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TITLE PAGE

Vesnarinone suppresses TNFα mRNA expression by inhibiting valosin-containing protein

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RUNNING TITLE PAGE

A running title:
Vesnarinone inhibits valosin-containing protein

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A list of nonstandard abbreviations used in the paper:
CHF: congestive heart failure
DOC: deoxycholic acid
FCS: fetal calf serum
HIF: hypoxia inducible factor
HIV: human immunodeficiency virus
IKK: IκB kinase
IL-1β: interleukin-1-beta
NEM: N-ethylmaleimide
NFκB: nuclear factor kappa B
PAGE: polyacrylamide gel electrophoresis
PCR: polymerase chain reaction
PDE3: phosphodiesterase III
PMSF: phenylmethylsulfonyl fluoride
RT-PCR: reverse transcription- polymerase chain reaction
TNFα: tumor necrosis factor alpha
VCP: valosin-containing protein

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Abstract

Vesnarinone is a synthetic quinolinone derivative used in the treatment of cardiac failure and cancer. It is also known to cause agranulocytosis as a side effect, which restricts its use, although the mechanism underlying agranulocytosis is not well understood. Here, we show that vesnarinone binds to valosin-containing protein (VCP), which interacts with poly-ubiquitinated proteins and is essential for the degradation of IκBα to activate NFκB. We show that vesnarinone degrade

to VCP impairs the degradation of IκBα and that the impairment of the degradation of IκBα is the result of the inhibition of the interaction between VCP and the 26S proteasome. These results suggest that vesnarinone suppresses NFκB activation by inhibiting the VCP-dependent degradation of poly-ubiquitinated IκBα, resulting in the suppression of TNFα mRNA expression.

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Introduction

Vesnarinone (3,4-dihydro-6-[4-(3,4-dimethosy-benzoyl)-1-piperazinyl]-2(1*H*)-quinolinone) is a quinolinone derivative developed as an inotropic agent for the treatment of congestive heart failure (CHF) (Cavusoglu et al., 1995) by modulating Ca^{2+} channels (Yatani et al., 1989). Vesnarinone is now known to have other activities, such as immunosuppressive activity (Matsui et al., 1994; Sato et al., 1995), the inhibition of HIV production, the reduction of endotoxemic lethality and the suppression of the growth of various tumor cell lines, including gastric cancer, lung cancer, adenoid squamous carcinoma and myeloid leukemia (Fujiwara et al., 1997; Nio et al., 1997; Honma et al., 1999; Kubo et al., 1999; Yokozaki et al., 1999). However, the induction of agranulocytosis has been reported as a side effect of vesnarinone, thereby representing a limitation of the usage of vesnarinone (Cohn et al., 1998).

At the molecular level, vesnarinone appears to enhance myocardial contractility by augmenting sodium-calcium exchange (Yatani et al., 1989), which may responsible for the treatment of CHF. Vesnarinone is also known to inhibit phosphodiesterase III (PDE3), resulting in an increase in the cyclic AMP concentration

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in cells, leading to vasodilation (Itoh et al., 1993). Although these pharmacological effects may be related to the treatment of CHF, the molecular basis of the side effect is not well understood.

Previously, we showed that vesnarinone impairs the production of TNFα in bone marrow stromal cells, an event that is essential for the differentiation of the cells into mature granulocytes (Hiramoto et al., 2004; Nabeshima et al., 1997). These finding are also supported by another study that showed that vesnarinone suppressed both the activation of the transcription factor NFκB and the expression of the TNFα gene, a target of NFκB (Manna and Aggarwal 2000). These findings raised the possibility that the inhibition of NFκB signaling by vesnarinone may cause the observed agranulocytosis.

NFκB is a key transcription factor that regulates many processes, including the immune response, inflammation and stress responses. When a cell is not stimulated, NFκB is sequestered in the cytosol through the formation of a complex with a member of the IκB family. However, once the cell is stimulated by factors such as TNFα and IL-1β, the IκB kinase (IKK) phosphorylates IκB, and phosphorylated IκB is then ubiquitinated and degraded by the proteasome. The released NFκB enters the

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nucleus and functions as a homo- or heterodimer transcription factor with a member of the NFκB family. Genes related to the immune response, inflammation and other processes are known to be targets of the NFκB transcription factors.

To investigate the mechanism by which vesnarinone inhibits the activation of NFκB, we attempted to purify a vesnarinone-binding protein with high-performance affinity magnetic beads (Nishio et al., 2008a; Shimizu et al., 2000), which are powerful tools for the identification of the molecular targets of many drugs, including thalidomide (Ito et al., 2010). Indeed, a valosin-containing protein (VCP) was identified as a result of our application of this method. It is thought that VCP plays important roles in ubiquitin-dependent protein quality control and intracellular signalling pathways (reviewed in Meyer et al., 2012).

We further showed that VCP is essential for the ubiquitin-dependent proteasome-mediated degradation of IκBα and that vesnarinone induces the accumulation of ubiquitinated IκBα resulting in the inhibition of NFκB activation by preventing the interaction between VCP and the 26S proteasome.

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Materials and Methods

Plasmid construction, antibodies, and materials

The human VCP and IκBα cDNAs were subcloned from a LP101 cell cDNA library into the mammalian expression vector pHyg-EF-2 (Nishizawa et al., 2003). Vectors encoding the VCP deletion mutants were created by PCR using mutagenic primers. Antibodies specific for FLAG (M2, mouse monoclonal, SIGMA), VCP (mouse monoclonal), IκBα (sc-371, rabbit polyclonal, Santa Cruz Biotechnology), phosphorylated IκBα (Cell Signaling Technology), actin, and ubiquitin (mouse monoclonal) were purchased from the indicated suppliers.

Affinity purification of vesnarinone-binding proteins using FG-beads

FG-EGDE beads were prepared as described previously (Nishio et al., 2008b). The vesnarinone amino derivative was incubated with the FG-EGDE beads in distilled water for 24 h at 37 ºC. The vesnarinone amino derivative-fixed FG-EGDE beads were washed three times with distilled water and stored at 4°C until use. The LP101 cell membrane extracts (Dignam et al., 1983) were incubated with the vesnarinone

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amino derivative-fixed beads for 4 h at 4°C, and the beads were then washed three times with binding buffer (10 mM Tris-HCl [pH 7.4], 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 mM DTT, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). The bound proteins were eluted with Laemmli dye or binding buffer containing the vesnarinone amino derivative. The eluted proteins were subjected to SDS-PAGE, silver stained and then subjected to in-gel digestion with trypsin. The peptide fragments were analyzed by quadrupole time-of-flight mass spectrometry, as described previously (Shimizu et al., 2000).

***In vitro* binding assays**

Lysates of 293T cells expressing the VCP mutants were incubated with the vesnarinone amino derivative-fixed FG-EGDE for 4 h at 4ºC and washed three times with binding buffer. The bound proteins were eluted with Laemmli dye and subjected to SDS-PAGE, followed by immunoblotting with an anti-FLAG antibody.

