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# Enhancement of TNF-α-mediated cell death in vascular smooth muscle cells through cytochrome *c*-independent pathway by the proteasome inhibitor

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**Abstract** **There is substantial evidence that cytokines induce apoptosis of vascular smooth muscle cells (VSMCs) in atherosclerosis. Its regulation, however, is not completely defined. The aim of this study is to investigate whether proteasome activity is related with apoptosis in VSMCs by tumor necrosis factor-α (TNF-α). Rat aorta smooth muscle cells were treated with TNF-α and proteasome inhibitor MG132 and then cell death was determined by morphology, viability, and DNA fragmentation. MG132 or TNF-α alone did not induce cell death. In contrast, co-treatment of TNF-α and proteasome inhibitor induced death and DNA degradation in VSMCs, suggesting proteasome inhibitor enhanced death activity of TNF-α. The death was not blocked by ascorbic acid but by nitric oxide synthase inhibitor $N^G$-monomethyl-L-arginine. Both caspase-3 and -8 were activated during the death by the proteasome inhibitor and TNF-α. The death was effectively blocked by the caspase-3 inhibitor z-DEVD-fmk, suggesting a role of caspase-3 in the death. Nonetheless, there were no significant alterations in the level of Bcl-2, Bcl-$X_L$, Bax and Bak by the proteasome inhibitor, nor any evidence of cytochrome (cyt) *c* release into cytosol from dying cells, suggesting that cyt *c* is not involved. These results suggest that proteasome inhibition potentiates TNF-mediated death in VSMCs in a cyt *c*-independent pathway. The present study proposes a new mechanism by which VSMCs undergo death by cytokines.**



*Key words:* Atherosclerosis; Proteasome; Vascular smooth muscle cell; Tumor necrosis factor

## 1. Introduction

Tumor necrosis factor (TNF)-α is a representative of the family of trimeric cytokines and cell surface proteins such as Fas ligand (FasL), CD40 ligand, and TNF-α-related apoptosis-inducing ligand [1]. Upon binding of ligands, members of the TNF-α receptor (TNFR) superfamily can induce cell death. The most widely accepted apoptotic pathway involves TNFR1-associated death domain protein (TRADD), Fas-associated death domain-containing protein (FADD), and caspases. Binding of TNF-α to its receptor, TNFR1, induces receptor trimerization and recruitment of several signaling proteins to the cytoplasmic domains of the receptors. Among the recruited proteins, TRADD, FADD, and caspase-8 are associated with cell death [2,3]. The recruited caspase-8 is activated by cleavage of the N-terminal prodomain, which in turn leads to activation of downstream caspases such as caspase-3 [4]. Although ligand binding of TNFR1 can trigger cellular apoptosis, there is evidence that TNF-α itself has little effect on the apoptosis of vascular smooth muscle cells (VSMCs) [5]. In addition to apoptosis, exposure of cells to TNF-α can result in activation of transcription factors AP-1 and NF-κB that induce gene expression [2,3]. The activation of NF-κB requires degradation of its inhibitor protein IκB. In response to TNF-α, the IκBs are phosphorylated by the IκB kinase complex, resulting in the ubiquitination, degradation by proteasome, and nuclear translocation of freed NF-κB [2]. Some genes induced by NF-κB act to suppress TNF-α-induced apoptosis, thereby explaining why the apoptotic response to TNF-α is usually dependent on inhibition of RNA or protein synthesis [6,7]. Still the mechanism of death in VSMCs by cytokines remains obscure.

