscle Cells.

Thoracic aortae from male Sprague-Dawley rats are excised rapidly and immersed in DMEM containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Connective tissue and adherent fat are cleaned away from the specimens. Isolated arteries are cut open, and the endothelium was removed by gently rubbing off the intimal surface with a pair of sharp scissors. Denuded aortae are cut into ~3-mm pieces and placed with the intimal face down into three 35-mm culture dishes (Iwaki, Osaka, Japan). DMEM containing 10% fetal calf serum and penicillin/streptomycin is gently added to the dishes to cover the tissues without disturbing the orientation of the explants. Vascular smooth muscle cells are allowed to proliferate from the tissue (7-10 days), and the tissues are removed using sterilized fine forceps and washed with culture medium. After reaching confluence in three 35-mm dishes, cells are harvested by brief trypsinization and subsequently cultured in T-75 flasks (Iwaki) (passage 1). Human aortic smooth muscle cells are purchased from Cascade Biologics (Portland, Oregon, USA). Cells are routinely propagated in culture dishes to 75–95% confluence, and used between passages 3–12. The purity of smooth muscle cells is evaluated by staining the cells with monoclonal antibodies to α-smooth

muscle actin. Cellular viability under all treatment conditions is determined by cell count, morphology, and trypan blue exclusion.

DNA Synthesis

To measure synthesis of new DNA, cells (1 x 10^5/well) were plated in six-well (35-mm) dishes 24 h before experiments as previously described (Liu et al., 2003). Cells were incubated with [^{3}H]thymidine (5μ Ci/ml). Following the treatment as indicated, cells were harvested by incubation at 4 °C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH, and radioactivity is determined by scintillation counting. Data were presented as the mean ± SEM of 9-12 determinations for six different cell preparations and normalized to the untreated sample x 100 (*i.e*. percentage of control).

RNA isolation and Northern Hybridization

Total RNA was obtained by using guanidine thiocyanate as described previously (Cheng et al., 2001). Total RNA was collected and examined by mini-gel agarose electrophoresis. Ten micrograms of RNA were mixed with loading buffer containing ethidium bromide. The sample mixture was loaded and separated on 1% agarose gels containing 3.7% formaldehyde. The RNA was transferred onto Nytran membrane (Schleicher and Schuell, Germany) by a vacuum blotting system (VacuGene XL, Pharmacia, Sweden) using 20X standard sodium citrate (SSC; 3M NaCl, 0.3 M sodium citrate, pH7) and immobilized by ultraviolet irradiation. After prehybridization, the membrane is hybridized with the 32P-labled 0.6 kb ET-1 cDNA probe. The membrane was washed and then exposed to X-ray film (Kodak X-Omat-AR) at -70℃. Autoradiographic results were scanned and analyzed using a densitometer (Computing Densitometer 300S, Molecular Dynamics).

Transfections

Cells were transiently transfected with different expression vectors by the calcium phosphate method as previously described (Cheng et al., 2001).

Chloramphenicol Acetyltransferase (CAT) Assays and β-Galactosidase Assays

The CAT and β-galactosidase assays were performed. The relative CAT activity was determined by normalizing the CAT value to its respective β-galactosidase activity. Cotransfected β-galactosidase activity was observed to vary by less than 10% within a given experiment and was not affected by any of the experimental manipulations described. As positive and negative controls, pBLCAT2 (with thymidine kinase promoter) and pBLCAT3 (without promoter) were included in every assay.

Assay of Intracellular ROS

ROS were measured using a previously described method (Cheng et al., 2001). Prior to the chemical treatment, cells were incubated in culture medium containing a fluorescent dye, DCF-DA of 30μM for 1 h to establish a stable intracellular level of the probe. The same concentration of DCF-DA was maintained during the chemical treatment. Subsequently, the cells were washed with PBS, removed from Petri dishes by brief trypsinization, and measured for 2',7'-dichlorofluorescein (DCF) fluorescence intensity. The DCF fluorescence intensity of the cells was an index of intracellular levels of ROS; and it was determined by fluorescence spectrophotometry with excitation and emission wavelengths at 475 and 525 nm, respectively.