Cell culture, transfection, and VCP knockdown

The 293T cells were maintained in Dulbecco's modified Eagle's medium containing 10% FCS in 5% CO_2, and the LP101 cells were maintained in IMDM

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containing 10% FCS in 5% CO_2. The knockdown of VCP in the 293T cells was performed using Lipofectamine RNAiMAX (Invitrogen).

Real-Time RT-PCR

The LP101 cells were treated with 5 ng/ml TNFα for 60 min, and the 293T cells were treated with 10 ng/ml TNFα for 60 min. The total RNAs were then prepared using Sepasol RNA I Super (Nakalai Tesque). The quantification of the TNFα or Glyceraldehyde-3-phosphate dehydrogenase mRNA levels was performed using the QuantiTect SYBR Green reverse transcription PCR master mix (Qiagen).

Coimmunoprecipitation assay and immunoblotting

Lipofectamine 2000 was used to transfect the 293T cells with pHyg-IκBα-His-FLAG or pcDNA-VCP-His-FLAG. At two days posttransfection, the cells were treated with 5 μM MG132 for 60 min, followed by stimulation with 10 ng/ml TNFα for 20 min. The cells were then harvested, washed twice with PBS, and lysed with RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 10 mM NEM, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin

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A, and 1 μg/ml leupeptin) or NP-40 lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% NP-40, 10 mM NEM, 20 mM NaF, 25 μM MG132, 1 mM PMSF, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin) at 4ºC for 30 min. The samples were centrifuged at 20,000 x *g* at 4ºC for 15 min, and the supernatants were incubated with anti-FLAG M2 affinity gel (SIGMA) for 2 h at 4ºC. The beads were then washed three times with the same buffer used as the binding buffer, and the bound proteins were eluted with a buffer containing the FLAG peptide (SIGMA), subjected to SDS-PAGE, and analyzed by immunoblotting.

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Results

Vesnarinone suppresses the TNFα-induced activation of NFκB

Previously, we reported that vesnarinone inhibits the production and the secretion of TNFα from human bone marrow stromal LP101 cells (Hiramoto et al., 2004; Nabeshima et al., 1997), but its molecular mechanisms were remained unclear. Based on our previous observations, we first examined whether the inhibition of TNFα secretion in LP101 cells is the result of the reduction of TNFα mRNA expression. After the treatment of LP101 cells with different concentrations of vesnarinone, the cells were induced with TNFα and the quantity of newly transcribed TNFα mRNA was determined by quantitative RT-PCR. As shown in Figure 1A, the expression of TNFα mRNA in the LP101 cells was induced by TNFα and reduced by the addition of vesnarinone in a dose-dependent manner. These results suggested that the observed inhibition of TNFα secretion is the result of decreased TNFα mRNA expression caused by the treatment with vesnarinone.

Next, we used human embryonic kidney 293T cells to examine whether the reduction of TNFα mRNA expression by vesnarinone is cell-type specific. A reduction of the induced TNFα mRNA was also observed in this cell line (Fig. 1B),

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suggesting that the vesnarinone-induced reduction of TNFα expression is not restricted to bone marrow stromal cells.

As NFκB is known to be the major transcription factor regulating TNFα expression, we next investigated whether vesnarinone inhibits NFκB-dependent transcription. A luciferase gene under the control of four tandem repeated NFκB binding sites was transfected into the 293T cells, and the effects of vesnarinone on the NFκB-driven reporter gene expression after TNFα treatment was examined. The TNFα-induced expression of the reporter gene was inhibited by the treatment with vesnarinone in a dose-dependent manner (Fig. 1C). These results suggested that vesnarinone inhibited NFκB-dependent transcriptional activation.

It is known that NFκB activity is regulated by specific inhibitory subunits, the IκB proteins, which are degraded by the ubiquitin-proteasome system during NFκB activation. If IκB is not properly degraded, NFκB cannot enter the nucleus and fails to activate NFκB-dependent transcription. To determine whether the inhibitory effect of vesnarinone is due to an effect on IκBα degradation, the cytoplasmic level of IκBα protein was measured by immunoblotting (Fig 1D). In the absence of vesnarinone, a reduction of IκBα protein was observed after 15 min of TNFα treatment as expected and

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the IκBα signal was recovered by 60 min. However, in the presence of vesnarinone, the inhibition of IκBα degradation was observed even after TNFα treatment (Fig. 1D). This result suggested that vesnarinone inhibits the IκBα degradation process.

As it is known that the degradation of IκB is triggered by IKK-mediated phosphorylation, we next examined whether IκB is phosphorylated after TNFα treatment. Interestingly, although the phosphorylation of IκBα was detected either in the presence or absence of vesnarinone at 15 min after the TNFα stimulation, the amount of phosphorylated IκBα rapidly decreased in the absence of vesnarinone, which correlated well with the degradation of IκB (Fig. 1D). Conversely, a substantial amount of phosphorylated IκBα was detected even after TNFα stimulation in the presence of vesnarinone. This result correlated well with the remaining amount of IκBα (Fig. 1D). These results suggested that vesnarinone does not inhibit the phosphorylation of IκBα but does inhibit the degradation of IκB at a specific point between IκB phosphorylation and degradation.

Identification of vesnarinone-binding proteins

To clarify the molecular mechanisms of the vesnarinone-induced

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inhibition of NFκB activation, we attempted to purify vesnarinone-binding proteins directly using high-performance affinity purification (Shimizu et al., 2000). The amino derivative of vesnarinone was immobilized on FG beads (Nishio et al., 2008b) via the epoxy group (Figure 2A) and then used for the purification of vesnarinone-binding proteins. The vesnarinone-fixed beads were incubated with extracts of human bone marrow stromal LP101 cells, and the binding proteins were directly purified. A 97 kDa protein was found to bind specifically to the vesnarinone-fixed beads, and the subsequent quadrupole time-of-flight mass spectrometry analysis identified the 97 kDa protein as a valosin-containing protein (VCP) (Fig. 2B). VCP is a member of the ATPases associated with diverse cellular activities possessing two ATPase domains and it is known to play a critical role in many cellular activities such as the ubiquitin-proteasome system, endoplasmic reticulum associated degradation of proteins, cell cycle, and DNA repair (reviewed in Meyer et al., 2012). The binding specificity of VCP to vesnarinone was examined by adding free vesnarinone to the elution buffer (Fig 2B). The addition of free vesnarinone led to the release of VCP from the beads in a concentration-dependent manner, suggesting that VCP is a vesnarinone-binding protein (Fig. 2B). The identification of VCP as a

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vesnarinone-binding protein was further confirmed by immunoblotting using an anti-VCP antibody (Fig 2B, bottom), which specifically reacted with the purified 97 kDa protein. In addition, the recombinant VCP (rVCP) protein overexpressed in *E. coli* also has the ability to bind to the vesnarinone-fixed beads, as shown in Figure 2C, indicating that vesnarinone binds to directly VCP.

