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Inhibition of Cardiac Hypertrophy by Triflusal (4-Trifluoromethyl Derivative of Salicylate) and Its Active Metabolite

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Running title: Triflusal inhibits cardiac hypertrophy

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ABBREVIATIONS: ANF, atrial natriuretic factor; APRT, adenosyl phosphoribosyl transferase; HTB, 2-hydroxy-4- trifluoromethylbenzoic acid.

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Abstract- Nuclear Factor (NF)-κB signaling pathway is an important intracellular mediator of cardiac hypertrophy. The aim of the present study was to determine whether triflusal (2-acetoxy-4-trifluoromethylbenzoic acid), a salicylate derivative used as antiplatelet agent, and its active metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB) inhibit cardiac hypertrophy *in vitro* and *in vivo* by blocking the NF-κB signaling pathway. In cultured neonatal rat cardiomyocytes HTB (300 μmol/L, a concentration reached in clinical use) inhibited phenylephrine(PE)-induced protein synthesis ([^{3}H]leucine uptake), induction of the fetal-type gene atrial natriuretic factor (ANF) and sarcomeric disorganization. Assessment of the effects of triflusal in pressure overload-induced cardiac hypertrophy by aortic banding resulted in a significant reduction in the ratio heart weight/body weight and in a reduction of the mRNA levels of the cardiac hypertrophy markers ANF and α-actinin compared with untreated banded rats. Electrophoretic mobility shift assay revealed an increase in the NF-κB binding activity in cardiac nuclear extracts of banded rats that was prevented by triflusal treatment. Interestingly, banded rats treated with oral triflusal showed enhanced protein levels of IκBα, which forms a cytoplasmic inactive complex with the p65-p50 heterodimeric complex, compared with untreated rats. Finally, HTB increased phospho-IκBα levels in neonatal cardiomyocytes and inhibited proteosome activity, suggesting that this drug prevented proteosome-mediated degradation of IκBα. These results indicate that triflusal, a drug with a well-characterized pharmacological and safety profile currently used as antiplatelet, inhibits cardiomyocyte growth by interfering NF-κB signaling pathway through a post-transcriptional mechanism involving reduced-proteosome degradation of IκBα.

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Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction, and cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia, and sudden death (Levy et al., 1990;Lorell and Carabello, 2000). Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role, since it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes (Purcell et al., 2001;Hirotani et al., 2002;Higuchi et al., 2002;Gupta et al., 2002). The transcription factor NF-κB can be activated by a wide array of exogenous and endogenous stimuli and plays a critical role in mediating immune and inflammatory responses. In resting cells, NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. After stimulation, a serine kinase cascade is activated leading to the phosphorylation of IκB. This event converts IκB in a substrate for ubiquitination and subsequent proteosome-mediated degradation, releasing the NF-κB heterodimer, which then translocates to the nucleus and regulates the expression of genes involved in inflammatory and immune processes.

Non-steroid anti-inflammatory drugs (NSAID), such as salicylates, are potent inhibitors of inflammatory processes, which act by blocking prostaglandin synthesis via inhibition of cyclooxygenase activity (Vane, 1971;Ferreira et al., 1971), and more recently their activity has been also associated to their ability to inhibit the activation of the transcription factor NF-κB (Kopp and Ghosh, 1994;Grilli et al., 1996;Oeth and Mackman, 1995;Pierce et al., 1996). Triflusal (2-acetoxy-4-trifluoromethylbenzoic acid) is a NSAID structurally related to the salicylate group of compounds, with

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a characterized pharmacological profile. Triflusal has an antiplatelet effect and has been largely used for the prevention and/or treatment of vascular thromboembolisms (McNeely and Goa, 1998). Once administered, triflusal is rapidly converted to its deacetylated metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB), which has a plasma half-life of 35 hours (McNeely and Goa, 1998). Recent studies have shown that triflusal and HTB block the inflammatory related transcription factor NF-κB more effectively than aspirin (Bayon et al., 1999;Hernandez et al., 2001;de Arriba et al., 1999;Acarin et al., 2000). Unlike aspirin, the effects of triflusal are found at concentrations reached in its therapeutic use as antiplatelet agent (McNeely and Goa, 1998;Bayon et al., 1999). The key role played by NF-κB activation in the development of cardiac hypertrophy (Gupta et al., 2002;Hirotani et al., 2002;Higuchi et al., 2002;Purcell et al., 2001) may suggest a potential role for triflusal in the inhibition of cardiac hypertrophy.

In this study, we examined the effects of HTB on phenylephrine(PE)-induced hypertrophy in neonatal rat cardiac myocytes and of triflusal in pressure overload-induced cardiac hypertrophy in rats. We found that these drugs inhibit cardiac hypertrophy by reducing the NF-κB signaling pathway through a post-transcriptional mechanism involving reduced-proteosome degradation of IκBα.

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Materials and Methods

Materials. Triflusal and HTB were from Uriach Laboratories. [γ-^{32}P]dATP (3000 Ci/mmol) and [^{3}H]leucine (50 Ci/mmol) were purchased from Amersham Pharmacia Biotech KK. Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories and Alexa flouro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were from Molecular Probes. MG-132 (carbobenzoxyl-L-leucil-L-leucil-L-leucinol) and ALLN (*N*-acetyl-leucyl-leucyl-norleucinal) were obtained from Calbiochem. All other chemicals were purchased from Sigma.