The cell number in each sample was counted and utilized to normalize the fluorescence intensity of DCF.

Western Blot Analysis

Rabbit polyclonal anti-phospho-specific ERK antibodies were purchased from New England Biolabs (Beverly, MA, U.S.A.). Anti-ERK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Western blot analysis was performed as previously described (Cheng et al., 2001).

Luciferase Assay

Cells plated on 3-cm-diameter culture dishes were transfected with the luciferase reporter construct possessing consensus AP-1 binding sites (AP-1-Luc) binding sites (Stratgene, La Jolla, CA, U.S.A.). After incubation for 24 h in serum-free DMEM, Cells were cultured under different treatments as indicated for 48 h. ECV304 were assayed for luciferase activity with a luciferase reporter assay kit (Strategene, La Jolla, CA, U.S.A.). The firefly luciferase activities as AP-1 transcriptional activity were normalized for transfection efficiency to its respective β-galactosidase activity and expressed as relative activity to control.

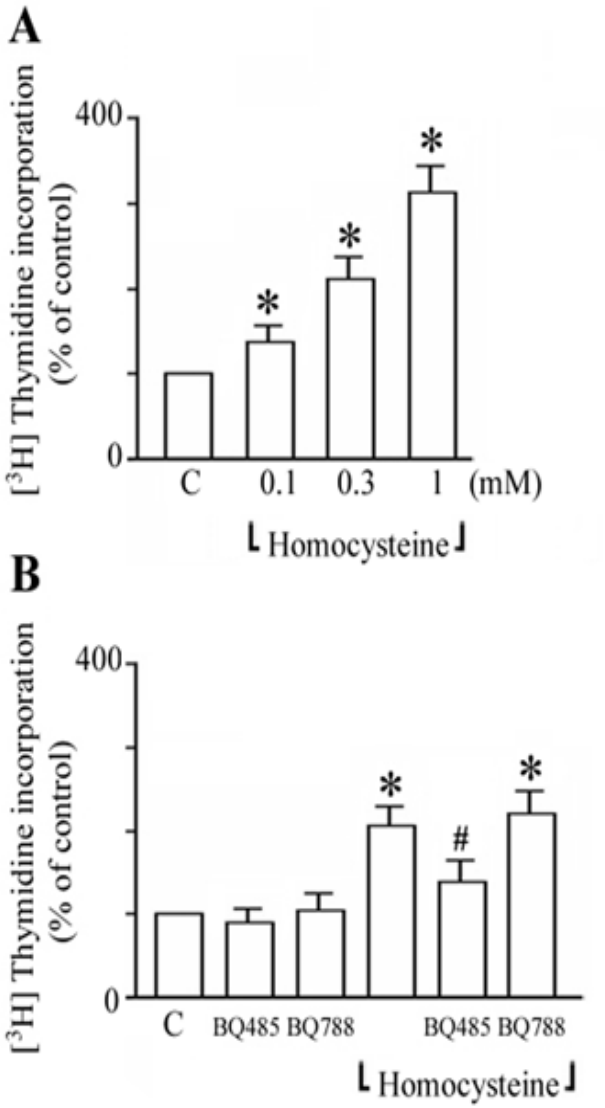
Statistical Analysis

Results are expressed as mean $\pm$ SEM of at least three experiments unless designated otherwise. Statistical analysis was performed using analysis of variance (ANOVA) and Student's t test as appropriate. A value of $p<0.05$ was considered to be statistically significant.

