Molecular Pharmacology Fast Forward. Published on April 13, 2006 as doi:10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Resveratrol Suppresses TNF-α–induced Fractalkine in Endothelial Cells

Sang-Ok Moon, Won Kim, Mi Jeong Sung, Sik Lee, Kyung Pyo Kang, Duk Hoon Kim,

Sang Yong Lee, June-No So, and Sung Kwang Park

Renal Regeneration Laboratory and Department of Internal Medicine

(S.O.M., W.K., M.J.S., S.L., K.P.K., D.H.K., S.K.P.)

Department of Diagnostic Radiology (S.Y.L.),

Research Institute of Clinical Medicine, Chonbuk National University Medical School,

Department of Pharmaceutical Engineering (J.N.S.), Woosuk University,

Jeonju,

Republic of Korea

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Resveratrol Suppresses Fractalkine Expression

To whom correspondence should be addressed:

Sung Kwang Park, M.D., Ph.D.

Renal Regeneration Laboratory and Department of Internal Medicine

Chonbuk National University Medical School

San 2-20 Keumam-dong, Jeonju

561-180

Republic of Korea

Phone: 82-63-250-1683

Fax: 82-63-254-1609

E-mail: parksk@chonbuk.ac.kr

The number of text pages: 34

The number of tables: 0

The number of figures: 6

The number of references: 40

The number of words in the abstract: 169

The number of words in the introduction: 639

The number of words in the discussion: 520

Abbreviations: HUVEC, human umbilical vein endothelial cell; EMSA, electrophoretic mobility shift assay.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Up-regulation of fractalkine is involved in vascular and tissue damage in inflammatory conditions. Resveratrol has been shown to have anti-inflammatory, antioxidant, and antitumor activities. Its regulatory effects on expression of fractalkine in vascular endothelial cells and fractalkine receptor, CX3CR1 in monocytes have not been studied. We evaluated the effects of resveratrol on fractalkine expression in human umbilical vein endothelial cells and CX3CR1 expression in THP-1 cells in response to treatment with tumor necrosis factor (TNF)-α. TNF-α significantly induced fractalkine mRNA and protein expression in endothelial cells. Resveratrol strongly suppressed TNF-α-induced fractalkine expression in endothelial cells through suppression of nuclear factor-κB and Sp1 activities. Resveratrol decreased the number of TNF-α-induced fractalkine-positive endothelial cells and CX3CR1-positive cells determined by flow cytometric analysis. Resveratrol suppressed TNF-α-stimulated monocytes adhesion to human umbilical vein endothelial cells. Immunohistochemical analysis revealed that resveratrol suppressed TNF-α-induced arterial endothelial fractalkine expression in heart, kidney, and intestine and decreased ED-1-positive cell infiltration in intestinal villi. Resveratrol may provide a new pharmacological approach for suppressing fractalkine/CX3CR1-mediated injury in inflammatory conditions.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Adherence of circulating inflammatory cells and migration to the subendothelial space is an initial process in inflammatory conditions (Cines et al., 1998; Gimbrone et al., 1997). Chemokines in endothelial cells and those receptors in inflammatory cells are critical in the initiation, maintenance, and resolution of inflammation (Fujiwara and Kobayashi, 2005). Fractalkine (CX3CL1) is the only CX3C-chemokine in which a soluble chemokine-like domain is fused to a mucin stalk that extends across the cell membrane into the cytoplasm (Bazan et al., 1997; Pan et al., 1997). The expression of membrane-bound fractalkine can be markedly induced on primary endothelial cells by inflammatory cytokines, such as interferon-γ, interleukin-1 and tumor necrosis factor (TNF)-α (Garcia et al., 2000). Fractalkine can function both as a chemoattractant and an adhesion molecule for its receptor, CX3CR1 (Haskell et al., 1999; Imai et al., 1997).

Expression of CX3CR1 and migration toward fractalkine have been demonstrated for monocytes/macrophages, some T cells, and natural killer (NK) cells (Fong et al., 1998; Foussat et al., 2000; Harrison et al., 1998; Imai et al., 1997). Thus, fractalkine expressed on inflamed endothelium may be a vascular gateway for CX3CR1-expressing cells by rapidly capturing them

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

from the blood and promoting their migration into tissue. Since up-regulation of fractalkine is involved in vascular and tissue damage in various diseases, such as atherosclerosis (Lesnik et al., 2003), glomerulonephritis (Segerer et al., 2002), cardiac allograft rejection (Robinson et al., 2000), HIV infection (Foussat et al., 2001), and rheumatoid arthritis (Nanki et al., 2004) down-regulation of fractalkine expression can be important in preventing and treating these diseases. Only a few substances-the soluble form of interleukin-6 receptor-α (Matsumiya et al., 2001), 15-deoxy-$\delta^{12,14}$-prostaglandin J_2 (Imaizumi et al., 2002) and hypoxia (Yamashita et al., 2003) were reported to inhibit fractalkine expression in endothelial cells. Recently, we have reported that α-lipoic acid reduces fractalkine-mediated inflammatory processes in endotoxemia (Ahn et al., 2004; Sung et al., 2005). It is well known that CX3CR1 is expressed in monocytes/macrophages and is the mechanism of monocytes/macrophages capture, firm adhesion, and activation (Fong et al., 1998). A polymorphism in the CX3CR1 gene was also reported to be associated with low CX3CR1 expression and reduced risk of acute coronary disease in humans (Combadiere et al., 2003). Because fractalkine and CX3CR1 are critical to inflammation, therapeutic interventions that target fractalkine in endothelial cells and CX3CR1 in monocytes/macrophages may open new avenues for controlling inflammatory diseases.

Resveratrol (trans-3,4',5-trihydroxy-*trans*-stilbene), a polyphenolic compound present in grapes

and red wine, has been reported to have a cardioprotective effect via an antioxidant effect (Fauconneau et al., 1997; Frankel et al., 1993), cancer chemopreventive activity (Jang et al., 1997), suppression of cellular smooth muscle proliferation/migration (Araim et al., 2002), and inhibition of platelet aggregation (Pace-Asciak et al., 1996). At concentrations present in human plasma after moderate wine consumption, resveratrol inhibits the expression of adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin by agonist-stimulated endothelial cells (Bertelli et al., 2001; Pendurthi and Rao, 2002). Ferrero et al (Ferrero et al., 1998) also have reported that resveratrol reduces granulocyte and monocyte adhesion to endothelial cells. The anti-inflammatory activity of resveratrol may be related to interference with the nuclear factor (NF)-κB signaling pathway, which regulates the expression of various genes involved in inflammation (Manna et al., 2000; Tsai et al., 1999). However, effects of resveratrol on fractalkine expression and its signal pathway in endothelial cells and on CX3CR1 expression in monocytes/macrophages have not been examined.

In this study, we examined whether resveratrol decreases the expressions of TNF-α-induced fractalkine in human umbilical vein endothelial cells (HUVECs) and TNF-α-induced CX3CR1 in THP-1 cells. Furthermore, we evaluated the role of resveratrol in TNF-α-induced endothelial fractalkine expression *in vivo*. Our results show that resveratrol prevents TNF-α-induced up-

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

regulation of fractalkine in endothelial cells and CX3CR1 in monocytes/macrophages, identifying a novel mechanism of resveratrol for preventing vascular inflammation.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Materials and cell culture.