Determination of the vesnarinone-binding region of VCP

We then determined the vesnarinone-binding region of VCP. VCP is known to have two ATPase domains, the D1 and D2 domains, which are followed by the N-terminal region of the poly-ubiquitin-recognition domain (Dai et al., 1998). Thus, we generated a FLAG-His-tagged full-length version and a series of deletion mutants of VCP that have either one or two ATPase domains and assessed the ability of the full-length and mutant recombinant VCP derivatives to bind to the beads. The full-length and deletion mutants consisting of amino acids 186-806, 1-481 and 149-494, which contain the D1 ATPase domain, bound to the vesnarinone-fixed beads. In contrast, two mutants consisting of residues 454-806 and 1-192, lacking the D1 domain, did not bind to the beads (Fig. 3). These results indicated that vesnarinone binds to the

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central region of VCP, which corresponds to the D1 ATPase domain. This result raised the possibility that VCP is required for NFκB activation and that the binding of vesnarinone to VCP causes a functional alteration in VCP, resulting in the impairment of NFκB activation.

VCP is required for NFκB-dependent gene activation

If vesnarinone inhibits VCP function, then the effect of vesnarinone should be similar to that of VCP malfunction. Thus, we knocked down VCP expression using a VCP-specific siRNA and compared the effect of vesnarinone on the transcriptional activation of NFκB target genes. A western blot analysis showed that the specific siRNA reduced the level of VCP protein by >90% at 2-4 days after the siRNA transfection, whereas it had little effect on the protein level of actin (Fig. 4).

After the induction of the cells with TNFα, the expression of the NFκB target genes TNFα, IκBα, and A20 was examined by quantitative PCR. When the VCP expression was knocked down by the VCP-specific siRNA, the induction of TNFα gene expression was significantly attenuated (Fig. 4B), comparable to the result of the vesnarinone treatment (Fig. 1). In addition to the attenuation of TNFα, the induced

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expression of the other NFκB target genes IκBα and A20 mRNA was also attenuated (Fig. 4C and D). These results suggest that both vesnarinone and VCP knockdown attenuate the expression of NFκB target genes in TNFα-stimulated cells by affecting NFκB activity.

This result, combined with the finding that phosphorylated IκBα is not degraded in the presence of vesnarinone (Fig. 1D), prompted us to examine the effect of the proteasome inhibitor, MG132, which is known to inhibit the activation of NFκB (Hellerbrand et al., 1998). When the cells were treated with MG132, the activation levels of TNFα, IκBα and A20 mRNA induced by TNFα were attenuated (Fig. 4) to the same level produced by the treatment with VCP siRNA. These results showed that the common effect of vesnarinone treatment and VCP knockdown are consistent with MG132 treatment, suggesting that vesnarinone inhibits the function of VCP, which is involved in a related proteasome degradation process.

Vesnarinone treatment enhances ubiquitinated IκBα accumulation

Due to the result that vesnarinone attenuates TNFα expression, we hypothesized that ubiquitinated-IκBα was not degraded, but rather, accumulated in the

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presence of vesnarinone or VCP knockdown. Thus, we examined the effect of vesnarinone on the accumulation of ubiquitinated IκB protein by performing a transient transfection of a FLAG-IκBα expression vector in 293T cells. The cells were incubated with various concentrations of vesnarinone, followed by FLAG-immunoprecipitation in a highly stringent buffer to purify and concentrate the ubiquitinated IκBα, which was detected by immunoblotting using anti-ubiquitin or anti-IκBα antibodies.

Predictably, the high molecular weight ubiquitinated protein detected using the anti-ubiquitin antibodies and the high molecular weight IκBα detected using the anti-IκBα antibodies were both enhanced in a dose-dependent manner by the treatment with vesnarinone (Fig. 5A, lanes 3-6). In addition, these signals produced similar pattern to the MG132 treatment (Fig. 5A, lane 7). Thus, we concluded that ubiquitinated IκBα accumulated after the vesnarinone treatment.

As the knockdown of VCP showed similar effects to vesnarinone, we examined whether the knockdown of VCP can also cause the accumulation of poly-ubiquitinated IκBα. As determined by western blotting, the knockdown of VCP induced the accumulation of ubiquitinated IκBα (Fig. 5B). These results suggest that

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VCP is significant for the degradation of IκBα and the activation of NFκB.

Vesnarinone prevents the interaction between VCP and the 26S proteasome

Because VCP is known to bind to ubiquitinated proteins such as such as IκBα, cyclin E, and HIF1α (Asai et al., 2002; Alexandru et al., 2008; Dai et al., 1998b; Cayli et al., 2009; Dai and Li, 2001; Yen et al., 2000a) and contributes to their degradation, we examined whether vesnarinone inhibits the interaction of VCP with ubiquitinated IκBα or the 26S proteasome. An expression vector encoding VCP-His-FLAG was transfected into 293T cells, and a co-immunoprecipitation assay was performed using an anti-FLAG antibody in the presence of MG132. As shown in Fig. 6A, in the presence of MG132, 20S C2, which is a component of the 26S proteasome was co-immunoprecipitated with VCP-His-FLAG(Fig. 6A, lane 4), whereas 20S C2was not precipitated without MG132 (Fig. 6A, lane 3). Interestingly, the quantity of the co-precipitated 20S C2 was reduced by increasing the amount of vesnarinone. These results suggested that vesnarinone inhibits the interaction between FLAG tagged VCP and proteosomal 20S C2 (Fig. 6A lanes 5-7).

When FLAG-IκBα-expressing cells were used for the co-immunoprecipitation assay,

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both VCP and 20S C2 were co-precipitated. In the presence of vesnarinone, the quantity of 20S C2 was reduced in a dose-dependent manner, whereas the quantity of VCP was unchanged. As the total amount of ubiquitinated IκB in the assay was almost the same, it can be concluded that vesnarinone inhibited the interaction between VCP and the 26S proteasome but not the interaction between VCP and ubiquitinated IκB.

Although we had tried to analyze the interaction between VCP and endogenous 20S C2 by co-immunoprecipitation using anti-20S C2 antibody, we failed to detect VCP or IκB with 20S C2, probably because endogenous proteosomal proteins expression was so abundant, and content of the VCP-interacted 20S C2 was quite limited in mammalian cells.

While co-immunoprecipitation assay is limited to show the interaction between VCP and ubiquitinated IκB, these results suggested that ubiquitinated IκB could interact with 20S C2 by mediating with VCP and that vesnarinone blocks proteosomal degradation of the ubiquitinated IκB by inhibiting the interaction between VCP and 20S C2.