結果與討論（含結論與建議）、

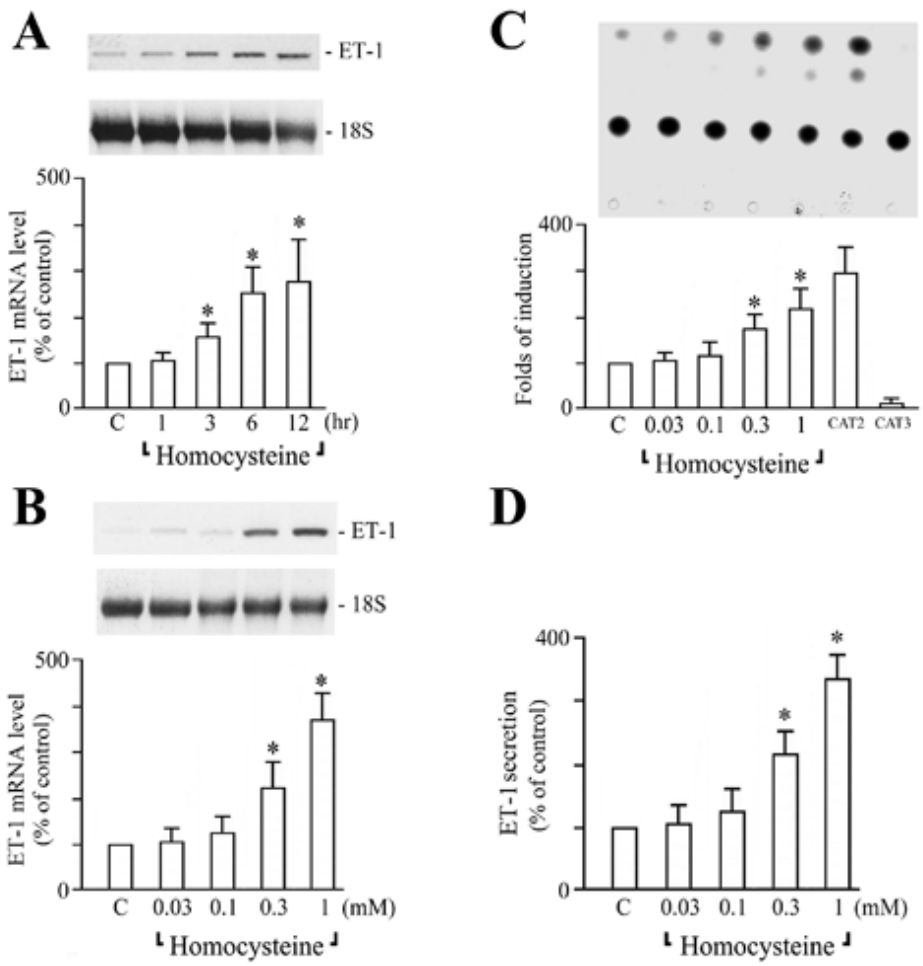
Role of Endogenous ET-1 in homocysteine-induced proliferation of smooth muscle cells

Homocysteine -stimulated rat aortic smooth muscle cell proliferation was assessed by analyzing DNA synthesis with [^{3}H]thymidine incorporation. Homocysteine increased DNA synthesis in rat aortic smooth muscle cells in a dose-dependent manner (Figure 1A). The maximum level of incorporation was 3.1-fold increase compared with the control. Homocysteine (0.3 mM)-stimulated DNA synthesis in SMCs was inhibited by ET_A receptor antagonist BQ485 (1μM) treatment (Figure 1B). These data suggest the possible role of endogenous ET-1 as an autocrine growth factor for the proliferation of SMCs under homocysteine stimulation.