Anti-fractalkine antibody (full length) and anti-CX3CR1 antibody were purchased from Torrey Pines BioLabs (Houston, TX). Anti-phospho-p65 (Ser536) and anti-p65 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-p65, anti-p50, and anti-Sp1 for gel supershift assay were from Santa Cruz Biotechnology (Santa Cruz, CA). Mitogen-activated protein kinase kinase 1/2 inhibitor PD98059, NF-κB inhibitor MG-132, protein kinase C inhibitor calpositin C and N-[2-(p-bromo cinnamyl amino) ethyl]-5-isoquinoline sulfonamide (H-89) were purchased from Calbiochem (San Diego, CA). Resveratrol, phosphatidylinositol 3′-kinase inhibitor wortmannin, NF-κB inhibitor pyrrolidine dithiocarbamate, TNF-α, media, sera, and most other biochemical reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. HUVECs were prepared from human umbilical cords by collagenase digestion as previously described (Ahn et al., 2004). THP-1 cells were from American Type Culture Collection (Manassas, VA).

RNase protection assay (RPA) and immunoblotting.

RPA and immunoblotting were performed as previously described (Ahn et al., 2004). The

membrane was reblotted with anti-actin antibody to verify equal loading of protein in each lane.

Monocyte isolation and adhesion assay.

The study protocol and informed consent forms were approved by the Chonbuk National University Hospital Review Board, and subjects were given informed consent. Human peripheral blood monocytes were isolated from fresh blood from healthy volunteers by Ficoll-Paque gradient centrifugation. Monocytes were further purified by negative selection using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) (Ancuta et al., 2003). Monocyte-endothelial adhesion was determined by previously used methods (Kim et al., 2003).

Mononuclear cells from circulating rat blood were separated by according to a previously described method (Mazzucchelli et al., 2004). Gradient centrifugation with a Histopaque-1083 (Sigma) was undertaken to optimize recovery of viable mononuclear cells. After centrifugation the opaque interface containing mononuclear cells was transferred to a new tube and washed twice with phosphate buffered saline by centrifugation at 250 × g for 10 min. Finally, viable cells were counted using the Trypan Blue exclusion test.

Flow cytometry.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

For flow cytometry analysis for fractalkine, HUVECs were treated as previously described (Sung et al., 2005). For flow cytometry analysis for CX3CR1, THP-1 cells and rat ED-1-positive monocytes/macrophages were used according to a previously described method (Sung et al., 2005).

Electrophoretic mobility shift assay (EMSA).

EMSA for NF-κB and Sp1 proteins were performed as previously described (Ahn et al., 2004). Signals were detected by chemiluminescent imaging according to the manufacturer's protocol (EMSA Gel-Shift kit; Panomics, Redwood City, CA).

Luciferase assay

HUVECs were plated at a density of 5.0 x 10^4 cells per well on 24-well plates and transfected using SuperFectant transfection reagent (Qiagen, Hilden, Germany) in serum-free medium according to the manufacturer's protocol. Cells were co-transfected with 1 μg/well pNF-κB-Luc or pSp1-Luc, firefly luciferase reporter constructs, and with 0.25 μg of a *Renilla* luciferase control reporter vector (pRL-TK; Promega, Madison, WI) to normalize transfection efficiency. At 6 h after transfection, the medium was changed and cells were further cultured for another 24 h. Cells were treated for an additional 6 h with stimulant and then lysed with Passive Lysis

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Buffer (Promega). Firefly and *Renilla* luciferases activities were assayed using a MiniLumat LB 9506 (Berthold Technologies, Bad Wildbad, Germany) with the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity represents the ratio of the activity of firefly to *Renilla* luciferase activity.

Animal experiments.

Inbred male Sprague-Dawley rats (180–200 g) were obtained from Orient (Charles River Korea, Seoul, Korea) and were maintained on standard laboratory diet and water *ad libitum*. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Chonbuk National University Medical School. The rats were divided into 3 groups: vehicle (0.1% DMSO; n=5), TNF-α (10 μg/kg; n=5), and TNF-α (10 μg/kg) plus resveratrol (50 mg/kg; n=5). Resveratrol was suspended in 0.3% carboxymethyl cellulose solution and given to rats by oral intubation at a dose of 50 mg in 0.5 mL of 0.3% solution/kg daily for 14 days.(Arichi et al., 1982) Control (vehicle) rats similarly received 0.3% carboxymethyl cellulose solution alone. Control buffer and TNF-α were then injected intravenously through the tail vein. Twelve hours after injection of vehicle or TNF-α, rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and sacrificed by cervical dislocation. Heart, kidney, and intestine were harvested for immunohistochemical analysis.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Immunohistochemical analysis of fractalkine and ED-1.

Immunohistochemical analysis were performed as previously described method (Sung et al., 2005).

Densitometric analyses and statistics.

Data are expressed as mean ± standard deviation (S.D.). Statistical significance was tested using Student's *t* test or one-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at $p<0.05$.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

TNF-α increases expression of fractalkine mRNA and protein in HUVECs.

We previously reported that TNF-α produces a maximal effect on the expression of fractalkine mRNA in HUVECs at 4 h (Ahn et al., 2004). Therefore, we examined the effect of various concentrations of resveratrol on TNF-α–induced fractalkine mRNA at this time. TNF-α (10 ng/mL) also increased the expression of fractalkine mRNA in a dose-dependent manner. Addition of TNF-α (10 ng/mL) maximally increased expression of fractalkine protein at 6 h, and the level continued to be higher than control for up to 24 h. The maximum mean increase in fractalkine was 9.5-fold.

Inhibitors Changed TNF-α-Stimulated Expression of Fractalkine mRNA.

We previously reported that NF-κB inhibitor MG-132 and pyrrolidine dithiocarbamate suppressed TNF-α-induced expression of fractalkine mRNA (Ahn et al., 2004). However, phosphatidylinositol 3′-kinase inhibitor wortmannin, mitogen-activated protein kinase kinase 1/2 inhibitor PD98059, protein kinase C inhibitor calpostin C, and protein kinase A inhibitor N-[2-(p-bromo cinnamyl amino) ethyl]-5-isoquinoline sulfonamide (H-89) did not produce any changes. These results suggested that TNF-α-stimulated expression of fractalkine might be

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

mediated mainly through activation of NF-κB pathways.

Resveratrol suppresses TNF-α-induced fractalkine mRNA and protein in HUVECs.

Because TNF-α produces a maximal effect on the expression of fractalkine mRNA in HUVECs at 4 h, we examined the effect of various concentrations of resveratrol on TNF-α-induced fractalkine mRNA at this time. Resveratrol suppressed TNF-α-induced expression of fractalkine mRNA in a dose-dependent manner (Fig. 1A). Resveratrol at 0.5 μmol/L suppressed approximately 20–30% of TNF-α-induced expression of fractalkine mRNA; higher concentrations of resveratrol (5 and 10 μmol/L) almost completely suppressed TNF-α-induced fractalkine mRNA expression. However, a high concentration of resveratrol (5 μmol/L) alone did not significantly affect on the mRNA levels of fractalkine. In agreement with the RPA data, resveratrol at 1 μmol/L suppressed approximately 30–40% of TNF-α-induced expression of fractalkine protein whereas resveratrol at 5 and 10 μmol/L almost completely suppressed TNF-α–induced fractalkine protein expression (Fig. 1B).

Resveratrol reduces the number of fractalkine-positive cells in TNF-α-stimulated HUVECs.

The number of fractalkine-positive cells was increased about 6-fold in HUVECs after stimulation with TNF-α; after resveratrol treatment, the number was decreased to the control level by flow

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

cytometry (Fig. 1C). These data suggest that resveratrol inhibits TNF-α-induced fractalkine expression in HUVECs.

Resveratrol reduces CX3CR1 protein and the number of CX3CR1-positive cells in TNF-α–stimulated THP-1 and ED-1-positive monocytes/macrophages.