Cell Culture. Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously (Kimura et al., 1994). The media was changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 hours before treatments. In this study phenylephrine (PE) was used to stimulate neonatal rat cardiomyocytes. Animal handling and disposal were performed in accordance with law 5/1995, 21st July, of the Generalitat de Catalunya.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA and protein analysis H9c2 cells in DMEM medium were treated with 500 μmol/L HTB for 24 hours.

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Incorporation of [^{3}H]leucine. To examine the effect of PE on protein synthesis, the incorporation of [^{3}H]leucine was measured essentially by the method of Thaik et al. (Thaik et al., 1995). Cultured neonatal rat ventricular myocytes were treated with PE in the presence or in the absence of drugs and coincubated with [^{3}H]leucine (1 μCi/mL) for 24 hours. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4ºC for 30 minutes to precipitate the protein. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted with scintillation counter.

Immunocytochemistry. Neonatal rat ventricular myocytes were fixed in ice-cold 100% methanol for 10 min. Anti-α–actinin antibody and anti-ANF polyclonal antiserum were added at dilutions 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 hour at room temperature. Secondary antibodies, Alexa flour 488 goat anti-rabbit and Alexa flour 568 goat anti-mouse, were used at a dilution of 1:300 in PBS containing 5% rat serum and incubated for 30 min at room temperature. Immunofluorescence was visualized using a confocal laser fluorescence microscope Olympus Fluoview FV500. Photographic images were taken from five random fields.

RNA preparation and analysis. Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described (Cabrero et al., 2002). The sequences of the sense and antisense primers used for amplification were: ANF, 5'-TCCTCTTCCTGGCCTTTTGGC-3' and 5'-AGACGGGTTGCTTCCCCAGTC-3'; α-actinin, 5'-GGCTGTGTTCCCATCCATCGT-3' and 5'-CCCGGTTAGCTTTGGGGTTCA-3'; IκBα, 5'-TGAAGGGAGACCTGGCCTTCC-3' and 5'-GTGGCCGTTGTAGTTGGTGGTGGC-3' and APRT

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(adenosyl phosphoribosyl transferase), 5'-GCCTCTTGGCCAGTCACCTGA-3' and 5'-CCAGGCTCACACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, α-actinin: 266 bp, IκBα: 263 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*), that did not result altered by the hypertrophic process.

Pressure overload-induced cardiac hypertrophy. Twenty-one male Sprague-Dawley rats (225 to 250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1ºC). They were fed standard diet (Panlab, Barcelona, Spain) for five days before the studies began. The animals were randomly distributed into three groups as follows: (1) sham-operated rats, (2) pressure overloaded rats and (3) pressure overloaded rats with triflusal. Five days before the surgical procedure, rats were fed either a control diet or a diet containing 0.05% (wt/wt) triflusal (which resulted in approximately 15 mg/kg/day). The diets were prepared as previously described (Cabrero et al., 2001). Throughout the study, the weight and daily food intake were measured. Triflusal treatment did not affect body weight. Pressure overload was induced by constriction of the abdominal aorta at the suprarenal level with 7-0 nylon strings by ligation with a blunted 25-gauge needle, which was then pulled out. For the age-matched sham operation, the identical procedure was performed except that the suture was not tied around the aorta. Hearts of the 18 rats (6 rats per group) that completed the study were harvested 15 days after the surgical operation. The pressure gradients achieved by the aortic banding process were not measured. The heart weight/body weight (HW/BW) ratio was calculated and the heart samples were frozen in liquid nitrogen and then stored at -80ºC. Animal handling and disposal were performed in accordance with the law 5/1995, 21st July, from the Generalitat de Catalunya.

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Immunoblotting. Cell lysates and nuclear extracts from hearts were obtained as previously described (Cabrero et al., 2002). Proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against IκBα, IκBβ and p65 (Santa Cruz Biotechnology, Inc), phospho-IκBα serine 32 (Cell Signalling) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies). Further, IκBα was identified by using a blocking peptide (Santa Cruz Biotechnologies) against this protein. Western blot data were normalized relative to β-tubulin protein levels for quantitative analysis

Electrophoretic mobility shift assay (EMSA). Isolation of nuclear extracts was performed as previously described. EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the nuclear factor-κB (NF-κB) nucleotide (5'AGTTGAGGGGACTTTCCCAGGC-3') and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3'). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5x kinase buffer, 1 μl of T4 polynucleotide kinase (10 U./μl), and 2.5 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) incubated at 37ºC for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) according to the manufacturer's instructions. Ten micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer (10 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA and 50 μg/ml poly(dI-dC)), in a final volume of 15 μl. Labeled probe (approximately 60.000 cpm) was added and the reaction was incubated for 15 min. at

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4ºC. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4ºC. Protein-DNA complexes were resolved by electrophoresis at 4ºC on a 5% acrylamide gel and subjected to autoradiography.