The ubiquitin/proteasome system is the main non-lysosomal route for intracellular protein degradation. It is instrumental to regulation of various cellular processes, such as cell cycle progression, antigen processing, and cell death [8]. The role of proteasome in cell death was investigated with various proteasome inhibitors. Proteasome inhibitors seem to be able to trigger both the intrinsic apoptotic pathway [9,10] and the receptor-mediated apoptotic pathway [11,12]. In human VSMCs, proteasome inhibitors increased susceptibility to FasL. The ability of proteasome inhibitors to sensitize vascular smooth muscle cells to FasL raised the question as to whether proteasome inhibitor can do the same in another member of the TNF family. The present study demonstrated that inhibition of proteasome function resulted in the enhancement of death activity of TNF-α in VSMCs without release of cytochrome (cyt) *c*.

## 2. Materials and methods

### 2.1. Cell culture

Rat aorta smooth muscle cells were grown in Dulbecco's modified Eagle's medium-high glucose (DMEM) (Life Technology, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 5 mM L-glutamine, plus 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere of 5% $CO_2$.

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*2.2. Antibodies and reagents*

MG132 was purchased from Calbiochem (La Jolla, CA, USA). z-Asp-Glu-Val-Asp-fluoromethylketone (z-DEVD.fmk) was obtained from Enzyme Systems Products (Livermore, CA, USA). Antibodies for Bcl-2, Bcl-$X_L$, Bax and Bak were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody for cyt *c* was from PharMingen (San Diego, CA, USA). Antibody for activated caspase-3 was obtained from Cell Signaling Technology (Beverly, MA, USA). Cytokines were obtained from R&D systems (Minneapolis, MN, USA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

*2.3. Measurement of viability*

Cell viability was determined by exclusion of trypan blue. At the indicated times after treatment with reagents, cells were detached with trypsin, pelleted, and resuspended in DMEM containing 1% FBS. After staining with trypan blue, viable cells in five random fields of view were counted. The percentage of treated viable cells was determined as a percentage of control viability.

*2.4. Enzyme assay of oligonucleosomes*

Oligonucleosomes in cells treated with appropriate reagents were quantitatively determined with the cell death detection ELISA$^{PLUS}$ kit (Roche, Indianapolis, IN, USA).

*2.5. Activities of caspase-8 and -3*

The activities of caspase-8 and -3 were examined using colorimetric methods following the manufacturer's instructions (Clontech, Palo Alto, CA, USA).

*2.6. Western blot analysis*

Cells were lysed in a lysis buffer (50 mM Tris–HCl, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The lysates were resolved on SDS–PAGE gels, followed by transfer to polyvinylidene difluoride membranes. Membranes were blocked in Tris-buffered solution (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk, and then incubated with primary antibodies for 1–2 h. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Chemiluminescent signals were imaged on X-ray films.

*2.7. Preparation of mitochondrial and S100 cytosol fractions*

Cells were washed with phosphate-buffered saline and incubated in buffer A (20 mM HEPES, pH 7.5/10 mM KCl/1.5 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/0.1 mM PMSF) containing 250 mM sucrose and homogenized by 10 strokes with a Teflon homogenizer. After centrifugation of homogenate twice at $750\times g$ for 10 min at 4°C, the supernatants were centrifuged at $10\,000\times g$ for 15 min at 4°C. The pellets were collected, resuspended in buffer A containing sucrose (250 mM), and kept frozen at −70°C. The resulting supernatants were further centrifuged at $100\,000\times g$ for 1 h at 4°C and the resulting supernatants (S100 cytosolic fraction) were also frozen at −70°C. Cyt *c* in mitochondrial and cytosolic fractions were immunologically probed by Western blotting.

## 3. Results

*3.1. Death in VSMCs by TNF-α is induced in the presence of proteasome inhibitor*

To investigate whether proteasome inhibition affected death signal by TNF-α, the proteasome inhibitor MG132 was utilized. MG132 efficiently blocked proteasome activity in eukaryotic cells [13,14]. The morphological appearance of VSMCs treated for 24 h with indicated reagents was examined (Fig. 1). Cells treated with TNF-α (A) or MG132 (B) alone remained attached to tissue culture plates. However, a large number of cells treated with both TNF-α and proteasome inhibitor (C) detached and were floating in the medium. The detachment was profoundly blocked by the caspase inhibitor z-DEVD (D). To assess the extent of cell death by the treatment, cell viability was determined at indicated time points