In Western blot analyses, TNF-α increased CX3CR1 protein level about 3.0-fold at 6 h in THP-1 cells (Fig. 2A). Resveratrol suppressed TNF-α-induced expression of CX3CR1 protein in a dose-dependent manner (Fig. 2A). Addition of resveratrol at 1 μmol/L suppressed approximately 17% of TNF-α-induced expression of CX3CR1 protein whereas higher concentrations of resveratrol (5 μmol/L) suppressed to the control level. However, a high concentration of resveratrol (5 μmol/L) alone did not significantly affect the protein levels of CX3CR1 (Fig. 2A).

The number of CX3CR1-positive cells was increased about 2.5-fold in THP-1 cells after stimulation with TNF-α; after resveratrol treatment, the number was decreased to the control level by flow cytometry (Fig. 2B). We also used flow cytometry to evaluate the change in the number of CX3CR1-positive cells in ED-1-positive monocytes/macrophages prepared from rats treated with TNF-α. Compared with the controls, the number of CX3CR1-positive cells in ED-1-positive monocytes/macrophages was increased about 2.7–fold at 12 h. Pretreatment with resveratrol (50 mg/kg per day) decreased the number of TNF-α-induced CX3CR1-positive cells

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

in ED-1-positive monocytes/macrophages by approximately 47%. These results indicate that resveratrol inhibits the TNF-α-induced CX3CR1 expression in THP-1 cells and in ED-1-positive monocytes/macrophages in rat blood.

Resveratrol suppresses TNF-α–induced monocyte adhesiveness to HUVECs.

Because the induction of fractalkine in endothelial cells induces monocyte adhesion (Ancuta et al., 2003), we examined whether resveratrol decreases monocyte adhesion to TNF-α-stimulated HUVECs. Stimulation of HUVECs with TNF-α (10 ng/mL) for 6 h induced a significant increase (~4.7-fold) in monocyte adhesion compared with treatment with control buffer but treatment with resveratrol led to a 58% decrease in monocyte adhesion (Fig. 3). Furthermore, treatment of TNF-α-stimulated cells with an anti-fractalkine antibody led to a 50% decrease in monocyte adhesion; resveratrol, or the anti-fractalkine antibody alone had no effect. These findings suggest that resveratol decreases monocyte adhesion to TNF-α-stimulated HUVECs through fractalkine expression.

Resveratrol suppresses TNF-α-induced NF-κB and Sp1 activities.

We previously demonstrated that TNF-α stimulated the expression of fractalkine mRNAs mainly through activation of NF-κB (Ahn et al., 2004). So, we examined the effect of resveratrol on NF-

κB activities using a phosphospecific anti-p65 antibody that only detects the p65 subunit of NF-κB when it is phosphorylated at Ser536. TNF-α-induced the phosphorylation of the p65 subunit (~3.1-fold) at 15 min; this phosphorylation was nearly completely inhibited by cotreatment with resveratrol (5 μmol/L) for 4 h (Figs. 4A and 4B); resveratrol alone had no effect. To identify NF-κB and Sp1-binding complexes induced by TNF-α, nuclear extracts of HUVECs were subjected to a supershift assay. As shown in Figures 4C and 4F, incubation with p65, p50 or Sp1 antibodies produced slowly migrating complexes, indicating that NF-κB p65/p50, p50/p50 and Sp1 complexes are activated by TNF-α treatment. We also used EMSA to examine whether resveratrol inhibits NF-κB and Sp1 activities in nuclear extracts of HUVECs stimulated with TNF-α (10 ng/mL). NF-κB (p65/p50) and Sp1 binding was increased in nuclear extracts from TNF-α-stimulated HUVECs, whereas cotreatment with TNF-α and resveratrol suppressed NF-κB and Sp1 binding (Figs. 4D and 4G). To examine NF-κB and Sp1-dependent transcriptional activities, we transiently transfected HUVECs with NF-κB or Sp1 responsive luciferase reporter constructs. The day after transfection, cells were treated with TNF-α, resveratrol, or TNF-α plus resveratrol for 6 h and then determined luciferase activity. Treatment with resveratrol reduced NF-κB and Sp1 transcriptional activities induced by TNF-α (Figs. 4E and 4H). These data suggest that resveratrol suppresses fractalkine expression through suppression of NF-κB and Sp1 activities in HUVECs.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Resveratrol suppresses TNF-α-induced fractalkine expression in cardiac, kidney, and small intestinal endothelial cells.

Similar to our previous report (Ahn et al., 2004), intravenous injection of TNF-α (10 μg/kg) markedly increased fractalkine expression at 12 h in arterial endothelial cells in the heart. In the kidney, TNF-α increased fractalkine expression in arterial endothelial cells but not in glomerular endothelial and tubular epithelial cells. In the small intestine, TNF-α increased fractalkine expression in arteriolar endothelial cells and villous endothelium but not in lymphatic endothelial cells and epithelial cells. Pretreatment with resveratrol (50 mg/kg per day) dramatically suppressed TNF-α-induced fractalkine expression in arterial endothelial cells in heart, kidney, and small intestine (Fig. 5). Resveratrol also decreased TNF-α-induced villous endothelial fractalkine expression (Fig. 5). These findings suggest that resveratrol suppresses TNF-α–induced fractalkine expression in cardiac, kidney, and small intestinal endothelial cells.

Resveratrol suppresses TNF-α-induced ED-1-positive cell infiltration in intestinal villi.

Immunohistochemical examination of rat intestinal villi revealed a 5.2-fold increase in ED-1-positive cell infiltration after TNF-α treatment (Fig. 6). Resveratrol treatment prevented TNF-α-induced accumulation of ED-1-positive cells in intestinal villi by about 60% whereas resveratrol

alone had no effect. There were significantly fewer infiltrated ED-1-positive cells in the jejunal sections from rats treated with TNF-α and anti-fractalkine antibody than treated with TNF-α and control antibody (Fig. 6). Anti-fractalkine antibody alone had no effect on ED-1-positive cell infiltration in jejunum. These results suggest that resveratrol decreases ED-1-positive macrophage infiltration into jejunum after TNF-α injection through regulation of fractalkine expression.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

In this study, we demonstrate that resveratrol suppresses endothelial fractalkine expression through the NF-κB-dependent signaling pathway and also decreases TNF-α-induced expression of CX3CR1 in monocytes. We also demonstrate that resveratrol decreases arterial endothelial fractalkine expression in heart, kidney, jejunum, and intestinal villous endothelial cells after TNF-α stimulation. Resveratrol also decreased TNF-α-induced ED-1-positive cell infiltration in intestinal villi. To our knowledge, this is a novel mechanism of the anti-inflammatory effect of resveratrol through the down-regulation of fractalkine.

Fractalkine can mediate adherence of leukocytes expressing CX3CR1, which include monocytes, NK cells, and some T lymphocytes (Fong et al., 1998; Foussat et al., 2000; Harrison et al., 1998; Imai et al., 1997). Atherosclerosis is a chronic inflammatory disease (Ross, 1999). Recruitment of circulating monocytes to the arterial intima can contribute to the formation of atherosclerotic lesions (Ross, 1999). A high expression of fractalkine in smooth muscle cells located in macrophage-rich areas of atherosclerotic plaques was observed in *apoE*$^{-/-}$ mice on a high-fat diet (Moatti et al., 2001). Moreover, CX3CR1$^{-/-}$ mice have less atheroma formation (Moatti et al., 2001). Thus, fractalkine/CX3CR1 plays an important role in inflammation and formation of

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

atherosclerosis. Our immunohistochemical analyses in heart, small intestine, and kidney revealed that administration of resveratrol suppresses TNF-α-induced fractalkine expression in cardiac, kidney, and small intestinal endothelial cells. Our data also demonstrated that resveratrol suppressed the CX3CR1 expression in monocytes after stimulation with TNF-α. Because fractalkine and CX3CR1 have an important role in inflammatory conditions, the down-regulation of fractalkine is important in preventing and treating these inflammatory diseases including atherosclerosis.