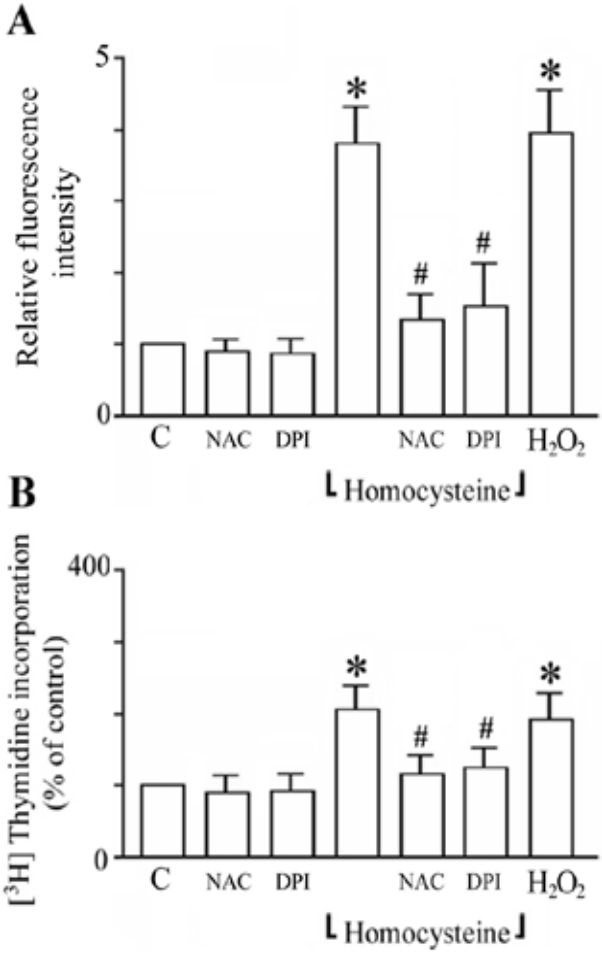
Homocysteine-induced ET-1 gene expression in smooth muscle cells

To examine whether homocysteine increases ET-1 mRNA levels in SMCs, we performed Northern blot analysis (Figure 2A and B). ET-1 mRNA was induced by homocysteine (0.3 mM) as early as three hours (Figure 2A). When SMCs were treated with homocysteine for 6hr, the homocysteine-induced ET-1 mRNA expression was dose-dependent with the maximum induction at 1 mM (Figure 2B). To determine whether the homocysteine-induced ET-1 expression is regulated at the transcriptional level, an ET-1 promoter construct containing the ET-1 promoter region (–4.4 kb) and the reporter gene CAT was constructed and transiently transfected into smooth muscle cells. SMCs exposed to 24hr of homocysteine (0.3 mM) significantly increased ET-1 promoter activity (Figure 2C). Dose-dependency of ET-1 expression by homocysteine had also been performed at the protein level (Figure 2D). Homocysteine dose-dependently increased ET-1 peptide secretion (Figure 2D). These data show that homocysteine directly induces ET-1 gene expression in SMCs.

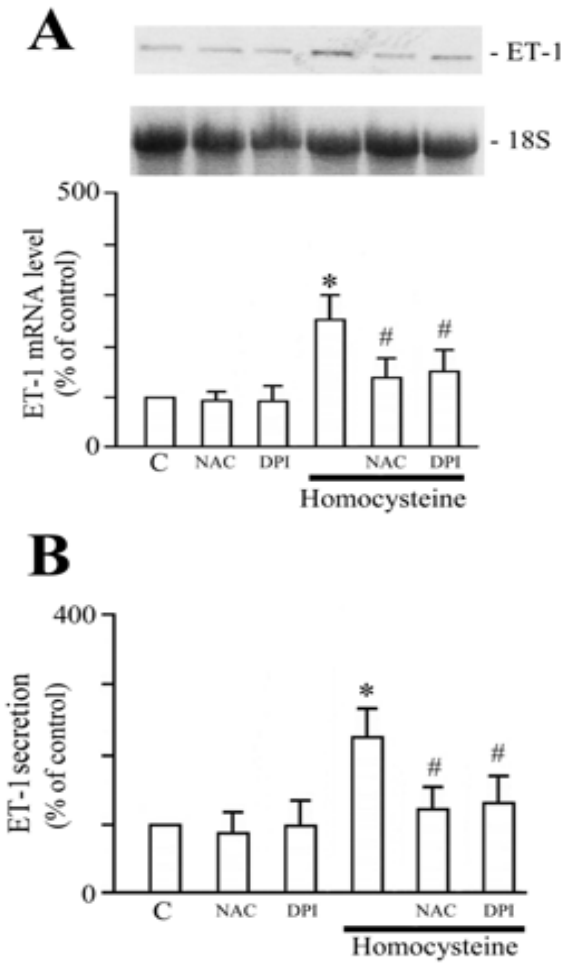


ROS mediate homocysteine-induced proliferation and ET-1 gene expression in smooth muscle cells