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Discussion

In this study, we showed that one of the molecular targets of vesnarinone is VCP (valosin-containing protein, also known as p97 and cdc48 in yeast), a member of the AAA (ATPase-associated with various activities) ATPase family (Dai and Li, 2001). VCP is known to bind ubiquitinated proteins, such as IκBα, cyclin E, and HIF1α and to contribute to the ubiquitin-dependent proteasome-mediated degradation of proteins (Asai et al., 2002; Alexandru et al., 2008; Dai et al., 1998b; Cayli et al., 2009; Dai and Li, 2001; Yen et al., 2000a). Vesnarinone induced the accumulation of ubiquitinated IκBα by inhibiting the interaction between VCP and the 26S proteasome, which was essential for the degradation of IκBα and the activation of NFκB, implying that vesnarinone inhibited the function of VCP.

It has been reported that ubiquitinated IκBα remained bound to the p65-containing complexes in cells treated with a proteasome inhibitor, which also supports our results and others (Roff et al., 1996; Didonato et al., 1996). These results suggested that vesnarinone is an NFκB pathway inhibitor and that vesnarinone suppresses the activation of NFκB and the expression of TNFα mRNA by inhibiting the interaction of VCP with the 26S proteasome. As NFκB is an essential transcription

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factor for TNFα activation, it is reasonable that the failure of NFκB activation by vesnarinone directly affects TNFα activation. This new finding can explain our previous data and other results (Manna and Aggarwal 2000) that showed that vesnarinone inhibits the TNFα activation in IL60 and other cell lines. Although it was shown that vesnarinone inhibited the TNFα expression by inhibiting NFκB activation in a previous study (Manna and Aggarwal 2000), we could explain this inhibition based on molecular mechanisms, showing that vesnarinone inhibits the IκB degradation mediated by VCP, which is a novel molecular target of vesnarinone. One of the important differences from the previous study is IκB phosphorylation status by vesnarinone treatment. While the previous study showed inhibition of IκB phosprylation by vesnarinone, we could observe IκB phosphorylation even after vesnarinone treatment. The difference may result of the difference of the cell lines, our finding revealed another mechanism of NFκB regulation by vesnarinone.

Vesnarinone is an inotropic agent for the treatment of congestive heart failure with several known modes of action. For example, vesnarinone is known to augment sodium-calcium exchange (Yatani et al., 1989), resulting in enhanced myocardial contractility; to inhibit phosphodiesterase III (PDE3), resulting in an increase of the

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cyclic AMP concentration; to increase intracellular calcium ions; to alter sodium and potassium channels; and to activate the phosphorylation of cell adhesion-related molecules. Our results presented here add a new role of vesnarinone to the above list of effects.

Our study also provides new insight into the regulation of NFκB activity. As NFκB is an important transcription factor in immune responses, inflammation, cell proliferation and other important biological processes, it has been considered as an important target for drug development. Many small molecules have been reported to be inhibitors of NFκB thorough their effects on the NFκB activation process.

One group of small molecule inhibitors targets the activity of NFκB. For example, gallic acid is reported to interfere with the binding activity of p65 (Choi et al., 2009). Proteasome inhibitors, such as PS-341 and PS-519, are known to inhibit protease activity directly (Sunwoo et al., 2001). In addition to the direct inhibition of the proteasome, the protein degradation pathway prior to proteasome entry is also a target of many compounds. For example, benzoquinones and herbimycin are known inhibitors of IκB kinase activity (Ogino et al., 2004) and sesquiterpene lactones are also hypothesized to interfere with the IκB kinase (Hehner et al., 1999). Because

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vesnarinone is supposed to inhibit the interaction between VCP and a proteasome component, it can be placed in a new category and no known chemicals have the same activity yet. Thus, our study raises the possibility of regulating the NFκB activity by a new target molecule and VCP can be considered a novel target of anti-inflammation and immune drugs.

Recently, multiple functions of VCP have been identified (Meyer et al., 2012) , including autophagy (Ju et al., 2009), endolysosomal sorting and regulating of proteins (Ritz et al., 2011), mitochondrial membrane protein turnover (Braun et al., 2006) and genome stability (Meerang et al., 2011). Although we only suggested the inhibition of the interaction between VCP and the 26S proteasome by vesnarinone in terms of NFκB inactivation, vesnarinone might affect many other biological processes within other cellular contexts. Indeed, the examination of the function of vesnarinone via VCP within the context of different cells is an intriguing avenue of study. For example, as a previous study suggests that VCP is required for the degradation of cyclin E, a cell cycle regulator (Dai and Li, 2001), vesnarinone may influence the proliferation of cancer cells by regulating the degradation of cyclin E. In fact, vesnarinone inhibits the growth of several cancer cell lines (Honma et al., 1999; Yokozaki et al., 1999), rising the

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possibility that vesnarinone might have anti-tumor activity by affecting the function of VCP and the degradation of cyclin E. As VCP is known to have multiple functions, the refined regulation of VCP may be useful in the development of drugs that will be used for aspects other than NFκB-related processes.

In this study, we used a high-performance affinity chromatography protocol developed in our laboratory (Shimizu et al., 2000) and identified VCP as a vesnarinone-binding protein. We have previously shown that several drug targets, including thalidomide, can be identified efficiently (Ito et al., 2010) by using this technique. The identification of VCP as a molecular target of vesnarinone might have been difficult without this technique.

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Footnotes

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Figure regends

Figure 1

Vesnarinone inhibits TNFα-induced expression of NFκB target genes

LP101 (A) or 293T (B) cells were preincubated at 37ºC for 12 h with different concentrations (0-300 μM) of vesnarinone, followed by a 60 min incubation with or without 10 ng/ml TNFα. The total RNA was prepared and analyzed by reverse transcription Q-PCR using primers specific for TNFα mRNA. The data represent the averages +/- S.D. of three independent experiments (*, $P < 0.05$). (C) NFκB-driven and control reporter plasmids were transfected into 293T cells; 12 h later, the cells were preincubated with different concentrations (0-300 μM) of vesnarinone for 12 h, followed by a 12 h incubation with or without 10 ng/ml TNFα. The cells were harvested, and the cell lysates were subjected to luciferase assays. (D) The 293T cells were preincubated with or without 300 μM vesnarinone for 12 h and treated with TNFα(10 ng/ml), followed by a 0-60 min incubation. The cells were harvested, and IκBα phosphorylation was analyzed by immunoblotting (upper panel). Lower graph shows each band intensity of p-IκB, IαB or 20S C2.

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Figure 2

Identification of VCP as a vesnarinone-binding protein

(A) Structure of the vesnarinone amino derivative and FG-beads. (B) Affinity purification of vesnarinone-binding proteins. Vesnarinone-binding protein was purified from LP101 cell extracts with vesnarinone immobilized FG-beads (fix-N-Ves). The bound protein was eluted by increasing concentrations (0-6 mM) of free N-vesnarinone (free-N-Ves). The eluted proteins were analyzed by silver staining (top) and immunoblotting (IB) using an anti-VCP antibody. When the free FG-beads were used (fix-N-Ves -), no specific binding protein was purified. (C) Direct binding of vesnarinone to VCP. Recombinant VCP-His-FLAG was incubated with vesnarinone-immobilized FG-beads, and the bound materials were immunoblotted.