Analysis of caspase-3 activity. For the determination of the caspase-3 activity we used the colorimetric substrate Ac-DEVD-*p*-nitroaniline (Oncogen) as described previously (Jorda et al., 2003). After treatment with 300μM HTB for 24 hours or 0.5mM H_2O_2 for 4 hours followed by 2h incubation in H_2O_2-free medium rat neonatal cardiomyocytes were collected in lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS and 0.1 mM EDTA, pH 7.4). Protein (50 μg/ml) was incubated with 200 M Ac-DEVD-*p*-nitroaniline in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol and 0.1 mM EDTA, pH 7.4) on 96-well plates at 37ºC for 24 h. Absorbance of the cleaved product was measured at 405 nm in microplate reader (Bio-Rad). Results are expressed as absolute absorbance measured (mean ± SD of 4 experiments).

Coimmunoprecipitation. Cell nuclear extracts were brought to a final volume of 0.5 mL with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, BSA 2% and 1 mg/ml nonfat milk for 6 h at 4ºC and incubated with 4 μg of anti-p65. Immunocomplex was captured by incubating the samples with protein A-agarose suspension overnight at 4ºC on a rocking platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of SDS-PAGE sample buffer and boiled for 5 min. at 100ºC. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody

against IκBα. The IκBα band was identified by using a blocking peptide (Santa Cruz Biotechnologies) against this protein.

Proteosome activity assay. Proteosome activity was quantified by stably transfecting HEK-293 cells with the proteosome sensor vector (BD Biosciences, San Jose, CA). This vector, designed for studies of proteosome function in mammalian cells, codes for a fluorescent protein (ZsGreen) fused to the mouse ornithine decarboxylase degradation domain (amino acids 422-461). This protein undergoes rapid degradation and it does not accumulate in cells until proteosome is inhibited, which is indicated by an increase in fluorescence. Cells were incubated during 3 hours (96-well plates, 5000 cells/well) in the absence or in the presence of HTB (300 μM) or the proteosome inhibitor ALLN (10 μM) and the change in fluorescence was measured using a fluorometer (excitation: 485 nm; emission 535 nm).

Statistical Analyses. Results were obtained from at least four independent experiments and presented as mean±S.D. Significant differences were established by two-sided Student's *t*-test or one-way ANOVA, according to the number of groups compared, using the computer program GraphPad Instat V2.03 (GraphPad Software Inc., San Diego, CA). Differences were considered significant at $P<0.05$.

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Results

HTB, but not Aspirin, Inhibits PE-induced Cardiac Hypertrophy in Neonatal Rat Cardiomyocytes

Cardiac hypertrophy is characterized by increase protein content (e.g. [^{3}H]leucine uptake), induction of fetal-type genes (e.g. ANF) and sarcomeric disorganization. Therefore, we first examined the effects of HTB on these parameters in a primary culture of neonatal rat cadiomyocytes, which have been largely used for this purpose. Cells were pretreated with either vehicle or drugs for 30 minutes and subsequently stimulated with 100 μmol/L PE for 24 hours. As shown in Figure 1A, [^{3}H]leucine incorporation was significantly increased by PE (1.5-fold, $P<0.001$) and this was inhibited by HTB at 300 μmol/L (-74%, $P<0.01$ compared with PE-induced cells), whereas this drug had no effect at 100 μmol/L. In contrast, 300 μmol/L aspirin (Acetyl Salicylic Acid, ASA) did not affect [^{3}H]leucine incorporation. PE-induced cardiac hypertrophy also led to 2-fold induction ($P<0.05$ vs control) in the mRNA levels of the sarcomere-associated protein α-actinin (Figure 1B). In contrast, in the presence of 300 μmol/L HTB PE-induced α-actinin expression was abolished ($P<0.05$ vs PE-stimulated cardiomyocytes). Immunostaining of cardiac myocytes for α-actinin and the fetal cardiac protein ANF clearly shown an increase in cardiac myocyte size and ANF protein expression following PE stimulation (Figure 1C). These changes were blocked in the presence of HTB in the culture medium.

Triflusal Treatment Inhibits Pressure Overload-Induced Cardiac Hypertrophy

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In order to evaluate whether our *in vitro* findings had physiological relevance we evaluated the effects of triflusal using the pressure overload model of cardiac hypertrophy. HW/BW ratio significantly increased (1.35-fold, $P<0.001$) after aortic constriction compared with sham-operated rats (Figure 2A). Treatment with triflusal attenuated the increase in the HW/BW ratio (1.12-fold, $P<0.01$ vs banded rats). Further, pressure overload enhanced mRNA levels of the cardiac hypertrophy markers ANF and α-actinin, compared with sham-operated rats (Figure 2B and C), and these changes were abolished by triflusal treatment. Collagen deposition was also evaluated by measuring the mRNA expression of type I and type III collagen. The expression of these genes was increased in banded rats and this induction was prevented in the presence of triflusal (data not shown).

Triflusal Treatment Inhibits NF-κB Activation in Pressure Overload-Induced Cardiac Hypertrophy

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes (Purcell et al., 2001;Gupta et al., 2002;Hirotani et al., 2002;Higuchi et al., 2002) and it has been reported that triflusal inhibits the activation of this redox transcription factor (Bayon et al., 1999;de Arriba et al., 1999;Hernandez et al., 2001;Acarin et al., 2000), we performed EMSA studies to investigate whether triflusal inhibited NF-κB activation in pressure overload-induced cardiac hypertrophy. These studies shown that the NF-κB probe formed two major complexes with cardiac nuclear proteins (complexes III and V, Figure 3A). NF-κB binding activity increased in banded rats, especially of complex III and V, compared with sham-operated rats (Figure 3B) and this effect was abolished by triflusal treatment. Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of this transcription factor. Addition of this antibody to incubation mixtures resulted in a supershifted, thus showing that specific NF-κB complexes contained p65 (Figure 3C). No

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changes were observed in the DNA binding of cardiac nuclear proteins from the different groups of rats to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (Figure 3D). Overall, these data demonstrate that triflusal inhibits NF-κB activation in pressure overload-induced cardiac hypertrophy and that this mechanism may contribute to the antihypertrophic effect of this compound.