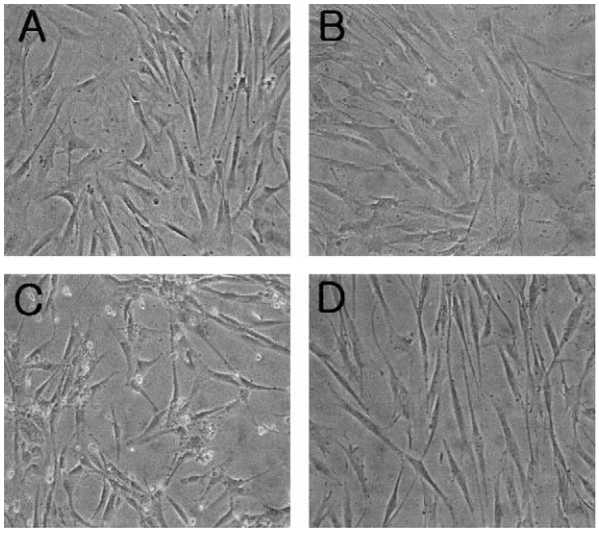


Fig. 1. Morphology of VSMCs treated with TNF-α and proteasome inhibitor. VSMCs were treated with proteasome inhibitor (0.1 μM) alone, or co-treated with TNF-α (50 ng/ml) and proteasome inhibitor with or without z-DEVD (50 μM). Cells were incubated for 24 h and photographed. Magnification 200×. A: TNF-α. B: MG132. C: MG132+TNF-α. D: MG132+TNF-α+z-DEVD.

(Fig. 2). TNF-α did not affect the viability, which is consistent with the findings by Geng et al. [5]. MG132 alone slightly affected viability without statistical significance, whereas simultaneous treatment with TNF-α and MG132 dramatically decreased viability in a time-dependent manner. Cell death was markedly increased at 24 h after treatment ($P < 0.01$). The data demonstrated that in the presence of proteasome inhibitor, TNF-α induced death of VSMCs. The cell death, however, was significantly blocked by z-DEVD ($P < 0.01$).

It was investigated whether degradation of DNA occurred by measuring the amount of mono- or oligonucleosomes comprising DNA fragments and histones (Fig. 3). Treatment with MG132 or TNF-α alone showed trivial effects on DNA fragmentation. Compared to cells treated with TNF-α or proteasome inhibitor alone, profoundly increased amounts of mono- or oligonucleosomes were detected in cells co-treated with TNF-α and proteasome inhibitor ($P < 0.01$). z-DEVD significantly inhibited the production of oligonucleosomes ($P < 0.01$). The data indicate that apoptosis might be induced by TNF-α, as the release of DNA fragments and histones is the key feature in apoptosis [15]. The death in VSMCs by oxidized low density lipoprotein and cytokines was blocked by ascorbic acid [16] and nitrogen oxide synthase inhibitor [5], respectively. Thus, it was investigated whether TNF-α-induced apoptosis was influenced by ascorbic acid or $N^G$-monomethyl-L-arginine (L-NMMA) (Fig. 3). Cells were pre-treated with either ascorbic acid or L-NMMA before they were exposed to MG132 and TNF-α. Pre-treatment with ascorbic acid did not alter death in VSMCs. The death, however, was significantly inhibited by the nitric oxide synthase inhibitor L-NMMA ($P < 0.05$).