Figure 3

Vesnarinone binds to the D1 domain of VCP

(A) Schematic structure of wild-type VCP and its derivatives used for the mutational study. VCP has an N-terminal poly-ubiquitin recognition domain, the D1-domain and the D2-domain. The results of the vesnarinone-binding assays are summarized to the

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right of the structure. (B) His-FLAG-tagged VCP deletion mutants were expressed in 293T cells and purified by using vesnarinone-immobilized beads. The input (left) and eluted (right) proteins were immunoblotted using an anti-FLAG antibody.

Figure 4

VCP knockdown suppresses the TNFα-induced expression of NFκB target genes

(A) Knockdown of VCP expression by siRNA. The 293T cells transfected with the VCP- or GFP-specific siRNA were incubated for different time periods (0, 20, 25, 30, 60, 90, 120, 180 min from the left) with 10 ng/ml TNFα, and the cell lysates were subjected to western blot analysis using anti-VCP and anti-actin antibodies (upper panel). Lower panel shows each band intensity of VCP or actin. (B-D) The 293T cells were transfected with VCP-specific siRNA or mock-transfected; 72 h later, the cells were treated with or without TNFα for 30 min. The total RNA was prepared and analyzed by reverse transcription Q-PCR using primers specific for the indicated mRNAs. The data represent the average +/- S.D. of three independent experiments (*, $P < 0.05$; **, $P < 0.01$).

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Figure 5.

Vesnarinone and VCP knockdown enhance IκBα protein accumulation

(A) Accumulation of polyubiquitinated IκB in vesnarinone-treated cells. Empty vectors (mock) or pcDNA3.1-FLAG-IκBα were transfected into 293T cells. The cells were then incubated with different concentrations (0-300 μM) of vesnarinone at 48 h after the transfection, and the cells were treated with or without 10 ng/ml TNFα for 30 min at 60 h after the transfection. The cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody. The lysates (input) and immunoprecipitates were immunoblotted.

(B) Accumulation of polyubiquitinated IκB in VCP knockdown cells. The cells were transfected with VCP-specific siRNA; 24 h later, the cells were transfected with pcDNA3.1-FLAG-IκBα. After 72 h, the cells were exposed to 10 ng/ml TNFα for 30 min. The cell lysates were subjected immunoprecipitation using an anti-FLAG antibody. The lysates (input) and immunoprecipitates were immunoblotted.

Figure 6

Vesnarinone inhibits the interaction between VCP and the proteasome.

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(A) Empty vector or pHyg-EF2-VCP-His-FLAG was transfected into 293T cells; 48 h later, the cells were treated with different concentrations (0-300 μM) of vesnarinone. Twelve hours later, the cells were treated with 5 μM MG132 for 60 min, followed by stimulation with 10 ng/ml TNFα for 20 min. The cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody. The lysates (input) and co-immunoprecipitates (IP:FLAG) were immunoblotted using either anti-FLAG or anti-20S C2. (B) Either an empty vector or pcDNA3.1-FLAG-IκBα was transfected into 293T cells; 48 h later, the cells were treated with different concentrations (0-300 μM) of vesnarinone. Twelve hours later, the cells were treated with or without 10 ng/ml TNFα for 30 min. The cell lysates were subjected to immunoprecipitation using an anti-FLAG antibody. The lysates (input) and co-immunoprecipitates (IP:FLAG) were immunoblotted. Co-immunoprecipitates were detected using anti-VCP, anti-FLAG, anti-20S C2 or anti-ubiquitin antibody.

Figure 1

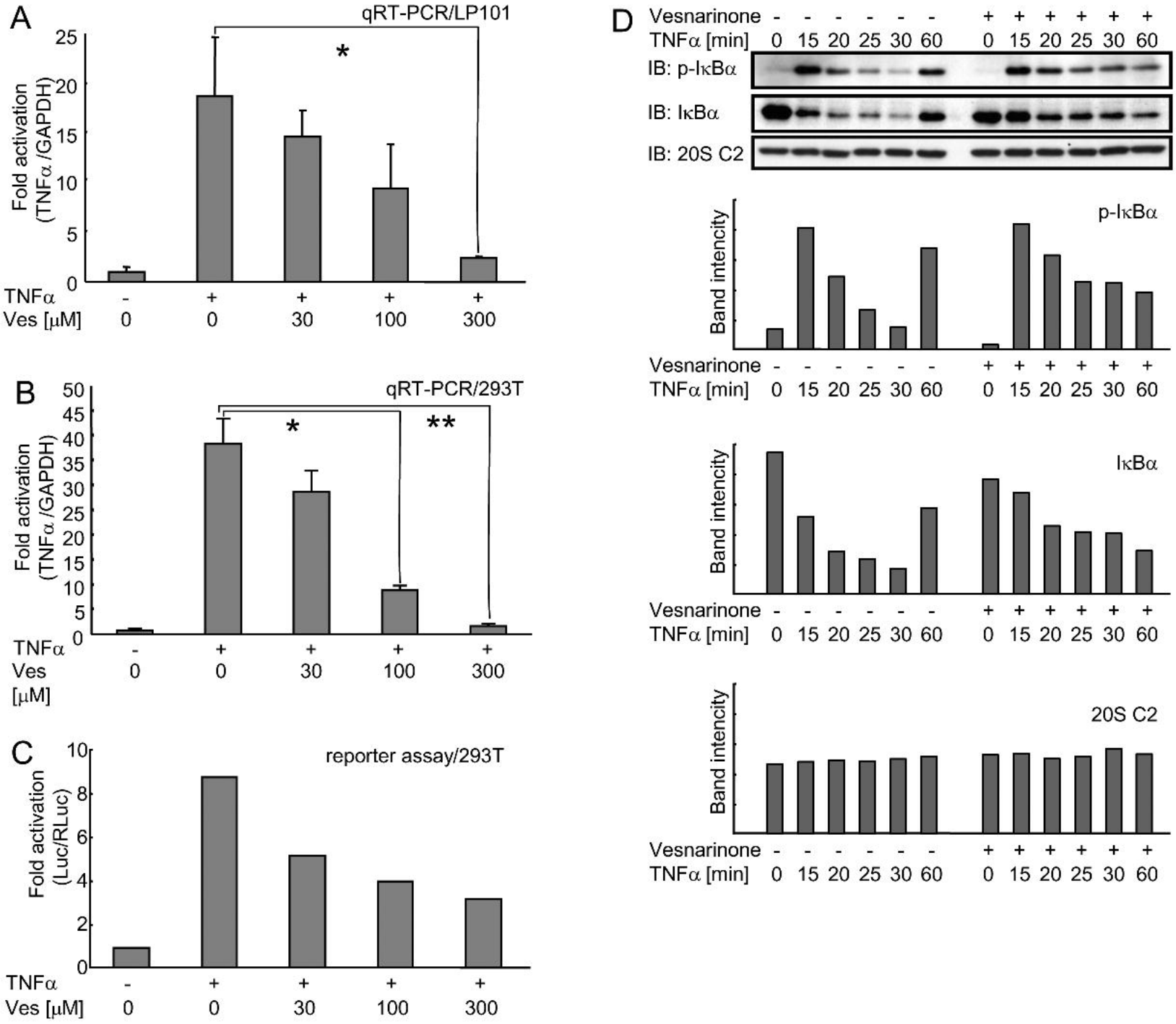
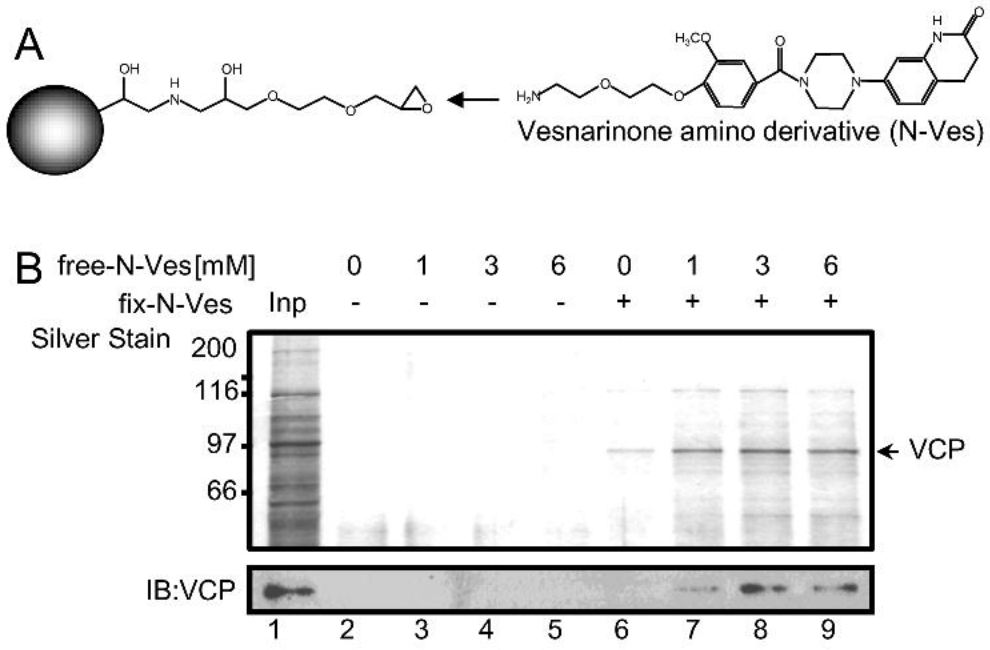