Triflusal treatment enhances IκBα levels in heart and cardiomyocytes in culture

Finally, we sought to determine the molecular mechanism by which triflusal inhibits NF-κB activation. Inhibition of NF-κB signaling may occur through different mechanisms. One of these mechanisms may involve enhanced expression of IκBα, which forms a cytoplasmic inactive complex with the p65-p50 heterodimeric complex. When we determined the protein levels of the p65 subunit of NF-κB, IκBβ and IκBα, we observed that triflusal did no affect the expression of these proteins, except of IκBα. Triflusal significantly increased the protein levels of this inhibitor of NF-κB, suggesting that this was the mechanism responsible for the inhibition of this transcription factor (Figure 4). Given that it has been reported that enhanced physical interaction between p65 and IκBα may lead to the removal of NF-κB proteins from the nucleus (Tam et al., 2000), we performed coimmunoprecipitation studies to evaluate this possibility. Nuclear extracts isolated from hearts were immunoprecipitated using anti-p65 antibody coupled to protein A-agarose beads. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-IκBα antibody. Data shown in Figure 5 demonstrate that triflusal enhanced the physical interaction of p65 with IκBα, suggesting that increased association between these proteins is a mechanism contributing to the reported reduction in NF-κB activity.

In order to assess whether HTB treatment resulted in increased expression of IκBα *in vitro*, we used the embryonic rat heart-derived H9c2 cells. Treatment of H9c2 cells with 500 μmol/L HTB for 24 hours

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resulted in a modest increase in the levels of IκBα mRNA (similar results were obtained when cells were treated with 300 μmol/L HTB for 24 hours, data not shown) (Figure 6A). In contrast, HTB treatment caused a huge increase in total IκBα protein levels (6.2-fold induction, $P<0.001$), suggesting that changes caused by triflusal on IκBα expression occur at the post-translational level. Since activation of NF-κB requires IκBα degradation, we next assessed whether HTB affected the protein levels of phospho-IκBα. Phosphorylation of IκBα triggers its polyubiquitinylation and proteosome-dependent degradation, thereby leading to NF-κB activation (Karin and Ben-Neriah, 2000), and proteosome inhibition leads to enhance phospho-IκBα protein levels (Ganesh et al., 2003). In cells exposed to HTB a large increase in phosphorylation of IκBα on serine 32 was detected (4.4-fold increase, $P<0.001$), suggesting that HTB inhibited proteosome-mediated dependent degradation of this protein (Figure 6B). We then examined whether similar changes occurred in neonatal rat cadiomyocytes. In cells exposed to PE the protein levels of IκBα were reduced (20%, $P<0.05$) (Figure 7A), but this reduction was not observed when cells were co-incubated with HTB. It is worth noting that 300μM HTB treatment did not enhance apoptosis in rat neonatal cardiomyocytes, since no changes were observed neither in the percentage of death cells (sub-G_1 fraction analysis by flow cytometry of propidium iodide staining) (data not shown) nor in the caspase-3 activity (Control: 0.379±0.009; HTB: 0.402 ±0.016; H_2O_2: 0.551±0.021***. Results are means of absorbances. ***$P<0.001$ vs. control cells) compared to control cells, whereas a 45% increase was observed in cardiomyocytes exposed to hydrogen peroxide. Further, we tested the effect of HTB and the proteosome inhibitor MG-132, which has been shown to block the degradation of IκBα (Grisham et al., 1999), on phospho-IκBα protein expression. Neonatal cardiomyocytes exposed to HTB and MG 132 (10 μM) showed enhanced phospho-IκBα protein levels (3.5 and 4-fold induction, respectively, $P<0.001$) compared to control cells (Figure 7B). To obtain direct evidence for inhibition of proteosome

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by HTB, we tested whether this drug inhibited *in vitro* the activity of the proteosome. Figure 7C shows that HTB inhibited proteosome activity to similar levels to those achieved by the proteosome inhibitor ALLN (10 μM).

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DISCUSSION

Whereas a plethora of signaling cascades have been implicated in the development of cardiac hypertrophy (Hunter and Chien, 1999), relatively little is known about the intrinsic mechanisms with the potential to inhibit or even reverse hypertrophy. In the present study we demonstrate that triflusal, which is currently used as an antiplatelet agent, and its main metabolite, HTB, inhibit PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes and in the pressure overload animal model of cardiac hypertrophy. Further, our findings indicate that the antihypertrophic effect of these salicylate-derived compounds reduces the NF-κB signaling pathway through a post-transcriptional mechanism involving reduced-proteosome degradation of IκBα.