Our results demonstrate that resveratrol significantly decreases TNF-α-induced mRNA and protein expression of fractalkine in HUVECs (Fig. 1). Our results indicate that 5 μmol/L resveratrol is sufficient to suppress most of the TNF-α-mediated fractalkine expression. Previous studies showed that as little resveratrol as 19 μmol/L can block the progression of carcinogenesis and induce terminal differentiation by 50% (Jang et al., 1997). Similarly, topical application of resveratrol at 25 μmol/L can inhibit 98% of skin tumors in mice induced by 7,12-dimethylbenz(*a*)anthracene plus 12-*O*-tetradecanoylphorbol-13-acetate (Jang et al., 1997). Considering that each gram of fresh grape skin contains 50–100 μg (200–400 μmol/L) resveratrol and red wine has 1.5–3 mg/L (Goldberg et al., 1995; Jang et al., 1997), the resveratrol concentration used in our studies is achievable *in vivo* by consumption of grapes or wine.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

A possible anti-inflammatory role of resveratrol could be related to an interference with the NF-κB signaling pathway, which regulates the expression of various genes involved in inflammation (Manna et al., 2000). However, it was reported that acute resveratrol treatment (30 min) does not inhibit NF-κB in HUVECs, but overnight treatment does (Pellegatta et al., 2003). Our results were similar to these data in acute treatment (30-60 min) with resveratrol, but longer treatment with resveratrol (5 μmoL/l for 4 h) blocked the phosphorylation of the p65 subunit of NF-κB (Fig. 4).

We demonstrate that reveratrol has a novel mechanism of the anti-inflammatory effect through the down-regulation of fractalkine and CX3CR1. Thus, our data add new evidence of resveratrol as an inflammation-modulating agent or adjunctive therapy in inflammatory diseases, including atherosclerosis.

ACKNOWLEDGMENTS

We thank Ms. Judith Dickson for her help in preparing the manuscript and Jung Eun Lee for her help with immunohistochemistry.

REFERENCES

Ahn SY, Cho CH, Park KG, Lee HJ, Lee S, Park SK, Lee IK and Koh GY (2004) Tumor necrosis factor-alpha induces fractalkine expression preferentially in arterial endothelial cells and mithramycin A suppresses TNF-alpha-induced fractalkine expression. *Am J Pathol* **164**:1663-1672.

Ancuta P, Rao R, Moses A, Mehle A, Shaw SK, Luscinskas FW and Gabuzda D (2003) Fractalkine preferentially mediates arrest and migration of CD16+ monocytes. *J Exp Med* **197**:1701-1707.

Araim O, Ballantyne J, Waterhouse AL and Sumpio BE (2002) Inhibition of vascular smooth muscle cell proliferation with red wine and red wine polyphenols. *J Vasc Surg* **35**:1226-1232.

Arichi H, Kimura Y, Okuda H, Baba K, Kozawa M and Arichi S (1982) Effects of stilbene components of the roots of Polygonum cuspidatum Sieb. et Zucc. on lipid metabolism. *Chem Pharm Bull (Tokyo)* **30**:1766-1770.

Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A and Schall TJ (1997) A new class of membrane-bound chemokine with a CX3C motif. *Nature* **385**:640-644.

Bertelli AA, Baccalini R, Battaglia E, Falchi M and Ferrero ME (2001) Resveratrol inhibits TNF alpha-induced endothelial cell activation. *Therapie* **56**:613-616.

Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM and Stern DM (1998) Endothelial cells in physiology and in the pathophysiology of vascular

disorders. *Blood* **91**:3527-3561.

Combadiere C, Potteaux S, Gao JL, Esposito B, Casanova S, Lee EJ, Debre P, Tedgui A, Murphy PM and Mallat Z (2003) Decreased atherosclerotic lesion formation in CX3CR1/apolipoprotein E double knockout mice. *Circulation* **107**:1009-1016.

Fauconneau B, Waffo-Teguo P, Huguet F, Barrier L, Decendit A and Merillon JM (1997) Comparative study of radical scavenger and antioxidant properties of phenolic compounds from Vitis vinifera cell cultures using in vitro tests. *Life Sci* **61**:2103-2110.

Ferrero ME, Bertelli AA, Pellegatta F, Fulgenzi A, Corsi MM and Bertelli A (1998) Phytoalexin resveratrol (3-4'-5-trihydroxystilbene) modulates granulocyte and monocyte endothelial adhesion. *Transplant Proc* **30**:4191-4193.

Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T and Patel DD (1998) Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. *J Exp Med* **188**:1413-1419.

Foussat A, Bouchet-Delbos L, Berrebi D, Durand-Gasselin I, Coulomb-L'Hermine A, Krzysiek R, Galanaud P, Levy Y and Emilie D (2001) Deregulation of the expression of the fractalkine/fractalkine receptor complex in HIV-1-infected patients. *Blood* **98**:1678-1686.

Foussat A, Coulomb-L'Hermine A, Gosling J, Krzysiek R, Durand-Gasselin I, Schall T, Balian A, Richard Y, Galanaud P and Emilie D (2000) Fractalkine receptor expression by T lymphocyte subpopulations and in vivo production of fractalkine in human. *Eur J Immunol* **30**:87-97.

Frankel EN, Waterhouse AL and Kinsella JE (1993) Inhibition of human LDL oxidation by resveratrol. *Lancet* **341**:1103-1104.

Fujiwara N and Kobayashi K (2005) Macrophages in inflammation. *Curr Drug Targets Inflamm*

Allergy **4**:281-286.

Garcia GE, Xia Y, Chen S, Wang Y, Ye RD, Harrison JK, Bacon KB, Zerwes HG and Feng L (2000) NF-kappaB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1beta, TNF-alpha, and LPS. *J Leukoc Biol* **67**:577-584.

Gimbrone MA, Jr., Nagel T and Topper JN (1997) Biomechanical activation: an emerging paradigm in endothelial adhesion biology. *J Clin Invest* **99**:1809-1813.

Goldberg DM, Hahn SE and Parkes JG (1995) Beyond alcohol: beverage consumption and cardiovascular mortality. *Clin Chim Acta* **237**:155-187.

Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB and Feng L (1998) Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. *Proc Natl Acad Sci U S A* **95**:10896-10901.

Haskell CA, Cleary MD and Charo IF (1999) Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction. Rapid flow arrest of CX3CR1-expressing cells is independent of G-protein activation. *J Biol Chem* **274**:10053-10058.

Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ and Yoshie O (1997) Identification and molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* **91**:521-530.

Imaizumi T, Matsumiya T, Tamo W, Shibata T, Fujimoto K, Kumagai M, Yoshida H, Cui XF, Tanji K, Hatakeyama M, Wakabayashi K and Satoh K (2002) 15-Deoxy-D12,14-prostaglandin J2 inhibits CX3CL1/fractalkine expression in human endothelial cells. *Immunol Cell Biol* **80**:531-536.

Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC and Pezzuto JM (1997) Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **275**:218-220.

Kim W, Moon SO, Lee S, Sung MJ, Kim SH and Park SK (2003) Adrenomedullin reduces VEGF-induced endothelial adhesion molecules and adhesiveness through a phosphatidylinositol 3'-kinase pathway. *Arterioscler Thromb Vasc Biol* **23**:1377-1383.