A
qRT-PCR/LP101
Fold activation (TNFα /GAPDH)
25
20
15
10
5
0
*
TNFα - + + + +
Ves [μM] 0 0 30 100 300
B
qRT-PCR/293T
Fold activation (TNFα /GAPDH)
50
45
40
35
30
25
20
15
10
5
0
*
**
TNFα - + + + +
Ves [μM] 0 0 30 100 300
C
reporter assay/293T
Fold activation (Luc/RLuc)
10
8
6
4
2
0
TNFα - + + + +
Ves [μM] 0 0 30 100 300
D
Vesnarinone - - - - - - + + + + + +
TNFα [min] 0 15 20 25 30 60 0 15 20 25 30 60
IB: p-IκBα
IB: IκBα
IB: 20S C2
p-IκBα
Band intencity
IκBα
20S C2

Figure 2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Vesnarinone amino derivative (N-Ves)
B
free-N-Ves[mM]
0 1 3 6 0 1 3 6
fix-N-Ves
Inp - - - - + + + +
Silver Stain
200
116
97
66
VCP
IB:VCP
1 2 3 4 5 6 7 8 9
C
Input (%)
Fixed N-Ves
0.1 0.3 1 - +
IB:FLAG

Figure 3

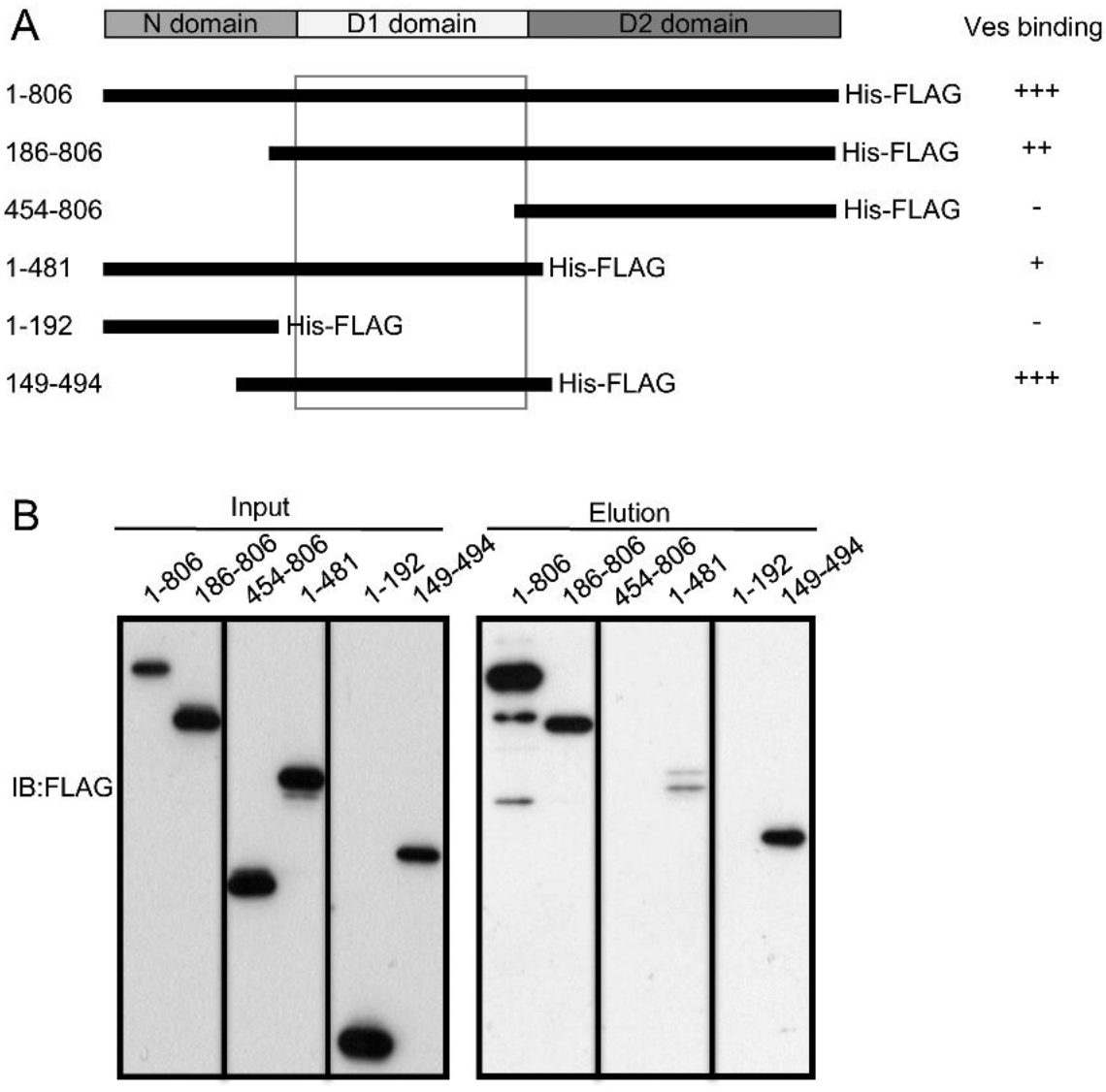