Recent studies by several groups have implicated the activation of NF-κB as a causal event in the cardiac hypertrophy response (Gupta et al., 2002;Hirotani et al., 2002;Purcell et al., 2001;Higuchi et al., 2002). Further, increased NF-κB activation was also observed in the myocardium of patients with congestive heart failure (Wong et al., 1998;Grabellus et al., 2002). This association between NF-κB and myocyte hypertrophy is interesting taking into account the hypothesis that inflammatory cytokines are involved in cardiomiopathic disease states and that NF-κB itself is regulated by several of these cytokines. Here we demonstrate that triflusal and its main metabolite inhibit NF-κB activation in cardiac cells and that this mechanism may be responsible for their antihypertrophic effect. Our findings are consistent with several studies reporting that prevention of NF-κB activation leads to inhibition of cardiac hypertrophy. Thus, both peroxisome proliferator-activated receptor γ (PPARγ) activators (Yamamoto et al., 2001) and antioxidants (Hirotani et al., 2002;Nakamura et al., 1998) can abolish the hypertrophic response of cardiomyocytes through inhibiting NF-κB activation. All these studies support the contention that NF-κB inhibition represents a potential therapeutic approach for preventing

or reversing cardiac hypertrophy. Therefore, the discovery that triflusal, a drug with a well-characterized pharmacological and safety profile currently used in therapy, inhibits cardiac hypertrophy may lead to the potential use of this agent in the treatment of this pathology. Triflusal is currently used in patients with vascular occlusive diseases and it is associated with an incidence of gastrointestinal bleeding lower than aspirin (Matias-Guiu et al., 2003;Lanas et al., 2003). The concentrations found to inhibit cardiac hypertrophy in this work (300 μmol/L) are easily reached after the approved dosage of triflusal (600-900 mg/day), where HTB plasma levels of near 1 mM are found (McNeely and Goa, 1998). The prevention on cardiac hypertrophy by triflusal treatment seems to be direct and not related to changes in blood pressure since triflusal inhibits cardiac hypertrophy *in vitro* and it has been reported that this drug affects neither blood pressure nor heart rate (Ferrari et al., 1996).

Inhibition of the activity of NF-κB can be performed at different stages. For instance, antioxidants can inhibit generation of reactive oxygen species, which are a stimulus for NF-κB activation. Interestingly, NF-κB is regulated by subcellular localization. It is retained in the cytosol by being bound to inhibitors of κB, IκB. In the present study we demonstrate that triflusal and HTB increase the expression of IκBα. Therefore, increased expression of this inhibitor of κB results in persistent binding to NF-κB, blocking its translocation to the nucleus and thus its activity. In addition, although the primary function of IκBα is to retain the NF-κB proteins in the cytoplasm, it has been reported that this inhibitor is also involved in the removal of NF-κB proteins from the nucleus (Tam et al., 2000). Thus, IκB has both cytoplasmic and nuclear roles in regulating the NF-κB pathway. Regarding the mechanism responsible for the enhanced expression of IκBα in cardiac cells our findings indicate that HTB increases phospho-IκBα protein levels, suggesting that this dug inhibits proteosome-dependent degradation of IκBα. It remains to study whether the effect of triflusal and its main metabolite on proteasome-mediated degradation is

general or specific for IκBα. However, a general effect of triflusal on proteasome-mediated degradation seems unlikely since this drug did not affect the protein levels of IκBβ, whose expression is increased when proteasome is inhibited (Stasiolek et al., 2000). Further studies are necessary to investigate whether triflusal may increase IκBα expression through additional mechanisms.

Previous studies have demonstrated that triflusal and HTB inhibit NF-κB activation in endothelial cells, in brain cells and *in vivo* (Acarin et al., 2000;Bayon et al., 1999;de Arriba et al., 1999;Hernandez et al., 2001) more effectively than aspirin. In our studies we tested the effect of aspirin at the same concentration that HTB. In contrast to the metabolite of triflusal, aspirin treatment did not affect [^{3}H]leucine incorporation. Thus, the introduction of the trifluoromethyl group in the 4-position of salicylates confers new properties to the molecule of triflusal.

In summary, in the present study we show that triflusal and HTB inhibit cardiac hypertrophy *in vitro* and *in vivo* through a mechanism that may involve inhibition of the NF-κB signaling pathway, an important intracellular mediator of this process. Therefore, these findings suggest that triflusal may become a therapeutic option to reduce cardiac hypertrophy.

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Legends for figures

Figure 1. The main metabolite of triflusal, HTB, inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μmol/L PE in the presence or absence of HTB (100 or 300 μmol/L) or aspirin (acetylsalicylic acid, ASA) (300 μmol/L) that were added 30 minutes before experiments. A, [^{3}H]leucine incorporation was determined by coincubating cardiac myocytes with 1.0 μCi/mL [^{3}H]leucine for 24 hours. Results are expressed as percentage compared with the control, which was arbitrarily set at 100% (mean ± SD, n=6). B, Analysis of the mRNA levels of α-actinin in PE-stimulated cardiomyocytes in the presence or absence of 300 μmol/L HTB. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 6 different experiments. C, Effects of PE with and without 300 μmol/L HTB on cardiac myocyte ANF protein expression and sarcomeric organization. Double immunofluorescent microscopy was performed using specific antibodies to α-actinin (upper panel, red color) and ANF (lower panel, green color). Experiments were performed three times with similar results.* $P<0.05$ and *** $P<0.001$ vs control. # $P<0.05$ and ## $P<0.01$ vs PE-stimulated cardiac myocytes. All blot data are representative of at least four separate experiments.

