

# Crotamine stimulates phagocytic activity by inducing nitric oxide and TNF- $\alpha$ via p38 and NF- $\kappa$ B signaling in RAW 264.7 macrophages

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Crotamine is a peptide toxin found in the venom of the rattlesnake *Crotalus durissus terrificus* and has antiproliferative, antimicrobial, and antifungal activities. Herein, we show that crotamine dose-dependently induced macrophage phagocytic and cytostatic activity by the induction of nitric oxide (NO) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Moreover, the crotamine-induced expression of iNOS and TNF- $\alpha$  is mediated through the phosphorylation of p38 and the NF- $\kappa$ B signaling cascade in macrophages. Notably, pretreatment with SB203580 (a p38-specific inhibitor) or BAY 11-7082 (an NF- $\kappa$ B inhibitor) inhibited crotamine-induced NO production and macrophage phagocytic and cytotoxic activity. Our results show for the first time that crotamine stimulates macrophage phagocytic and cytostatic activity by induction of NO and TNF- $\alpha$  via the p38 and NF- $\kappa$ B signaling pathways and suggest that crotamine may be a useful therapeutic agent for the treatment of inflammatory disease. [BMB Reports 2016; 49(3): 185-190]

## INTRODUCTION

Crotamine is a low molecular weight cationic polypeptide that is found in the venom of the South American rattlesnake *Crotalus durissus terrificus* and possesses several interesting biological activities (1, 2). We have successfully produced the toxin as a pure and active form from *Escherichia coli* (*E. coli*) (3). The toxin selectively inhibits the three human voltage-gated potassium channels, hKv1.1, hKv1.2, and hKv1.3 (3, 4). It has been reported that hKv1.3 channel blockade enhances the phagocytic function of

RAW264.7 macrophages (5), suggesting that crotamine may enhance macrophage phagocytosis.

Macrophages recognize invading pathogens and are key to mounting an appropriate immune response (6, 7). Once activated by stimuli, macrophages upregulate the production of nitric oxide (NO) and various cytokines. Nitric oxide is a free-radical gas that is synthesized by the inducible nitric oxide synthase (iNOS) and mediates a diverse set of functions, including vasodilatation and immunoresponses (6, 8). It has also been identified as a major effector molecule in the destruction of cancer cells by activated macrophages (9). In addition, the involvement of NO during macrophage-mediated killing inhibits the proliferation of tumor cells both *in vitro* and *in vivo* (10-12).

One of the key cytokines upregulated by activated macrophages is TNF- $\alpha$ , which stimulates apoptosis of a wide variety of cells, including tumor cells and cells infected with virus or bacteria (12). The transcription factor NF- $\kappa$ B is a major survival factor in preventing TNF- $\alpha$  induced apoptosis (13) and its regulation is a key component in several phosphorylation steps in the mitogen-activated protein kinase (MAPK) pathway (7, 14). NF- $\kappa$ B plays a critical role in the activation of immune cells by increasing the expression of various cytokines that are essential for immune responses (15).

In this study, we examined how crotamine affects macrophage function. We show for the first time that crotamine enhances macrophage phagocytosis and cytostatic activity, and that it increases NO and TNF- $\alpha$  production by upregulating iNOS and TNF- $\alpha$  expression. Finally, we show that these expression changes are mediated by phosphorylation of p38 and NF- $\kappa$ B and that direct blockage of these pathways obliterates crotamine's activity.

## RESULTS

### Effects of crotamine on phagocytosis and cytostasis of macrophages

The cytotoxic effects of crotamine on RAW 264.7 cells were measured by an MTT assay. Crotamine at 1.5  $\mu$ M did not sig-