*3.2. TNF-α and proteasome inhibitor activate caspase-3 and -8*

Since caspase-3 inhibitor blocked the death and DNA fragmentation by TNF-α, it was investigated whether TNF-α and proteasome inhibitor activated caspases. The activities of caspase-3 and -8 in the cell lysates were determined at indicated

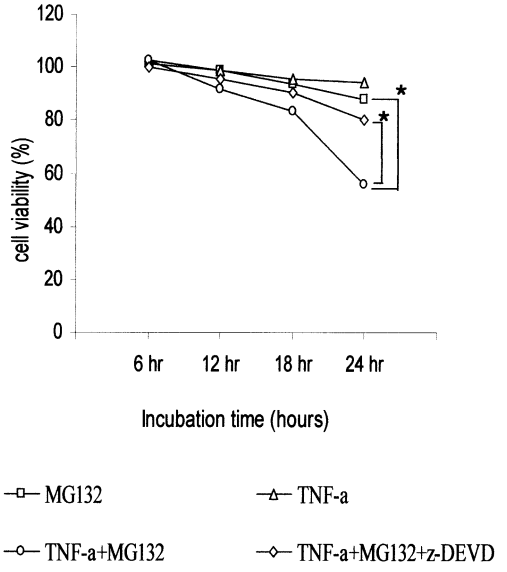


Fig. 2. Viability of VSMCs treated with TNF-α and proteasome inhibitor. Rat VSMCs were incubated for 6, 12, 18, and 24 h with MG132 (0.1 μM) alone, or co-incubated with TNF-α (50 ng/ml) and MG132 in the presence or absence of z-DEVD (50 μM). Viable cells were counted after staining with trypan blue. The viability of untreated control cells was counted as 100%. The viability of treated cells was expressed as a percentage of control cells. Data represent average of four experiments. $^{*}P < 0.01$ at 24 h.

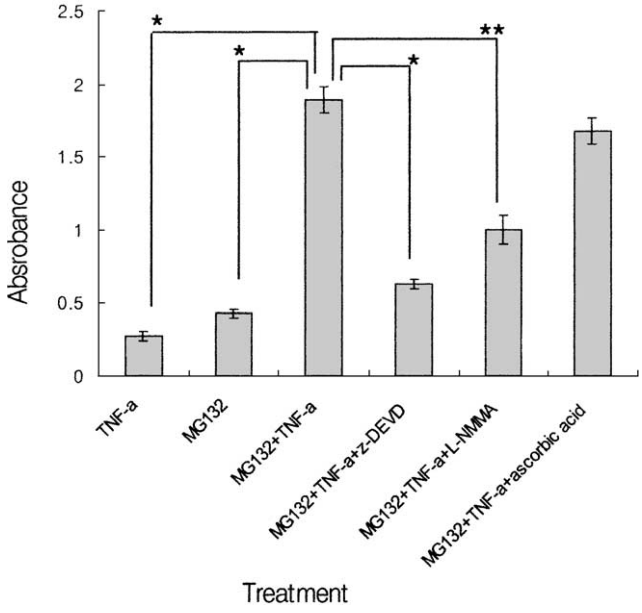


Fig. 3. Enrichment of nucleosomes in dying VSMCs. After pretreatment with or without ascorbic acid (100 μM) or L-NMMA (500 μM) for 12 h, VSMCs were exposed for 24 h to proteasome inhibitor alone, or TNF-α and proteasome inhibitor in the absence or presence of z-DEVD. The fragmented nucleosomes in cell culture supernatant and cell lysate were analyzed using ELISA$^{PLUS}$. The extent of DNA fragmentation was expressed as absorbance at 405 nm. The data are presented as mean ± S.D. of triplicate cultures. The concentrations of proteasome inhibitor, TNF-α, and z-DEVD.fmk were 0.1 μM, 50 ng/ml, and 50 μM, respectively. $^{*}P < 0.01$, $^{**}P < 0.05$.

time points (Fig. 4A). The caspase-8 activity was detected at 6 h post treatment, reached a maximum at 12 h after treatment, and decreased thereafter. The increased caspase-8 activity was followed by activation of caspase-3. The caspase-3 activity was observed at 9 h post treatment and increased up to 20 h after treatment. Furthermore, the active (cleaved) form of caspase-3 was detected in VSMCs by Western blot analysis after cells were co-treated with TNF-α and MG132 (Fig. 4B). The cleaved caspase-3 was detected at 9 h after the co-treatment. Thereafter, the immunoreactive cleaved caspase-3 increased in a time-dependent manner.