Figure 2. Triflusal inhibits pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. Treatment with triflusal was initiated 5 days before operation and continued for 15 days as food admixture at a concentration of 0.05% (wt/wt). At 15 days after operation, hearts were excised and weighed. A, Analysis of the HW/BW ratio in sham-operated rats, in banded rats (aortic banding, AB) and in banded rats treated with triflusal (AB+TRIF). Analysis of the mRNA levels of ANF (B) and α-actinin (C) in pressure overload-induced cardiac hypertrophy.

Data are expressed as mean ± S.D. of 6 different experiments. *** P<0.001 vs sham-operated rats. ## P<0.01 vs banded rats.

Figure 3. Treatment with triflusal prevents NF-κB activation in pressure overload-induced cardiac hypertrophy. A, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear protein extract (NE) shows four specific complexes (I to V), based on competition with a molar excess of unlabeled probe. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and cardiac NE from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with triflusal (AB+TRIF). C, Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. Supershifted immune complex (IC) is denoted. D, Autoradiograph of EMSA performed with a ^{32}P-labeled Oct-1 nucleotide. All blot data are representative of at least three separate experiments.

Figure 4. Triflusal treatment increases the protein levels of IκBα. Cardiac protein extracts from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with triflusal (AB+TRIF) were assayed for western-blot analysis with p65 (A), IκBβ (B), IκBα (C) and β-tubulin (D) antibodies. All blot data are representative of at least three separate experiments.

Figure 5. Triflusal enhances protein-protein interaction between the p65 subunit of NF-κB and IκBα. Nuclear cardiac protein extracts (equalized by protein concentrations) from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with triflusal (AB+TRIF) were subjected to immunoprecipitation using anti-p65 antibody coupled to protein A-agarose beads. Immunoprecipitates

were subjected to SDS-PAGE, and immunoblotted with anti-IκBα antibody. Arrowheads represent the IκBα or IgG signal.

Figure 6. The active metabolite of triflusal, HTB, induces the expression of IκBα in the embryonic rat heart-derived H9c2 cells. Analysis of the mRNA levels (A) and protein levels (B) of IκBα and phospho-IκBα in H9c2 myocytes in the presence or absence of 500 μmol/L HTB for 24 hours. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 6 different experiments.

Figure 7. The main metabolite of triflusal, HTB, enhances phospho-IκBα protein levels in neonatal cardiomyocytes. A, Analysis of the IκBα protein levels in neonatal cardiomyocytes. Cells were pre-treated with HTB (300 μmol/L) for 24 hours and then stimulated with 100 μmol/L PE for 30 min. Blot data are representative of three separate experiments Data are expressed as mean ± S.D. of 3 different experiments. B, Analysis of total and phospho-IκBα protein levels in neonatal cardiomyocytes treated with HTB (300 μmol/L) or MG 132 (10 μmol/L) for 24 hours. Blot data are representative of three separate experiments. Data are expressed as mean ± S.D. of 3 different experiments. Phospho-IκBα protein levels were normalized to control values. C, Effects of HTB and ALLN on proteosome activity. Each point represents the mean ± S.D. of more than 8 determinations. Proteosome activity is expressed in relative fluorescence units (RFU). ***P<0.001, ** P<0.01 and * P<0.05 vs. control (CT). $^{\#}$ P<0.05 vs. PE-stimulated cells.

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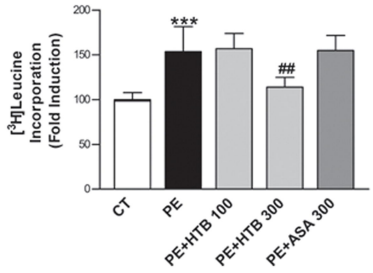

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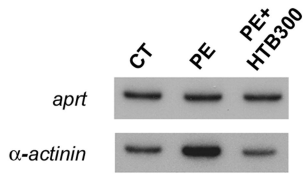

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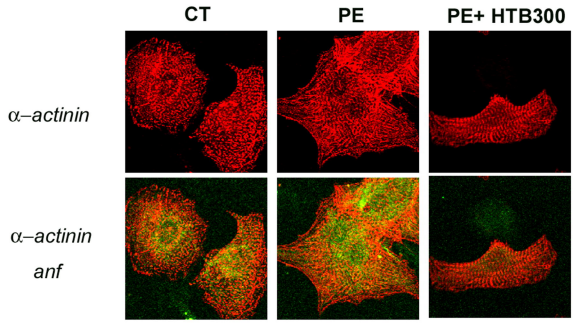