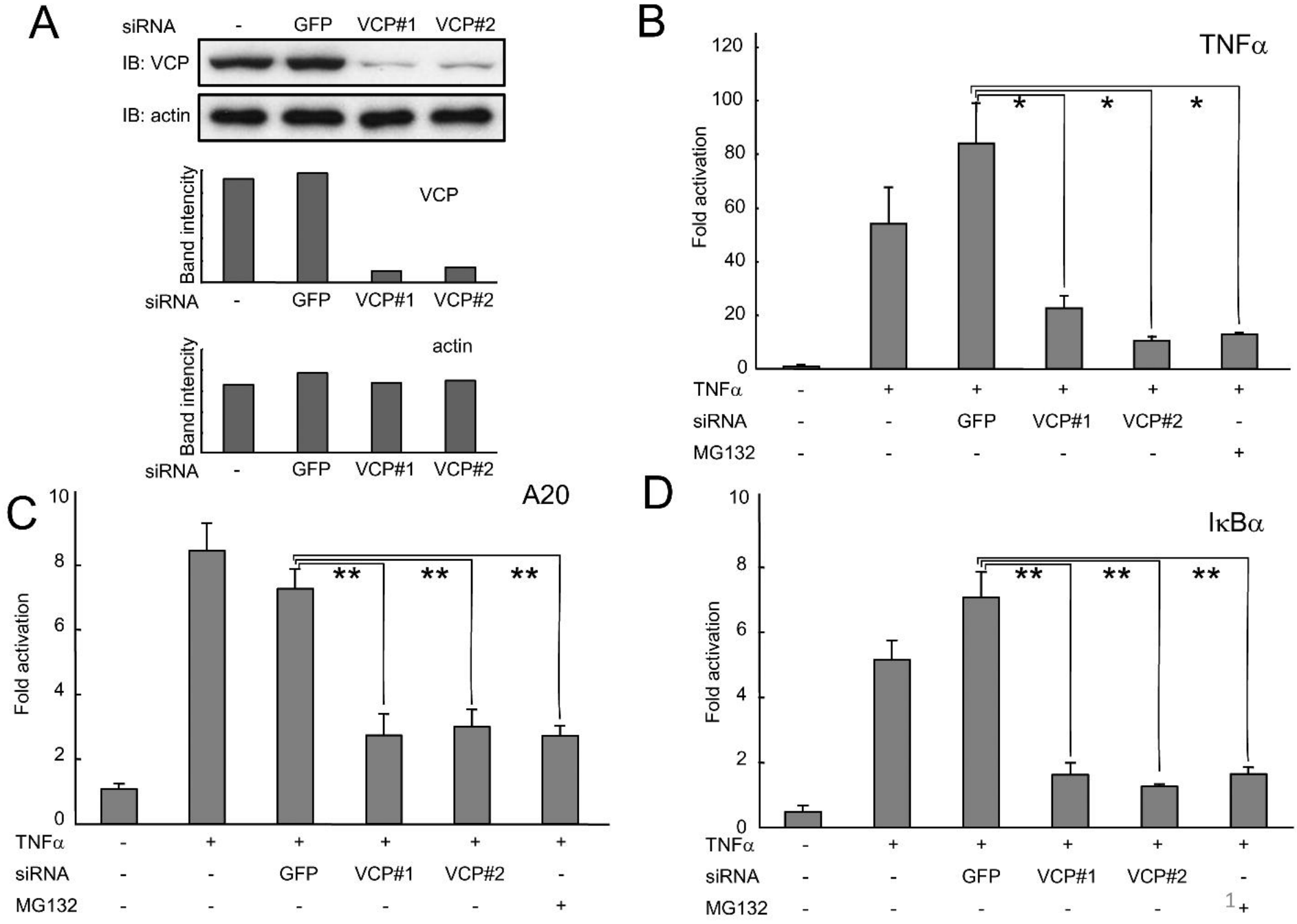

A
N domain
D1 domain
D2 domain
Ves binding
1-806
His-FLAG
+++
186-806
His-FLAG
++
454-806
His-FLAG
-
1-481
His-FLAG
+
1-192
His-FLAG
-
149-494
His-FLAG
+++
B
Input
Elution
1-806
186-806
454-806
1-481
1-192
149-494
IB:FLAG

Figure 4


A
siRNA - GFP VCP#1 VCP#2
IB: VCP
IB: actin
Band intencity
VCP
siRNA - GFP VCP#1 VCP#2
Band intencity
actin
siRNA - GFP VCP#1 VCP#2
B
TNFα
Fold activation
120
100
80
60
40
20
0
*
*
*
TNFα - + + + + +
siRNA - - GFP VCP#1 VCP#2 -
MG132 - - - - - +
C
A20
Fold activation
10
8
6
4
2
0
**
**
**
TNFα - + + + + +
siRNA - - GFP VCP#1 VCP#2 -
MG132 - - - - - +
D
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Fold activation
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**
TNFα - + + + + +
siRNA - - GFP VCP#1 VCP#2 -
MG132 - - - - - +