To confirm previous observations that homocysteine induces intracellular ROS in SMCs, we measured intracellular ROS level by analyzing the fluorescent product DCF, a peroxidative product of DCF-DA. SMCs treated with homocysteine (0.3 mM) had significantly higher ROS levels than those cells treated with vehicle only (Figure 3A). The increase of ROS was completely blocked by pretreatment of cells with antioxidants such as N-acetylcysteine (NAC) or diphenyleneiodonium (DPI), a flavoprotein containing the NADH/NADPH oxidase inhibitor. Olmesartan (1μM), NAC (10mM) and DPI (10μM) all showed a significant reduction in ROS production (Figure 3A). To elucidate the involvement of ROS in the homocysteine-induced proliferation, SMCs were pretreated with NAC or DPI for 30min followed by homocysteine treatment. SMCs pretreated with NAC (10mM) or DPI (10μM) significantly suppressed homocysteine-induced [^{3}H]thymidine uptake (Figure 3B).

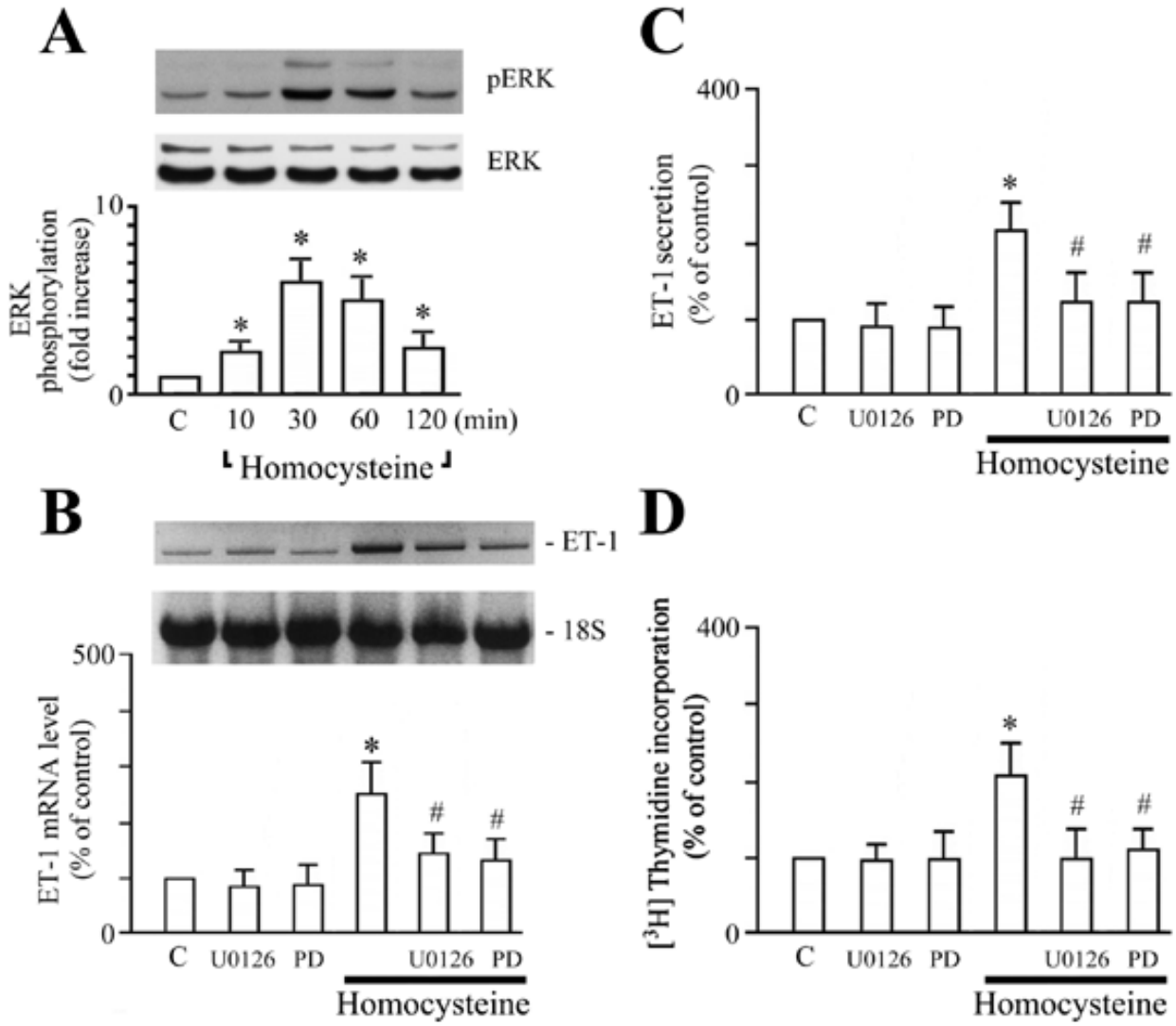


To further examine the ROS involvement in the homocysteine-induced ET-1 gene expression, SMCs were preincubated with an antioxidant NAC or DPI for 30min and then treated with homocysteine. As shown in Figure 4A, SMCs pretreated with NAC (10mM) or DPI (10μM) significantly suppressed homocysteine-induced ET-1 mRNA level. Similarly, cells pretreated with NAC or DPI also suppressed homocysteine-increased ET-1 secretion (Figure 4B). These findings suggest that intracellular ROS generation apparently mediate homocysteine-induced proliferation and ET-1 gene expression in rat aortic SMCs.



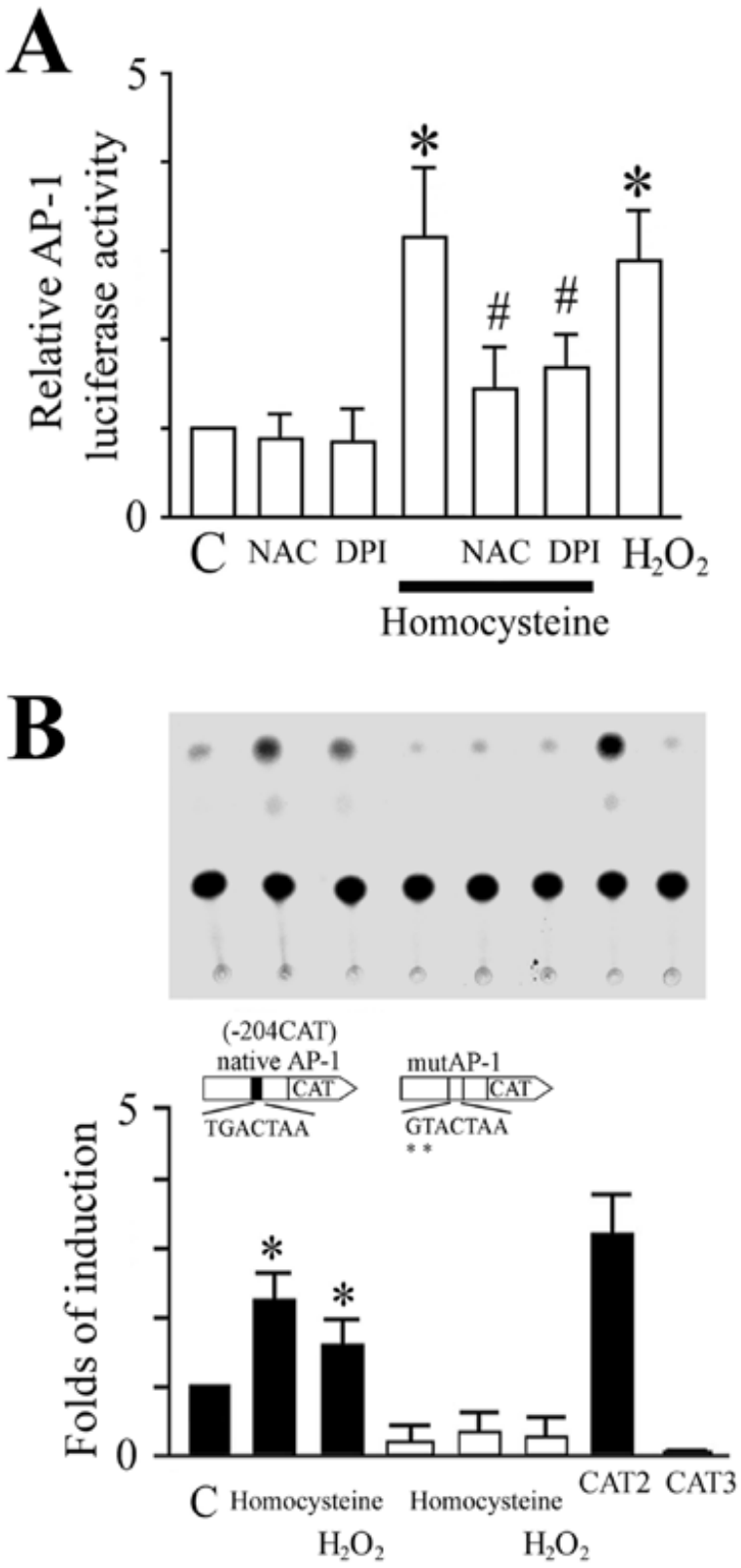
Homocysteine-induced ET-1 gene expression is mediated via ERK pathway