Figure 1

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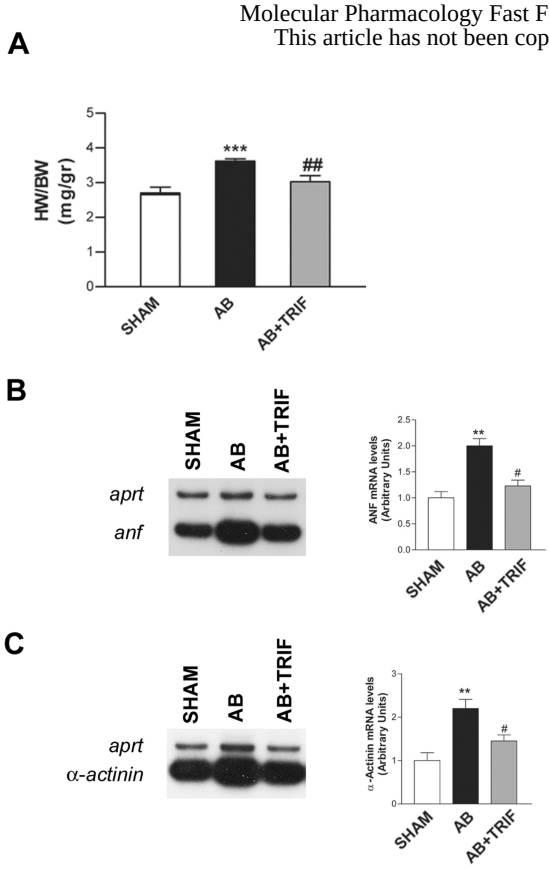

Figure 2

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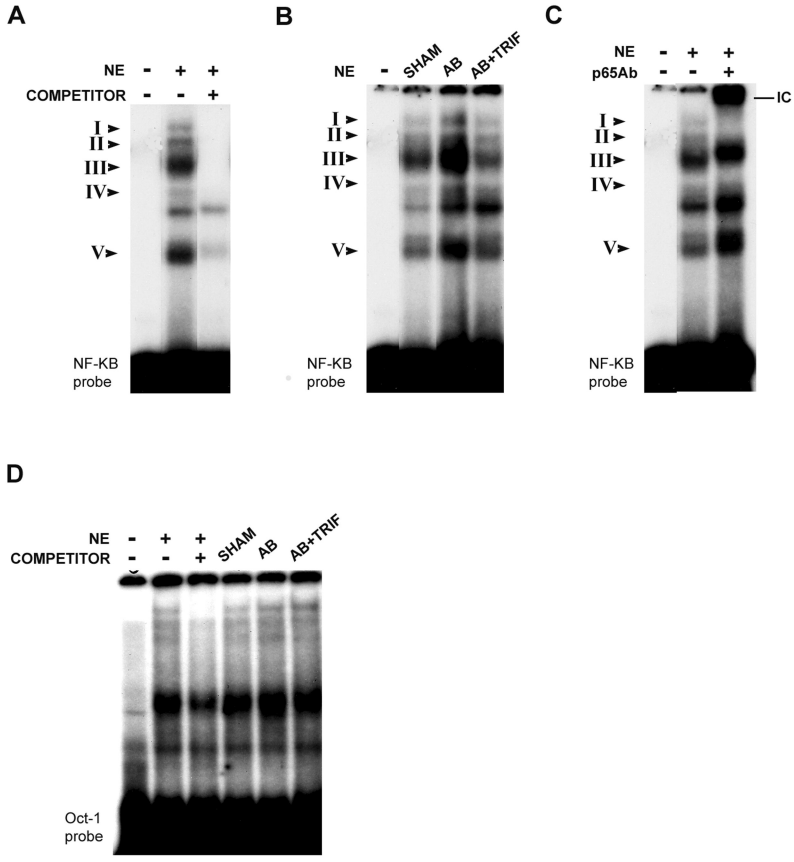

Figure 3

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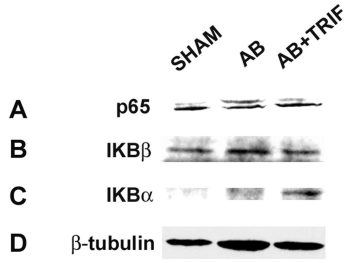

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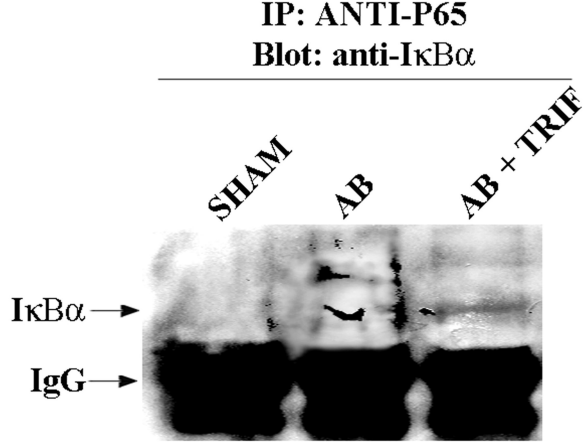