Figure 5

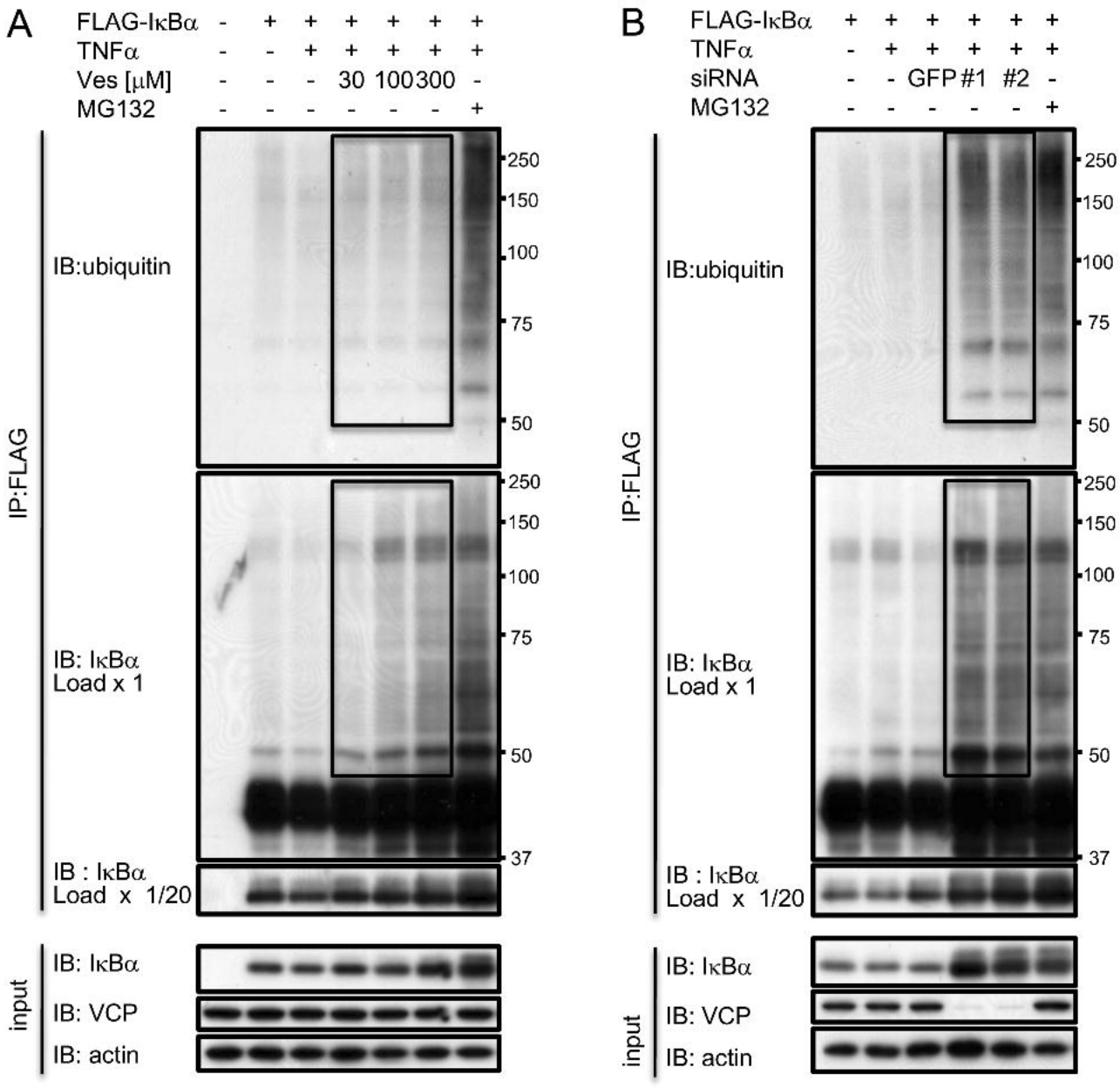
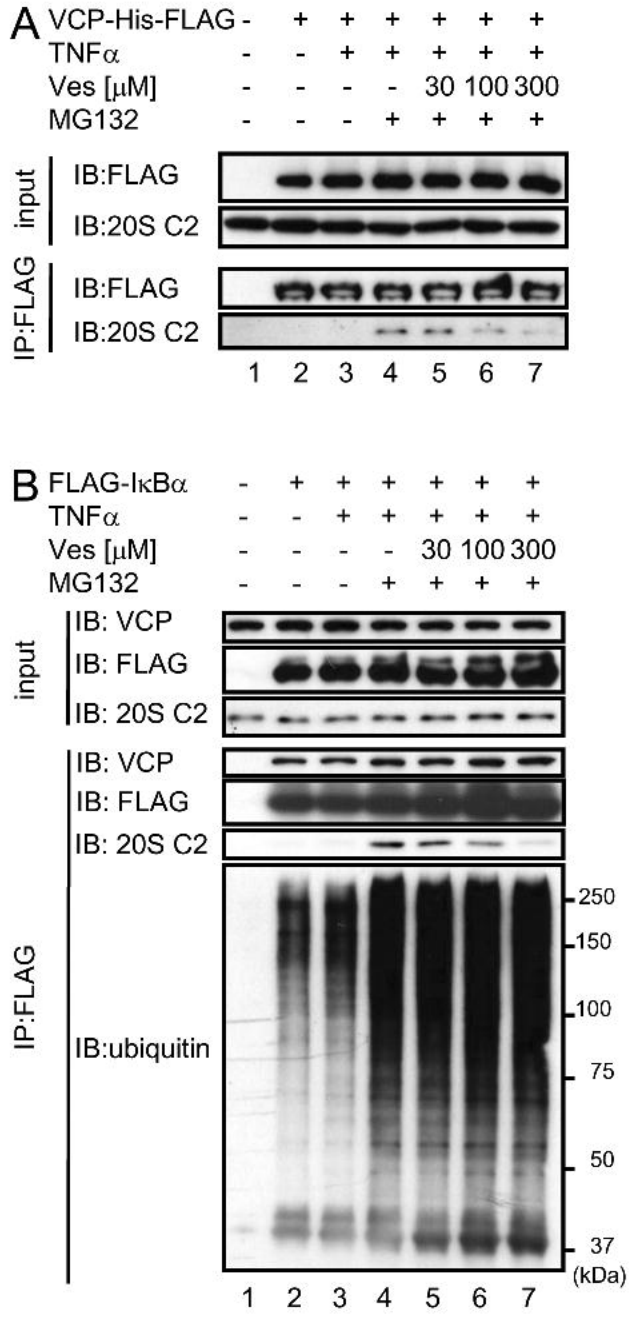

A
FLAG-IκBα - + + + + + +
TNFα - - + + + + +
Ves [μM] - - - 30 100 300 -
MG132 - - - - - - +
IP:FLAG
IB:ubiquitin
IB: IκBα
Load x 1
IB : IκBα
Load x 1/20
input
IB: IκBα
IB: VCP
IB: actin
250
150
100
75
50
37
B
FLAG-IκBα + + + + + +
TNFα - + + + + +
siRNA - - GFP #1 #2 -
MG132 - - - - - +
IP:FLAG
IB:ubiquitin
IB: IκBα
Load x 1
IB : IκBα
Load x 1/20
input
IB: IκBα
IB: VCP
IB: actin
250
150
100
75
50
37

Figure 6


A
VCP-His-FLAG - + + + + + +
TNFα - - + + + + +
Ves [μM] - - - - 30 100 300
MG132 - - - + + + +
input
IB:FLAG
IB:20S C2
IP:FLAG
IB:FLAG
IB:20S C2
1 2 3 4 5 6 7
B
FLAG-IκBα - + + + + + +
TNFα - - + + + + +
Ves [μM] - - - - 30 100 300
MG132 - - - + + + +
input
IB: VCP
IB: FLAG
IB: 20S C2
IP:FLAG
IB: VCP
IB: FLAG
IB: 20S C2
IB:ubiquitin
250
150
100
75
50
37
(kDa)
1 2 3 4 5 6 7