Lesnik P, Haskell CA and Charo IF (2003) Decreased atherosclerosis in CX3CR1-/- mice reveals a role for fractalkine in atherogenesis. *J Clin Invest* **111**:333-340.

Manna SK, Mukhopadhyay A and Aggarwal BB (2000) Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol* **164**:6509-6519.

Matsumiya T, Imaizumi T, Fujimoto K, Cui X, Shibata T, Tamo W, Kumagai M, Tanji K, Yoshida H, Kimura H and Satoh K (2001) Soluble interleukin-6 receptor alpha inhibits the cytokine-Induced fractalkine/CX3CL1 expression in human vascular endothelial cells in culture. *Exp Cell Res* **269**:35-41.

Mazzucchelli R, Amadio M, Curreli S, Denaro F, Bemis K, Reid W, Bryant J, Riva A, Galli M and Zella D (2004) Establishment of an ex vivo model of monocytes-derived macrophages differentiated from peripheral blood mononuclear cells (PBMCs) from HIV-1 transgenic rats. *Mol Immunol* **41**:979-984.

Moatti D, Faure S, Fumeron F, Amara Mel W, Seknadji P, McDermott DH, Debre P, Aumont MC, Murphy PM, de Prost D and Combadiere C (2001) Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. *Blood* **97**:1925-

1928.

Nanki T, Urasaki Y, Imai T, Nishimura M, Muramoto K, Kubota T and Miyasaka N (2004) Inhibition of fractalkine ameliorates murine collagen-induced arthritis. *J Immunol* **173**:7010-7016.

Pace-Asciak CR, Rounova O, Hahn SE, Diamandis EP and Goldberg DM (1996) Wines and grape juices as modulators of platelet aggregation in healthy human subjects. *Clin Chim Acta* **246**:163-182.

Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC and Gearing D (1997) Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature* **387**:611-617.

Pellegatta F, Bertelli AA, Staels B, Duhem C, Fulgenzi A and Ferrero ME (2003) Different short- and long-term effects of resveratrol on nuclear factor-kappaB phosphorylation and nuclear appearance in human endothelial cells. *Am J Clin Nutr* **77**:1220-1228.

Pendurthi UR and Rao LV (2002) Resveratrol suppresses agonist-induced monocyte adhesion to cultured human endothelial cells. *Thromb Res* **106**:243-248.

Robinson LA, Nataraj C, Thomas DW, Howell DN, Griffiths R, Bautch V, Patel DD, Feng L and Coffman TM (2000) A role for fractalkine and its receptor (CX3CR1) in cardiac allograft rejection. *J Immunol* **165**:6067-6072.

Ross R (1999) Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**:115-126.

Segerer S, Hughes E, Hudkins KL, Mack M, Goodpaster T and Alpers CE (2002) Expression of the fractalkine receptor (CX3CR1) in human kidney diseases. *Kidney Int* **62**:488-495.

Sung MJ, Kim W, Ahn SY, Cho CH, Koh GY, Moon SO, Kim DH, Lee S, Kang KP, Jang KY

and Park SK (2005) Protective effect of alpha-lipoic acid in lipopolysaccharide-induced endothelial fractalkine expression. *Circ Res* **97**:880-890.

Tsai SH, Lin-Shiau SY and Lin JK (1999) Suppression of nitric oxide synthase and the down-regulation of the activation of NFkappaB in macrophages by resveratrol. *Br J Pharmacol* **126**:673-680.

Yamashita K, Imaizumi T, Hatakeyama M, Tamo W, Kimura D, Kumagai M, Yoshida H and Satoh K (2003) Effect of hypoxia on the expression of fractalkine in human endothelial cells. *Tohoku J Exp Med* **200**:187-194.

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTE

This work was supported by grants from the National Research Laboratory Program of Korea Science and Engineering Foundation (S.K.P.) and Korea Research Foundation grant KRF-2004-041-E00031 (W.K.).

S.O.M. and W.K. contributed equally to this work.

Address correspondence to:

Sung Kwang Park, M.D., Ph.D.

Renal Regeneration Laboratory and Department of Internal Medicine

Chonbuk National University Medical School

San 2-20 Keumam-dong, Jeonju

561-180

Republic of Korea

E-mail: parksk@chonbuk.ac.kr

Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

LEGENDS FOR FIGURES

Fig. 1. Resveratrol suppressed TNF-α-induced fractalkine expression. **A,** RPA of fractalkine mRNA in TNF-α-stimulated HUVECs cotreated with resveratrol (Res). HUVECs were incubated for 4 h with control buffer (CB), Res (5 μmol/L), TNF-α (10 ng/mL), or TNF-α plus various concentration of Res. Total RNA (10 μg) isolated from the cells was subjected to RPA. Densitometric analyses are presented as the relative ratio of fractalkine mRNA level to cyclophilin mRNA level. **B,** Western blot analyses of fractalkine protein in TNF-α-stimulated HUVECs cotreated with Res. HUVECs were incubated for 6 h with CB, TNF-α (10 ng/mL), or TNF-α plus the indicated amount of Res. Western blot was probed with an anti-fractalkine antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. Densitometric analyses are presented as the relative ratio of each protein to actin. The ratio relative to CB is arbitrarily presented as 1. Bars represent the mean ± S.D. from 3 experiments. * $p<0.05$ *versus* CB; $^{\#}$ $p<0.05$ *versus* TNF-α only. **C,** Analysis of fractalkine expression in HUVECs detected by flow cytometry. HUVECs incubated with control buffer (CB), Res (5 μmol/L), TNF-α (10 ng/mL), or TNF-α (10 ng/mL) plus Res (5 μmol/L) for 6 h.

Fig. 2. Resveratrol decreased the number of CX3CR1-positive cells in TNF-α-stimulated THP-1 cells. **A,** Western blot analyses of CX3CR1 protein in TNF-α-stimulated THP-1 cells cotreated

with resveratrol (Res). THP-1 cells were incubated for 6 h with CB, TNF-α (10 ng/mL), or TNF-α plus the indicated amount of Res. Western blot was probed with an anti-CX3CR1 antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. Densitometric analyses are presented as the relative ratio of each protein to actin. The ratio relative to CB is arbitrarily presented as 1. Results were similar in 3 independent experiments. Bars represent the mean ± S.D. * $p<0.05$ versus CB; # $p<0.05$ versus TNF-α only. **B,** Analyses of CX3CR1 expression in HUVECs detected by flow cytometry. THP-1 cells incubated with control buffer (CB), Res (5 μmol/L), TNF-α (10 ng/mL), or TNF-α (10 ng/mL) plus Res (5 μmol/L) for 6 h.

Fig. 3. Resveratrol suppressed TNF-α-induced endothelial adhesiveness for monocytes. **A,** Fluorescent microscopic findings of endothelial adhesiveness for monocytes. Bar = 50 μm. **B,** Quantification of monocytes adhesion to HUVECs. Monocytes, fluorescently labeled with calcein-AM (50 μmol/L), were added to confluent monolayers of HUVECs, which were treated with TNF-α (10 ng/mL) for 6 h and were also treated with control buffer (CB), resveratrol (Res, 5 μmol/L), or anti-fractalkine antibody (20 μg/mL). More monocytes were found in HUVECs treated with TNF-α than with CB. Note that treatment with TNF-α plus Res or anti-fractalkine antibody decreased the TNF-α-induced increase of monocyte adhesion to HUVECs. Bars

represent the mean ± S.D. from 4 experiments. * p <0.05 *versus* CB; # p <0.05 *versus* TNF-α.