### 3.3. Proteasome inhibition does not affect Bcl-2 family proteins

The susceptibility to apoptosis is determined by the ratio of the positive regulators (Bak, Bax, and Bcl-$X_S$) to negative regulators (Bcl-2, Bcl-$X_L$, Mcl-1, and A1) of the Bcl-2 family [17]. It has been reported that proteasome inhibition was followed by accumulation of pro-apoptotic Bax in Jurkat cells [18]. Therefore, it was determined whether proteasome inhibition potentiated TNF-α-mediated death by modifying the amount of anti- or pro-apoptotic Bcl-2 family proteins (Fig. 5A). To investigate this, the levels of Bcl-2 family proteins in response to the proteasome inhibitor were examined. As shown in the figure, there was no evidence of significant changes in immunoreactivity levels of Bcl-2, Bcl-$X_L$, Bax, and Bak in cells treated with MG132.

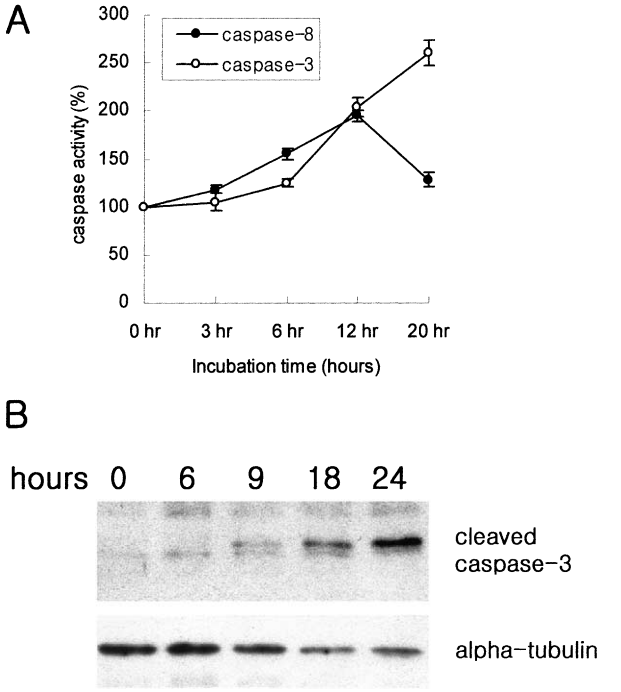


Fig. 4. Activation of caspase-3 and -8. A: Cell lysates were prepared from the VSMCs treated with 0.1 μM MG132 and 50 ng/ml TNF-α for indicated periods of time. The activities of caspase-3 and -8 in the lysates were determined by colorimetric methods. The activities of untreated control cells were counted as 100%. The activities of caspase-3 and -8 in the treated cells were expressed as a percentage of control cells. B: Cells were co-treated with MG132 and TNF-α. The lysates were prepared at indicated time points and were subjected to Western blot analysis with an antibody which recognizes the active form of caspase-3.