To study whether intracellular signaling pathways were involved in homocysteine-induced ET-1 gene expression in smooth muscle cells, we examined the effect of homocysteine on ERK pathway and determined the effect of ERK inhibitors on homocysteine-induced ET-1 expression. We first determined whether homocysteine increases phosphorylation of ERK1/2 in smooth muscel cells (Figure 4A). Both DPI and NAC significantly inhibited homocysteine-induced phosphorylation of ERK1/2 (Figure 4B). These data suggest that ERK1/2 are redox-sensitive signaling pathways activated by homocysteine in smooth muscle cells. We next determined the role of redox-sensitive activation of ERK in homocysteine-induced ET-1 gene expression. PD98059 (PD) and U0126, specific inhibitors of MKK-1 (MEK), inhibited augmentation of ET-1 expression stimulated with homocysteine (Figure 4C D). These findings suggest that activation of ERK is a necessary step for ET-1 gene expression induced with homocysteine.



Identification of homocysteine-responsive regulatory elements in the ET-1 promoter

The ET-1 promoter contains a number of AP-1 and GATA sites, which can be regulated by multiple activation pathways (10,14). Pretreating cells with antioxidants, NAC or DPI attenuated the homocysteine-stimulated AP-1-mediated reporter activity (Figure 5A). These results clearly indicate that ROS mediate the transcriptional activity of AP-1 induced by homocysteine in smooth muscle cells. We further examined whether AP-1 site is essential for the induction of ET-1 gene by homocysteine. In cells transfected with reporter construct -204CAT containing both GATA and AP-1 sites with two-bp mutation in the AP-1 site, the homocysteine-induced ET-1 promoter activity was completely abolished. In addition, the basal promoter activity also decreased as compared with control (Figure 5C). These findings suggest that the AP-1 binding element is essential for the induction of ET-1 gene by homocysteine.



參考文獻、

Ammarguellat, F., I.I. Larouche and E.L. Schiffrin, 2001, Myocardial Fibrosis in DOCA-Salt Hypertensive Rats : Effect of Endothelin ET(A) Receptor Antagonism, Circulation 103, 319.

Au-Yeung, K.K., C.W. Woo, F.L. Sung, J.C. Yip, Y.L. Siow and K. O, 2004, Hyperhomocysteinemia activates nuclear factor-kappaB in endothelial cells via oxidative stress, Circ Res 94, 28.