Fig. 4. Resveratrol suppressed TNF-α–induced NF-κB and Sp1 activities. **A,** Western blot analyses of phospho-p65 subunit of NF-κB in protein extracts of HUVECs. HUVECs were pretreated with resveratrol (Res, 5 μmol/L) for the indicated time and treated for 15 min with control buffer (CB) or TNF-α (10 ng/mL). **B,** Densitometric analyses are presented as the relative ratio of each protein to the p65 subunit of NF-κB. The ratio relative to CB is arbitrarily presented as 1. Bars represent the mean ± S.D. from 3 experiments. * p<0.05 *versus* CB; # p <0.05 *versus* TNF-α only. **C and F,** Gel supershift assay demonstrating specific activation of NF-κB (C) or Sp1 (F) by TNF-α. Nuclear extracts of HUVECs treated with TNF-α (10 ng/mL) were pre-incubated for 1 h with antibodies to p65, p50 or Sp1. Comp, 100× cold NF-κB competitor probe; Cold-pb, unlabeled probes; SS, supershift; NS, nonspecific bands; FP, free probes. **D and G,** EMSA of NF-κB (D) or Sp1 (G) in nuclear extracts of HUVECs. HUVECs were incubated with CB, Res (5 μmol/L), or TNF plus Res for 2h. **E and H**, Luciferase activity of NF-κB (E) or Sp1 (H). HUVECs were transiently transfected using reporter plasmids, and 24 hours post-transfection, cells were treated with Res (5 μmol/L), TNF-α (10 ng/mL), or TNF-α plus Res. Whole cell lysates were assayed for luciferase activity. * p <0.05 *versus* CB; # p <0.05 *versus* TNF-α only.

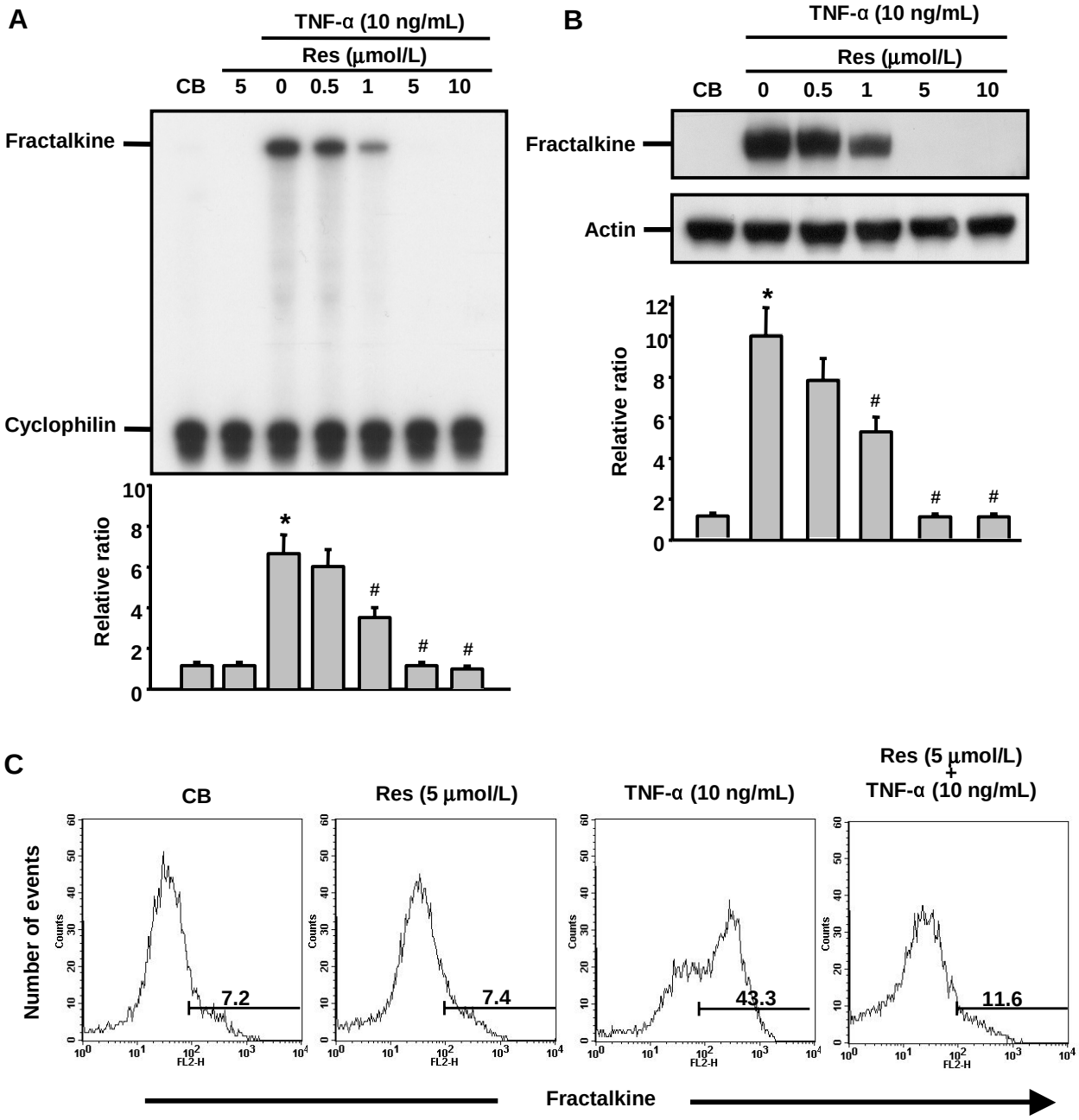
Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 5. Resveratrol suppressed TNF-α-induced fractalkine expression *in vivo*. **A,** Immunohistochemical analyses of arterial endothelial fractalkine expression in rat heart, kidney, small intestinal wall, and intestinal villi. Rats were given control buffer (CB), TNF-α (10 μg/kg), or TNF-α plus resveratrol (Res, 50 mg/kg per day). Bar = 50 μm **B,** Semiquantitative analysis of fractalkine expression in heart, kidney, and small intestine. For each section, 3–5 endothelial portions of the tissue were graded on a scale from 1 (no staining) to 5 (very strong). Bars represent the mean ± S.D. from 3 experiments. * p <0.05 *versus* CB; # p <0.05 *versus* TNF-α only.

Fig. 6. Resveratrol inhibits ED-1-positive cell infiltration in intestinal villi. **A,** Immunohistochemical analyses of ED-1 in rat intestinal villi. Rats were given control buffer (CB), TNF-α (10 μg/kg), or TNF-α plus resveratrol (Res, 50 mg/kg per day), TNF-α plus control antibody (rabbit IgG, 500 μg/kg) or TNF-α plus anti-fractalkine antibody (500 μg/kg). Five days after injection of CB, TNF-α, Res, or antibodies, jejunums were harvested for immunohistochemical analyses. Bar = 50 μm **B,** Quantitative analysis of ED-1-positive cells in intestinal villus. Bars represent the mean ± S.D. from 3 experiments. * p <0.05 *versus* CB; # p <0.05 *versus* resveratrol; † p <0.05 *versus* TNF-α plus control antibody.