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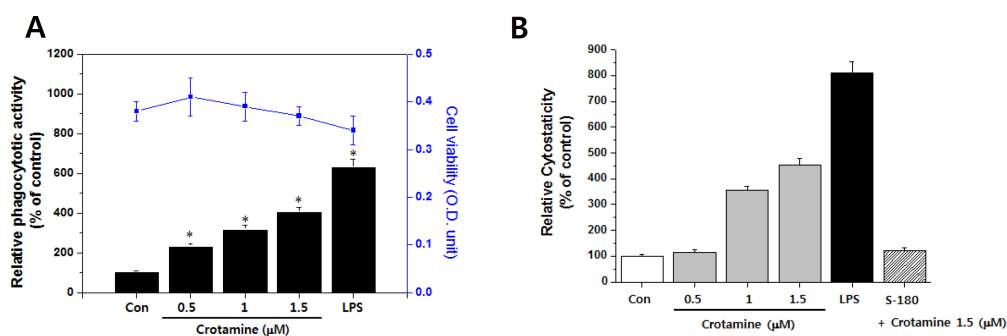
nificantly affect cytotoxicity, as it resulted in 95% cell viability (Fig. 1A). To determine how crotamine affects the phagocytic activity of macrophages, the uptake of FITC-labeled *E. coli* particles was compared between crotamine-treated and untreated macrophages. The stimulatory effects of crotamine on macrophage phagocytosis were measured by internalization of the FITC-labeled *E. coli* particles and were estimated based on the mean fluorescence intensity. Macrophage phagocytosis increased after crotamine treatment in a dose-dependent manner (Fig. 1A). These findings indicate that crotamine increased macrophage phagocytosis, which is one of the functions of macrophages.

To assess other functional activities stimulated by crotamine, the tumor cytostaticity of crotamine-stimulated macrophages was tested against S-180 cells (Fig. 1B). Macrophages showed strong cytostatic activity against the S-180 tumor cells when stimulated with crotamine. However, crotamine at 1.5  $\mu$ M concentration did not exhibit any direct killing activity against S-180

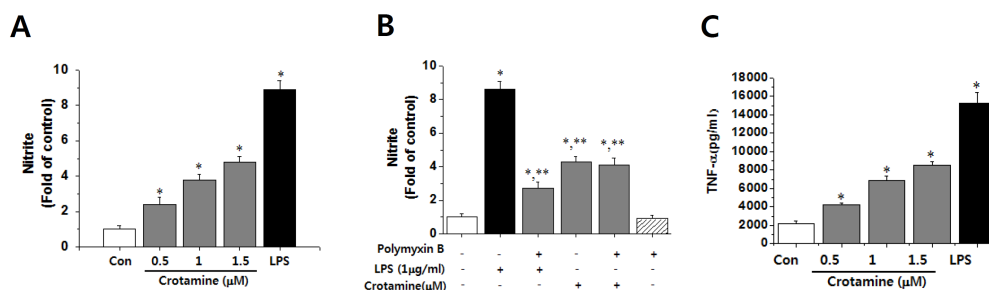
cells. These findings indicate that crotamine enhances macrophage cytostaticity against tumor cells.

### Effects of crotamine on NO production and TNF- $\alpha$ secretion

To further determine whether crotamine could activate macrophages, we assessed NO production in RAW 264.7 cells. Cells were incubated with crotamine for 24 h, and the NO concentration in culture supernatants was measured using the Griess reaction. With crotamine stimulation, NO synthesis by RAW 264.7 cells was augmented in a dose-dependent manner (Fig. 2A). Crotamine or LPS in the absence of polymyxin B were previously used as controls (16). Fig. 2B shows that polymyxin B efficiently inhibited NO production induced by LPS, but that it had no effect on crotamine. This finding suggested that the NO produced by crotamine stimulation did not arise from LPS contamination in the crotamine preparation. Moreover, to assess the effects of crotamine on TNF- $\alpha$  production by activated macrophages, we incubated RAW 264.7 cells with increasing concen-



**Fig. 1.** Effects of crotamine on phagocytosis and cytostasis of macrophages. (A) RAW 264.7 cells were treated with 0.5, 1 or 1.5  $\mu$ M crotamine for 24 h. After FITC labeled-*E. coli* were added, cells were incubated for 3 h. Supernatants containing unphagocytosed bacteria were removed, and fluorescence was measured using a fluorescence microplate reader. Cell viability was assessed using MTT assays. \*Significantly different from control cells ( $P < 0.01$ ). (B) RAW 264.7 cells were incubated with crotamine or medium alone for 48 h. Cells were then washed twice and further co-cultured with S-180. The cultures were incubated for 24 h, and macrophage cytostaticity was measured according to the MTT method. \*Significantly different from control cells ( $P < 0.01$ ).