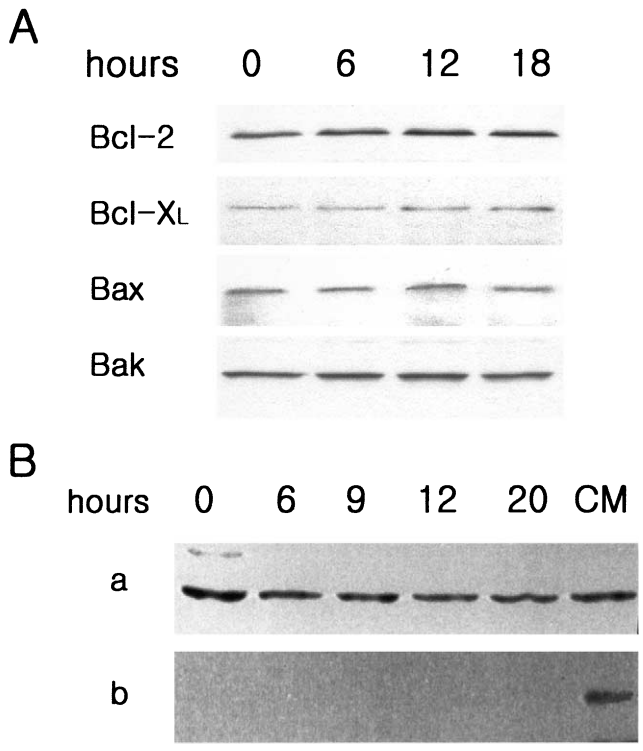


Fig. 5. Immunoblot analysis of Bcl-2 family proteins and cyt *c* in VSMCs. A: The cells were incubated with 0.1 μM MG132 for indicated periods of time. The cells were lysed as described in Section 2. The lysates were subjected to Western blot analysis using antibodies against Bcl-2 family proteins. B: Cells were treated with TNF-α (50 ng/ml) and MG132 (0.1 μM) for indicated periods or with a cytokine mixture (CM) of TNF-α, interferon-γ, and IL-1β as described [21]. Fractions of mitochondria and S100 cytosol were prepared. The presence of cyt *c* in the fraction was examined.

### 3.4. Cyt c is not released from dying VSMCs

TNF-α and Fas treatment can trigger cleavage of Bid by caspase-8, and the cleaved Bid inserts into the mitochondrial membrane, resulting in cyt *c* release [19,20]. The released cyt *c* activates effector caspases including caspase-3 and -7 [21]. We observed activation of caspase-8 and -3 in dying cells. Thus, it was investigated whether cyt *c* played a role in the activation of caspase-3 by examining release of cyt *c* into cytosol (Fig. 5B). Cyt *c* was detected in mitochondrial fractions and the cyt *c* in mitochondrial fraction was not changed before and after treatment with TNF-α and MG132 (panel a). The immunoreactive cyt *c*, however, was not detected in S100 cytosolic fractions (panel b). As a positive control, the cytosolic fraction of VSMCs treated with a cytokine mixture of TNF-α, interferon-γ, and interleukin (IL)-1β was included. Liu et al. [21] reported that the treatment with the cytokine mixture caused release of cyt *c* into cytosol. In agreement with the finding by Liu et al. [21] cyt *c* was detected in the cytosolic fraction of the cytokine-treated cells. The data suggest that cyt *c* is not involved in the activation of caspase-3 while VSMCs undergo death by TNF-α and proteasome inhibition.

## 4. Discussion

Proteasome inhibition can be pro- or anti-apoptotic depending on cell type and proliferative state of the cells. For example, proteasome inhibitors protected cells from apoptosis induced by some but not all stimuli. Programmed cell death of sympathetic neurons upon deprivation of nerve growth factor was delayed by proteasome inhibitors [22]. In thymocytes, proteasome inhibition blocked apoptosis induced by ionizing radiation, glucocorticoids, or phorbol esters [23]. In contrast, proteasome inhibitors elicited apoptosis in Rat-1 cells, PC12 cells [9], and human leukemic HL60 cells [10]. This study showed that proteasome inhibition is pro-apoptotic in VSMCs, as the proteasome inhibitor sensitized cells to TNF-α-mediated death. The data indicate that proteasome inhibitor can regulate cell viability by activation of receptor-mediated death pathways. This finding concurs with that by Masdehors et al., who reported that proteasome inhibitors sensitized chemoresistant and radioresistant human CLL lymphocytes to TNF-α-induced apoptosis [11]. Apoptosis by Fas, a member of the TNF receptor family, in human VSMCs was induced by the proteasome inhibitors MG132 and MG115 [12]. Taken together, these results indicate that proteasome inhibition can alter viability of cells either by triggering intrinsic apoptosis or by activating receptor-mediated death or both.