Figure 5

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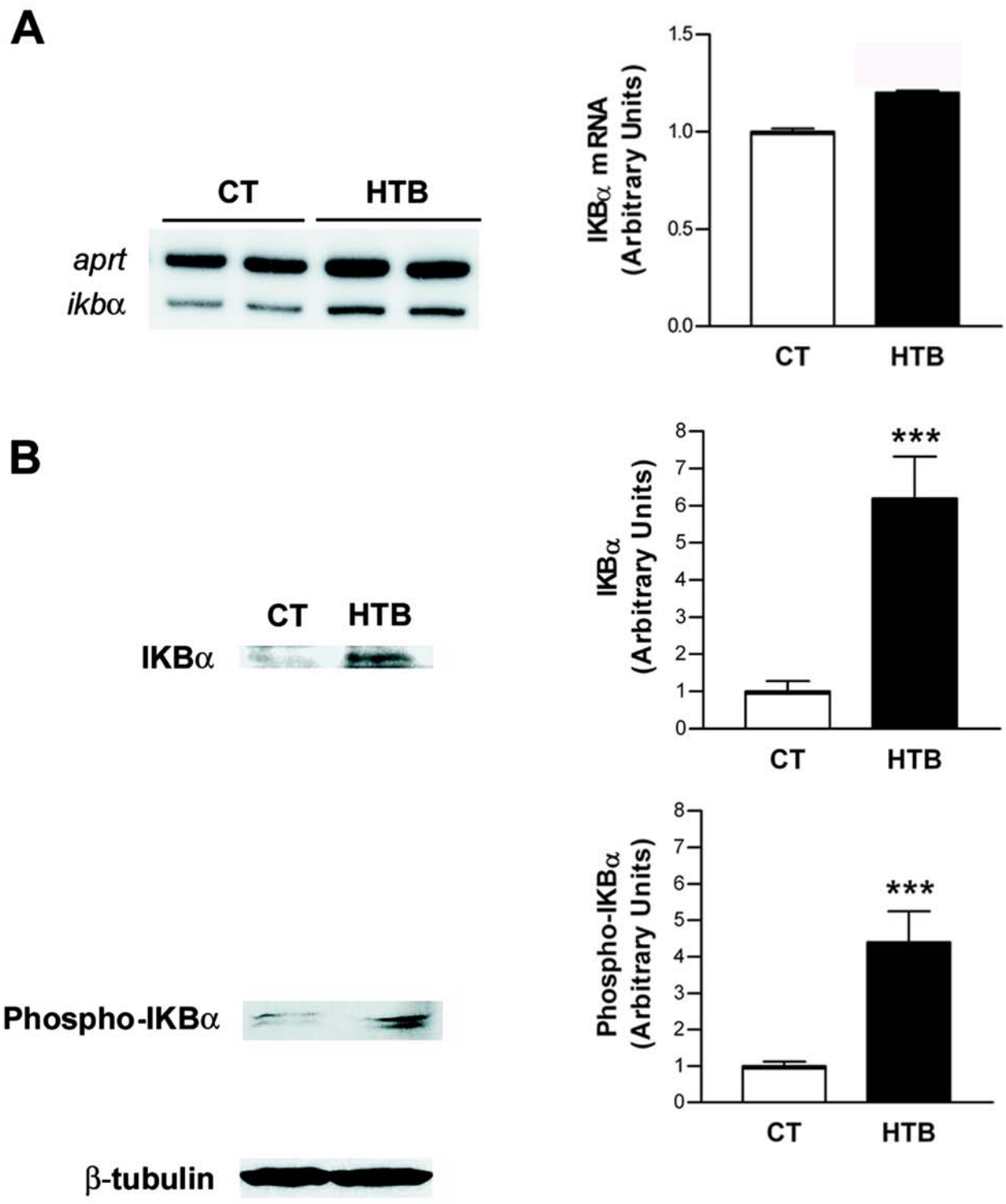

Figure 6

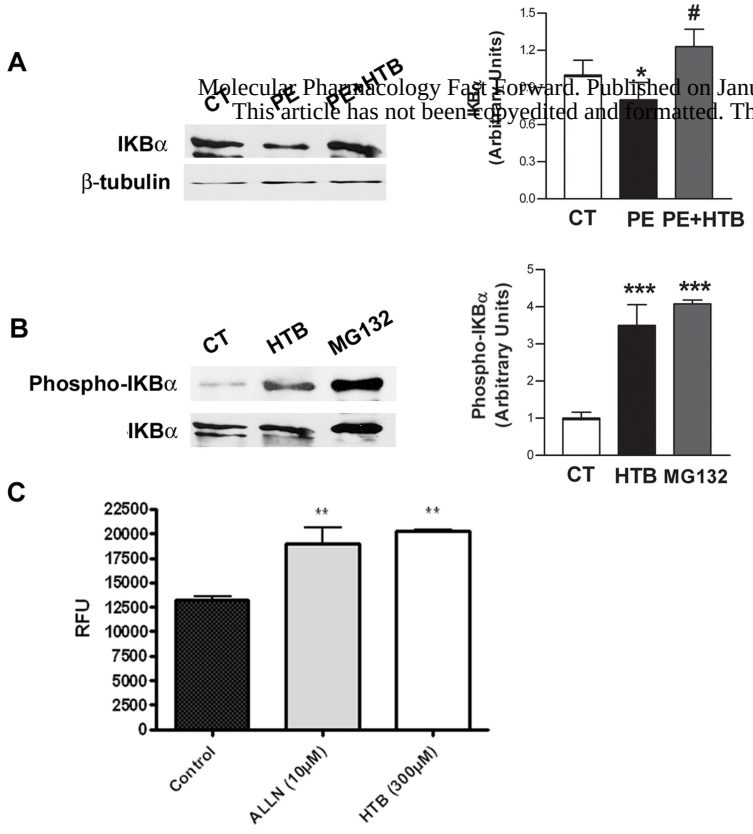

Figure 7