**Fig. 2.** Effects of crotamine on NO and TNF- $\alpha$  production in macrophages. (A) RAW 264.7 cells were cultured for 24 h with the indicated concentrations of crotamine, or with LPS (1  $\mu$ g/ml). Levels of NO production were determined by measuring the accumulation of nitrite in the culture medium. (B) RAW 264.7 cells were cultured with crotamine or LPS in the presence or absence of polymyxin B. Supernatants were harvested 24 h later and assayed for NO. (C) RAW 264.7 cells were cultured for 6 h in the presence of media alone or with crotamine. The amounts of TNF- $\alpha$  released into the culture medium were measured by immunoassays. \* $P < 0.01$ , significantly different from control cells. \*\* $P < 0.01$ , significantly different from LPS-treated cells.

trations of crotamine. Then, using ELISA, these cytokines were monitored in culture supernatants. Crotamine increased TNF- $\alpha$  secretion in supernatants in a dose-dependent manner (Fig. 2C).

### Effects of crotamine on TNF- $\alpha$ and iNOS gene and protein expression in macrophages

We have shown that crotamine induces the secretion of TNF- $\alpha$  and NO from macrophages. We now wanted to see if crotamine regulates NO and TNF- $\alpha$  secretion at the mRNA and protein levels. Using a real-time quantitative RT-PCR and western blot assay, we found that crotamine dose-dependently increased both the amount of protein produced (Fig. 3A, B) and the expression levels of TNF- $\alpha$  and iNOS (Fig. 3C, D) in a dose-dependent manner. The control  $\beta$ -actin was constitutively expressed and was unaffected by crotamine treatment. These results show that crotamine regulates the transcriptional activation of TNF- $\alpha$  and iNOS.

### Effects of crotamine on NF- $\kappa$ B and p38 activation

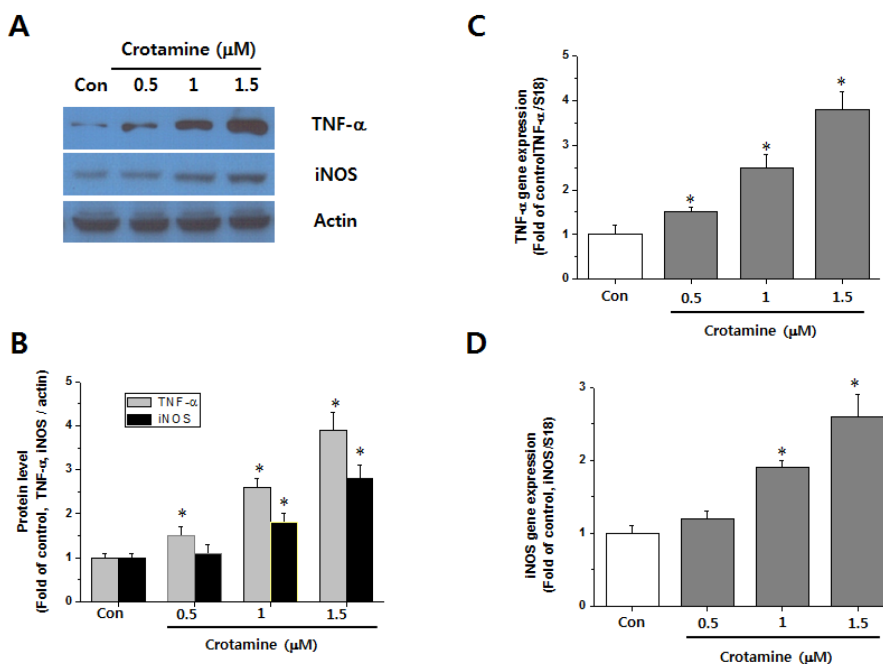
The role of crotamine in TNF- $\alpha$  and iNOS gene expression was examined further by investigating the effects of crotamine on NF- $\kappa$ B dependent gene expression using a luciferase reporter assay for crotamine-induced NF- $\kappa$ B-dependent luciferase activity (Fig. 4A). We found dose-dependent effects on gene expression, which were consistent with the results for NO and TNF- $\alpha$  protein and mRNA expression levels. Additionally, crotamine increased the levels of the NF- $\kappa$ B subunit p65 in the nucleus (Fig. 4C) by immunoblot analysis. With cell activation, the phosphorylation and subsequent degradation of I $\kappa$ B- $\alpha$  leads to NF- $\kappa$ B activation. I $\kappa$ B- $\alpha$  was also rapidly degraded when macrophages

were treated with crotamine (Fig. 4B). Anti-lamin B and  $\beta$ -actin antibodies were used as controls. We can therefore conclude that crotamine-induced NF- $\kappa$ B activation is a consequence of increased I $\kappa$ B- $\alpha$  degradation.