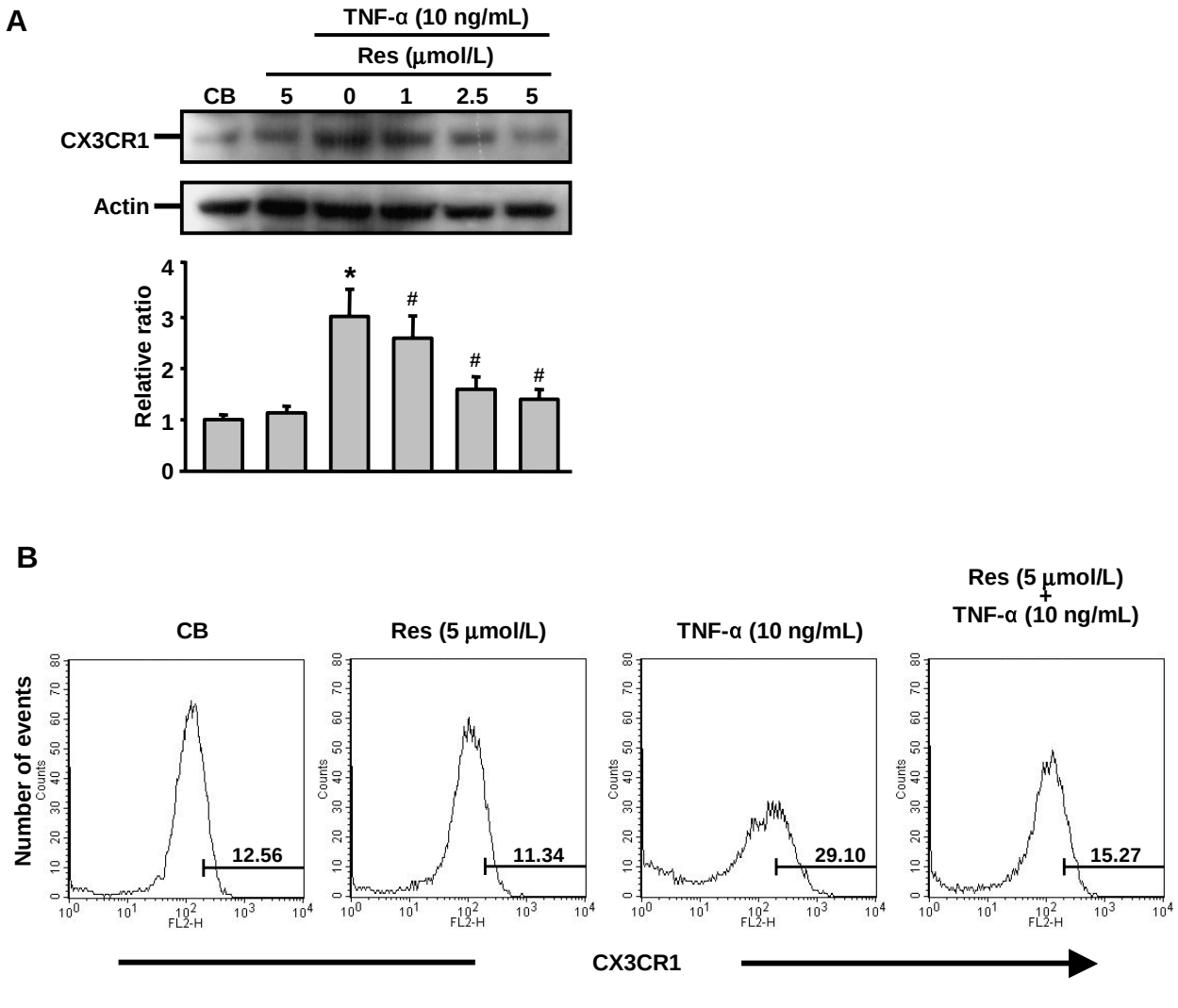
Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1. Moon SO



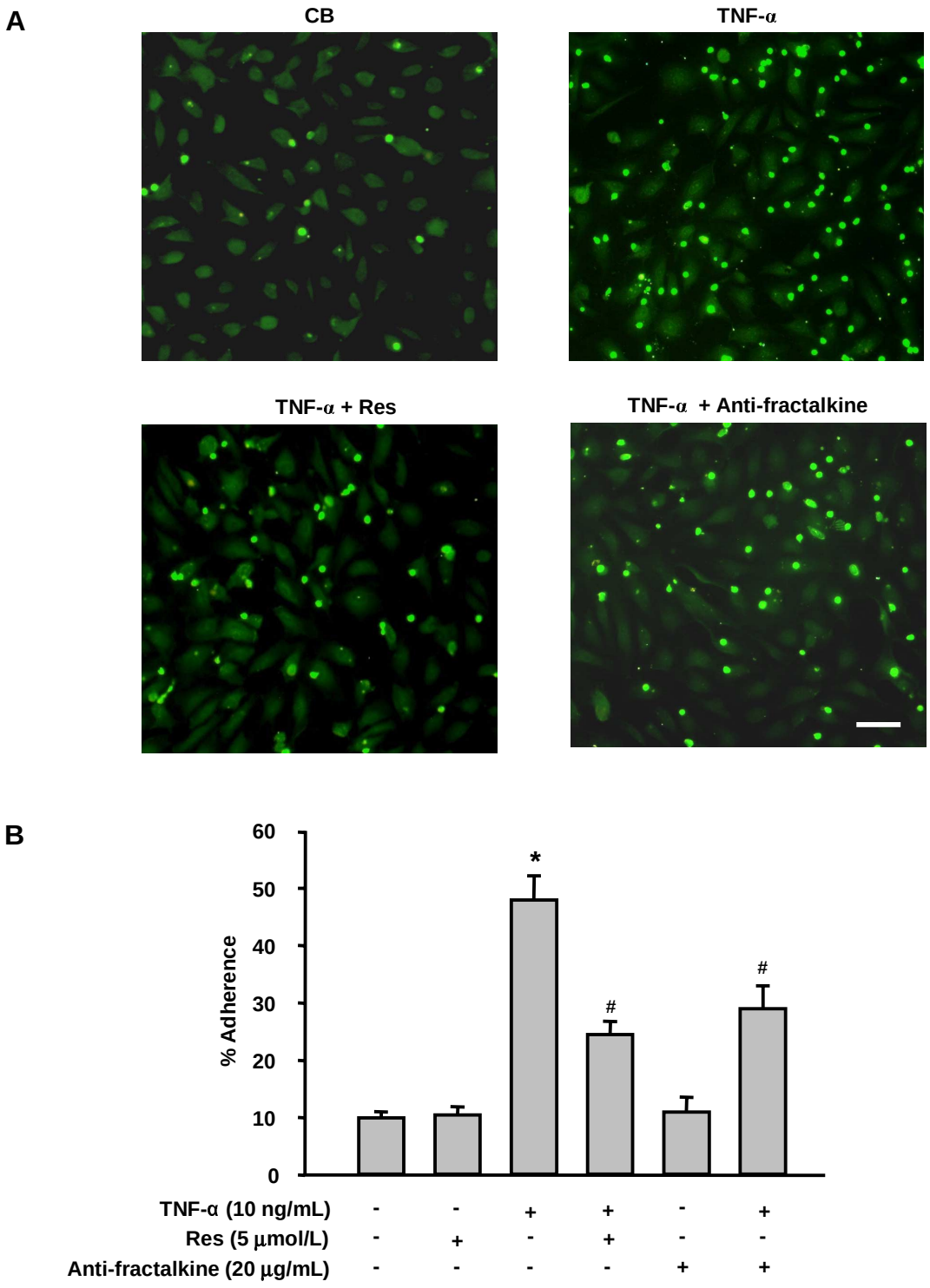
Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2. Moon SO