It is well known that proteasome inhibitors inactivate NF-κB transcriptional activity by preventing degradation of IκB [13]. The inactivation of NF-κB would contribute to death in VSMCs. This view is supported by the fact that overexpression of IκB triggered apoptosis of VSMCs by TNF-α [24]. We were interested in what mechanisms other than NF-κB proteasome inhibition would induce death in VSMCs by TNF-α. Apoptosis is regulated by inciting signals, the Bcl-2 family of proteins, and effector caspase-8 and is determined by the relative amounts of pro- and anti-apoptotic proteins. It was previously reported that proteasome inhibitors did not change the level of caspase-3 and -8 in human VSMCs [12]. The alteration in the expression of Bcl-2 family protein appeared to be related to apoptosis of VSMCs in atherosclerosis. In the apoptotic cells of endarterectomy and atherectomy specimens, there was increased expression of pro-apoptotic Bax and Bak coupled with a paucity of Bcl-2 and lack of Bcl-$X_L$ [25]. Bax induced cyt *c* release from mitochondria. In contrast, Bcl-2 prevented release of cyt *c* [26]. Bak accelerated cell death following IL-3 withdrawal and inhibited the protective action of both Bcl-2 and Bcl-$X_L$ [27,28]. It is, however, unknown what factors regulate the expression of Bcl-2 family proteins. Furthermore, little is known as to whether proteasome inhibition influences the expression of Bcl-2 family in VSMCs. The investigation of the presence or absence of a single protein from this group in cells would not provide complete information on the regulation of apoptosis as the roles of individual proteins are likely to be specific for different cells [29]. Therefore, we investigated whether proteasome is a regulatory factor of the Bcl-2 family in VSMCs. When the expression of four proteins – Bcl-2, Bcl-$X_L$, Bak, and Bax – was determined, there was no appreciable change of the proteins by proteasome inhibitor. The data suggest that proteasome inhibition is not associated with degradation of the Bcl-2 family in VSMCs.

We showed that TNF-α activated caspase-8 and -3 and that the death in VSMCs by TNF-α was suppressed by caspase-3 inhibition. These results suggest that induction of caspase-3 activity is a candidate for the mechanism underlying the sensitization to TNF-α by proteasome inhibitor. Caspase-3 can be activated at least by two pathways [4,19]. The pathway,

type I or type II, depends not only on the apoptotic stimuli but also on the cell type. In addition, there is cross-talk between the two pathways. In type I cells, large amount of caspase-8 formed at the death-inducing signal complex are sufficient to activate caspase-3 and cell death. The anti-apoptotic protein Bcl-2 has no effect on the activation of caspase-8 and -3. In type II cells, caspase-3 is primarily activated downstream of mitochondria, and apoptosis is blocked by Bcl-2. Activated caspase-8 elicits release of cyt *c* in the intermembrane space of mitochondria into cytosol. The released cyt *c* binds with other molecules to form apoptosome complex. The apoptosome complex activates effector caspases such caspase-3 and -7 via caspase-9 [30]. We determined whether mitochondria played a role in the death by detecting release of cyt *c* into cytosol. The data in the present study suggest that mitochondria are unlikely to be involved in the death of VSMCs by proteasome inhibition and TNF-α, as cyt *c* was not released from mitochondria of dying cells.

In the current study, we have investigated the relation between the proteasome system and the TNF pathway and report that (i) proteasome inhibition induces TNF-α-dependent death in VSMCs by up-regulation of caspase activity; (ii) the expression of Bcl-2 family proteins is not influenced by proteasome inhibition; and (iii) cyt *c* is not released from the dying VSMCs into cytosol.

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