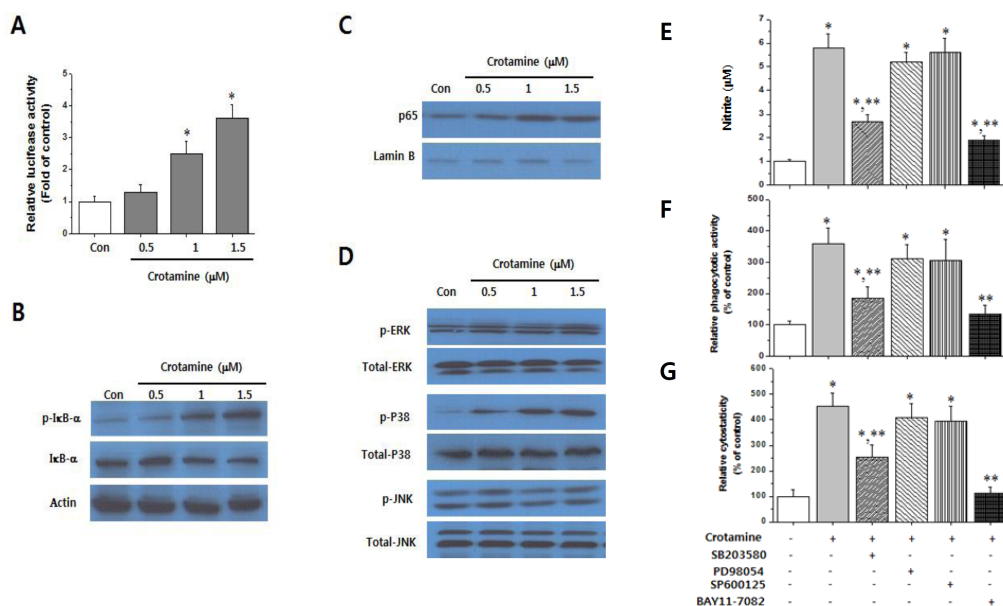
The MAPK pathway is a major signaling pathway for cytokines that are associated with macrophages (17). We therefore examined whether the effect of crotamine-induced NO release and TNF- $\alpha$  production depended on MAPK activation. Western blot analyses were done to detect phosphorylation-specific activation after stimulation with crotamine of the MAP kinases ERK, p38, and JNK. Our results showed that crotamine significantly increases the phosphorylation of p38, but not of JNK or ERK (Fig. 4D).

### Effects of crotamine on the induction of NO and TNF- $\alpha$ by p38 and NF- $\kappa$ B mediated signaling

To further investigate the roles of NF- $\kappa$ B and p38 in NO and macrophage-related TNF- $\alpha$  expression induced by crotamine, we tested the I $\kappa$ B- $\alpha$  kinase inhibitor BAY 11-7082. BAY 11-7082 inhibits the phosphorylation and subsequent degradation of I $\kappa$ B- $\alpha$ , which is an endogenous NF- $\kappa$ B inhibitor. Pretreatment of macrophages with BAY 11-7082 reversed NO production (Fig. 4E), phagocytosis (Fig. 4F) and tumor cytotoxicity (Fig. 4G) induced by crotamine. Additionally, SB203580 which is a p38 inhibitor, effectively inhibited NO production as it reversed NO production, phagocytosis, and tumor cytotoxicity (Fig. 4E-G). Together, crotamine-stimulated NO production, phagocytosis and tumor cytotoxicity are mediated by p38 activation and the NF- $\kappa$ B signaling pathways.



**Fig. 3.** Effects of crotamine on protein and mRNA expression levels of TNF- $\alpha$ , and iNOS in macrophages. (A) RAW 264.7 cells were treated with crotamine for 18 h and then lysed. Expression of levels of TNF- $\alpha$  and iNOS were measured by western blotting. (B) After the optical density of protein immunoreactivity was measured and normalized to that of internal control  $\beta$ -actin. RAW 264.7 cells were treated with crotamine for 3 h and then were lysed and total RNA was prepared for analysis of TNF- $\alpha$  (C) and iNOS (D) gene expression. PCR amplification of the house-keeping gene, S18, was performed for each sample to assess mRNA expression in exposed cells compared with the expression of unexposed cells at each time point by real-time PCR. \*Significantly different from control cells ( $P < 0.01$ ).