Cheng, T.H., N.L. Shih, S.Y. Chen, S.H. Loh, P.Y. Cheng, C.S. Tsai, S.H. Liu, D.L. Wang and J.J. Chen, 2001, Reactive oxygen species mediate cyclic strain-induced endothelin-1 gene expression via Ras/Raf/extracellular signal-regulated kinase pathway in endothelial cells, J Mol Cell Cardiol 33, 1805.

Cheng, T.H., N.L. Shih, S.Y. Chen, D.L. Wang and J.J. Chen, 1999, Reactive oxygen species modulate endothelin-I-induced c-fos gene expression in cardiomyocytes, Cardiovasc Res 41, 654.

Fanapour, P.C., B. Yug and M.S. Kochar, 1999, Hyperhomocysteinemia: an additional cardiovascular risk factor, Wmj 98, 51.

Graf, K., Y.S. Do, N. Ashizawa, W.P. Meehan, C.M. Giachelli, C.C. Marboe, E. Fleck and W.A. Hsueh, 1997, Myocardial osteopontin expression is associated with left ventricular hypertrophy, Circulation 96, 3063.

Hirotani, S., K. Otsu, K. Nishida, Y. Higuchi, T. Morita, H. Nakayama, O. Yamaguchi, T. Mano, Y. Matsumura, H. Ueno, M. Tada and M. Hori, 2002, Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy, Circulation 105, 509.

Hong, H.J., P. Chan, J.C. Liu, S.H. Juan, M.T. Huang, J.G. Lin and T.H. Cheng, 2004, Angiotensin II induces endothelin-1 gene expression via extracellular signal-regulated kinase pathway in rat aortic smooth muscle cells, Cardiovasc Res 61, 159.

Kawana, M., M.E. Lee, E.E. Quertermous and T. Quertermous, 1995, Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene, Mol Cell Biol 15, 4225.

Lapointe, N., C. Blais, Jr., A. Adam, T. Parker, M.G. Sirois, H. Gosselin, R. Clement and J.L. Rouleau, 2002, Comparison of the effects of an angiotensin-converting enzyme inhibitor and a vasopeptidase inhibitor after myocardial infarction in the rat, J Am Coll Cardiol 39, 1692.

Liu, J.C., J.J. Chen, P. Chan, C.F. Cheng and T.H. Cheng, 2003, Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol, Hypertension 42, 1198.

Majors, A., L.A. Ehrhart and E.H. Pezacka, 1997, Homocysteine as a risk factor for vascular disease. Enhanced collagen production and accumulation by smooth muscle cells, Arterioscler Thromb Vasc Biol 17, 2074.

Pages, G., P. Lenormand, G. L'Allemain, J.C. Chambard, S. Meloche and J. Pouyssegur, 1993, Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation, Proc Natl Acad Sci U S A 90, 8319.

Piacentini, L., M. Gray, N.Y. Honbo, J. Chentoufi, M. Bergman and J.S. Karliner, 2000, Endothelin-1 stimulates cardiac fibroblast proliferation through activation of protein kinase C, J Mol Cell Cardiol 32, 565.

Tanaka, K., M. Honda and T. Takabatake, 2001, Redox regulation of MAPK pathways and cardiac hypertrophy in adult rat cardiac myocyte, J Am Coll Cardiol 37, 676.

Wang, D.L., C.C. Tang, B.S. Wung, H.H. Chen, M.S. Hung and J.J. Wang, 1993, Cyclical strain increases endothelin-1 secretion and gene expression in human endothelial cells, Biochem

Biophys Res Commun 195, 1050.
Woo, D.K., S.J. Dudrick and B.E. Sumpio, 2000, Homocysteine stimulates MAP kinase in bovine aortic smooth muscle cells, Surgery 128, 59.
Yanagisawa, M. and T. Masaki, 1989, Molecular biology and biochemistry of the endothelins, Trends Pharmacol Sci 10, 374.

計畫成果自評、

研究內容與原計畫完全相符、順利達成預期目標、研究成果之學術價值甚高、即將投稿至國外 SCI 學術期刊發表。