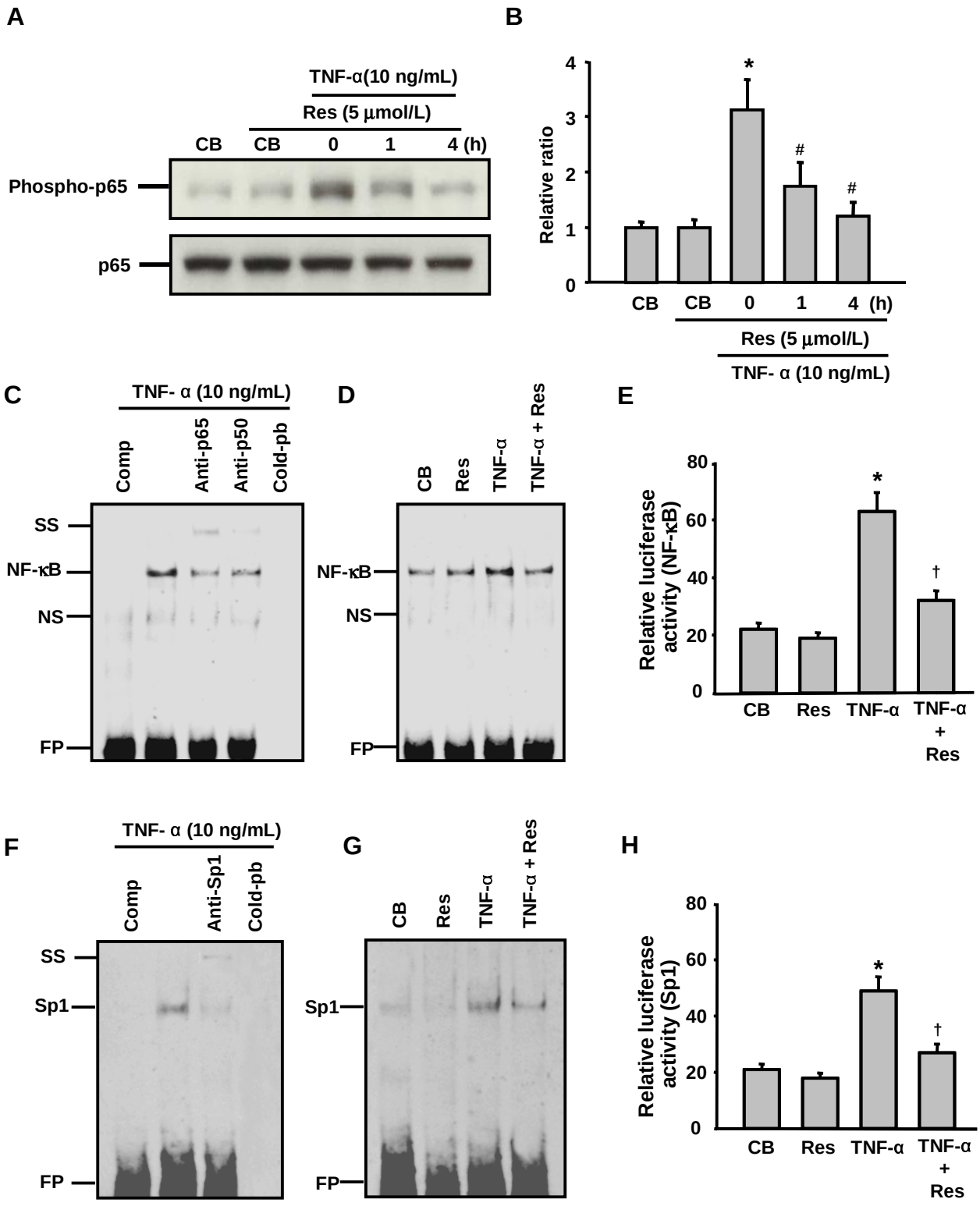
Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Moon SO



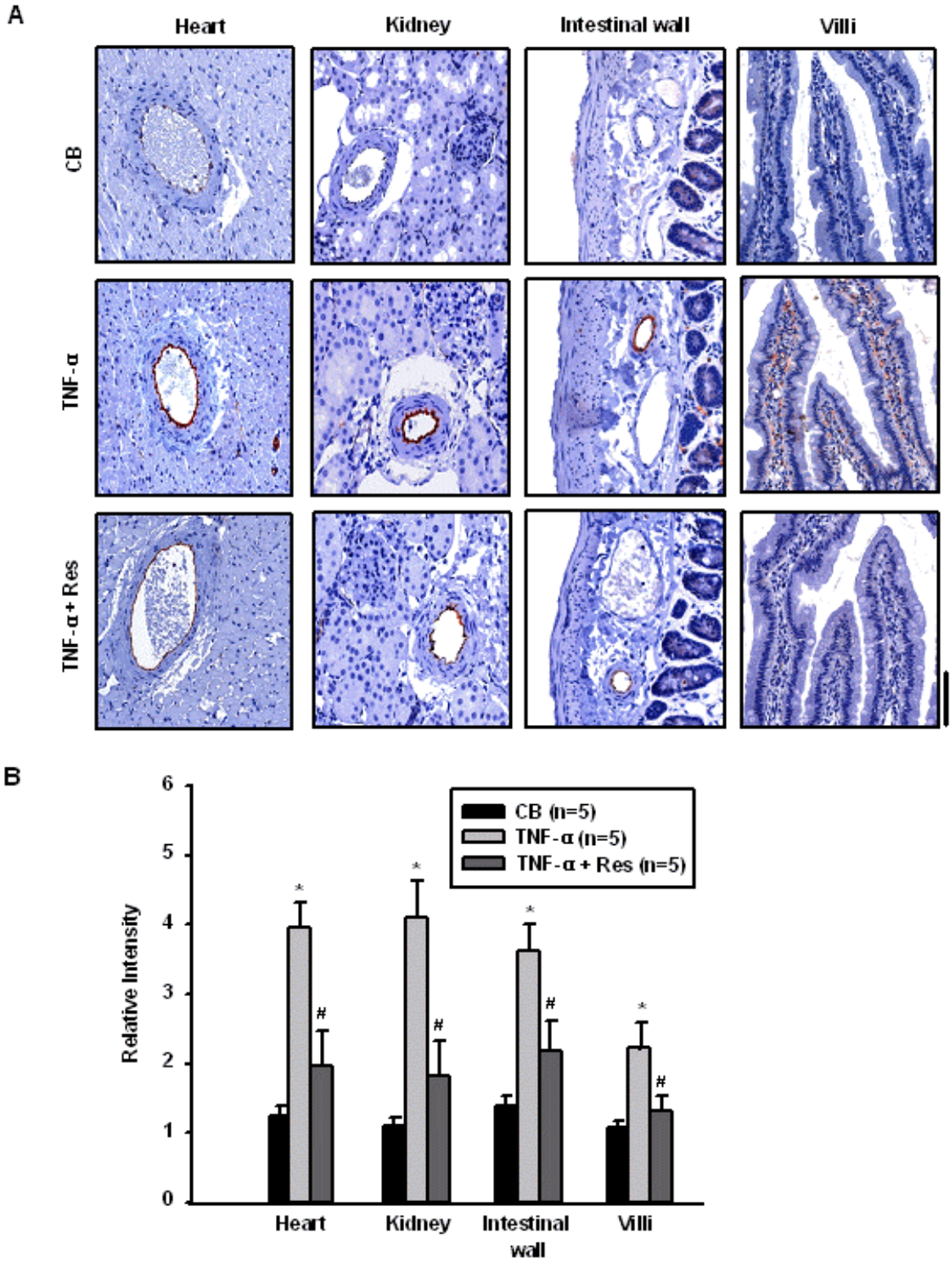
Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4. Moon SO



Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. Moon SO



Molecular Pharmacology Fast Forward. Published on April 13, 2006 as DOI: 10.1124/mol.106.022392
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6. Moon SO