**Fig. 4.** Crotamine induces NF-κB transactivation and p38 phosphorylation. (A) RAW 264.7 cells were transiently co-transfected with pGL3-NF-κB-Luc and pCMV-β-gal. After 4 h, cells were treated with the indicated concentrations of crotamine for 18 h. Cells were harvested, and cellular luciferase and β-galactosidase activities were determined. (B) Crotamine induces the phosphorylation and degradation of IκB-α in macrophages. RAW 264.7 cells were treated with the indicated various concentrations of for 30 min. Western blot analysis was then performed. (C) Cells were treated with crotamine for 20 min, and protein levels of p65 were determined immunochemically using its specific antibody. (D) RAW 264.7 cells were treated with crotamine for 30 min to assess MAPK expression. Cell extracts were analyzed to assess MAPK activation by western blotting using antibodies specific to phosphorylated and total ERK, p38 and JNK. NF-κB and p38 specific inhibitors reverse crotamine-mediated NO production, phagocytosis and cytotoxicity in macrophages. Cells were pretreated with PD98059 (20 μM), SB203580 (20 μM) and SP600125 (10 μM) or BAY 11-7082 (10 μM) for 30 min and then cultured for 24 h with crotamine (1.5 μM). (E) Levels of NO production were determined by measuring the accumulation of nitrite in culture medium. (F) After FITC labeled-E.coli were added, cells were incubated for 3 h. Supernatants containing unphagocytosed bacteria were removed, and fluorescence was measured using a microplate reader. (G) Macrophage cytotoxicity was measured according to the MTT method. \*P < 0.01, significantly different from control. \*\*P < 0.01, significantly different from crotamine.

## DISCUSSION

Crotamine is classified as a small basic myotoxin (18) and has specific cytotoxicity *in vitro* against various neoplastic cells and also was shown to inhibit tumor (19). However, the effects of crotamine on macrophage-related immune functions remain unclear. Therefore, to better understand the effects of crotamine, we investigated its immunostimulatory effects, in particular, its augmentation of macrophage function, including NO production, cytokine production, phagocytosis, and cytotoxicity.

The macrophage is an important cell type that is central to cell-mediated and humoral immunity due to its actions as an antigen-presenting, microbicidal, and tumoricidal cell (20). Macrophage phagocytosis represents the initial response to invading microorganisms and enhances the innate immune response. We found that crotamine induces phagocytic activity in RAW 264.7 cells. During phagocytosis, activated macrophages produced NO, which is related to the cytolytic function against various pathogens in macrophages (21).

In this study, we showed that crotamine induced NO and cytokine production in macrophages and that this contributes to the peptide's immunostimulatory activity in RAW264.7 cells.

Moreover, crotamine also enhanced the cytostatic activity of macrophages such as inhibiting the growth of S-180 cells. Cytotoxicity against tumor cells is dependent upon the activation of macrophages, and these mechanisms are closely correlated with the production of several cytokines, such as TNF-α and IL-1β. We found that TNF-α levels were increased through increasing level of protein and genes expression. Our results thus elucidate the possible roles of macrophages in the anti-tumor activities of crotamine.

NF-κB plays a key role in the transcription of macrophage-related cytokines (18, 22). We sought to determine whether crotamine induces TNF-α and iNOS expression in macrophages through the NF-κB signaling pathway. Using a luciferase assay, we found that crotamine does indeed increase the level of NF-κB activation in macrophages, implicating the NF-κB signaling pathway. In resting macrophages, the NF-κB is localized to the cytosol by interactions with IκB inhibitory proteins (22). After exposure to pro-inflammatory stimuli, such as NO, TNF-α and IκB is phosphorylated by IκB kinases α and β, ubiquitinated, and degraded. The liberated NF-κB dimers (p65/p50) translocate to the nucleus, where transcription of the target genes occurs (23). We showed that crotamine induced the translocation of the

NF- $\kappa$ B subunit p65 into the nucleus and the subsequent degradation of I $\kappa$ B- $\alpha$ . Therefore, crotamine-induced NF- $\kappa$ B activation results from increased I $\kappa$ B- $\alpha$  degradation. The specific I $\kappa$ B $\alpha$  kinase inhibitor, BAY 11-7082, inhibited the NO production and phagocytic activity induced by crotamine. BAY 11-7082 inhibits the activation of NF- $\kappa$ B by preventing the phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$ . The effects of crotamine were similar to those obtained with BAY 11-7082.

Activated p38 is a key player involved in signaling pathways for macrophage-related cytokine release, phagocytosis and inflammation (24-26). In our study, crotamine significantly increased the phosphorylation of p38, but not ERK and JNK. Our data also showed that specific p38 inhibitors effectively inhibited the NO production and the phagocytic activity induced by crotamine.

Overall, our study shows that crotamine augments RAW 264.7 cells to secrete cytokines and stimulates macrophage phagocytic activity and NO production. Crotamine increased both, the gene expression and protein levels of TNF- $\alpha$  and iNOS, as well as, increased the phosphorylation and degradation of I $\kappa$ B- $\alpha$ , which is required for NF- $\kappa$ B activation. Thus, our results suggest that crotamine induces macrophage activation and immunostimulatory activity via the NF- $\kappa$ B signaling pathway, and that crotamine increases the secretion of NO and TNF- $\alpha$  by macrophages, thereby modulating the host's immune response. Crotamine may be useful in the clinic as a peptide therapeutic to activate macrophages.

## MATERIALS AND METHODS

### Materials

Materials used are listed in Supplementary section S1.

### Preparation of crotamine

Crotamine was prepared as described previously (3) with minor modifications. Pure crotamine was obtained after applying onto HiTrap SP and HiLoad 26/600 Superdex 200 column with Tris buffer. Finally, the endotoxins were removed by 1% Triton X-114 (27).

### Cell cultures

The RAW 264.7 cell line was obtained from the ATCC (Bethesda, MD). The cell culture method can be found in Supplementary section S2.

### Proliferative activity of macrophages

RAW 264.7 cells used for the MTT-based assay were measured according to the manufacturer's instructions. Further details can be found in Supplementary section S3.

### Macrophage phagocytosis

The phagocytosis assay was performed using a commercially available IncuCyte phagocytosis assay kit (Essen Bioscience, Michigan) and were performed according to the manufacturer's

instructions. Cells were collected in cold PBS and analyzed using a Microplate-Fluorometer (Molecular Devices, Menlo Park, CA).

### Macrophage cytostaticity

RAW 264.7 cells ( $5 \times 10^5$  cells/well) on 48-well culture plates were incubated with crotamine for 48 h. After washing, cell cytostaticity against tumor cells was assayed by co-culture with S-180. The cultures were incubated for 18 h. Macrophage cytostaticity was measured according to the MTT method. Cytostaticity (%) =  $[1 - (\text{absorbance at 575 nm of experiment}/\text{absorbance at 575 nm of control})] \times 100$ .

### NO assay

NO was measured as its stable oxidative metabolite, nitrite (NO $_x$ ), as previously described (28).

### Measurement of TNF- $\alpha$ production

For cytokine immunoassays, cells were cultured for 6 and 24 h at a density of  $5 \times 10^5$  cells/well in 48-well plates. Supernatants were removed at the indicated times, and TNF- $\alpha$  production was quantified by sandwich immunoassays using a protocol supplied by R&D Systems.

### RNA preparation and mRNA analysis by real-time quantitative PCR

Total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY). Accumulated PCR products were directly detected by monitoring the increase in reporter dye (SYBR<sup>®</sup>). The expression levels of TNF- $\alpha$  and iNOS in the treated cells were compared to those in control cells at each time point using the comparative cycle threshold (C $_t$ ) method (17, 28). Primers used are listed in Supplementary Table 1. The quantity of each transcript was calculated as described in the instrument manual and was normalized to the amount of S18, a house-keeping gene.

### Transient transfection and luciferase activity assay

For transient transfections, cells were seeded at  $5 \times 10^5$  cells/well in a 48-well plate. The expression vector that contained the NF- $\kappa$ B luciferase reporter construct (pNF- $\kappa$ B-LUC, Stratagene, Grand Island, NY) or the empty vector were transfected with 0.5  $\mu$ L of serum- and antibiotic free LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). After 6 h, the medium was replaced with basal medium. Cells were treated with crotamine for 18 h and then lysed. Luciferase and  $\beta$ -galactosidase activities (normalized control) were measured in cellular extracts as described previously (29).

### Western blotting

Cells were harvested and washed three times with cold phosphate-buffered saline (PBS). Cytoplasmic and nuclear protein fractions were extracted using NE-PER extraction reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL).

## Statistical analysis

Statistical methods can be found in Supplementary section S4.

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