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

Anna Planavila, Ricardo Rodríguez-Calvo, Alberto Fernández de Arriba, Rosa M. Sánchez, Juan C. Laguna, Manuel Merlos, and Manuel Vázquez-Carrera

Pharmacology Unit, Department of Pharmacology and Therapeutic Chemistry, Faculty of Pharmacy, University of Barcelona, Spain (A.P., R.R.-C., R.M.S., J.C.L., M.M., M.V.-C.); and the Department of Pharmacology, Uriach Research Center, Barcelona, Spain (A.F.d.A., M.M.)

ABSTRACT

The 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 μM, 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 of heart weight to 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. It is noteworthy that banded rats treated with oral triflusal, compared with untreated rats, showed enhanced protein levels of IκBα, which forms a cytoplasmic inactive complex with the p65-p50 heterodimeric complex. 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 with the NF-κB signaling pathway through a post-transcriptional mechanism involving reduced-proteosome degradation of IκBα.

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, because it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes (Purcell et al., 2001; Gupta et al., 2002; Higuchi et al., 2002; Hirotani 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,

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ABBREVIATIONS: NF-κB, nuclear factor-κB; HTB, 2-hydroxy-4-trifluoromethylbenzoic acid; PE, phenylephrine; ANF, atrial natriuretic factor; MG-132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; APRT, adenosyl phosphoribosyl transferase; bp, base pair(s); HW/BW, heart weight/body weight; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate.

which then translocates to the nucleus and regulates the expression of genes involved in inflammatory and immune processes.

Nonsteroidal anti-inflammatory drugs, such as salicylates, are potent inhibitors of inflammatory processes; they act by blocking prostaglandin synthesis via inhibition of cyclooxygenase activity (Ferreira et al., 1971; Vane, 1971), and their activity has been also associated with their ability to inhibit the activation of the transcription factor NF-κB (Kopp and Ghosh, 1994; Oeth and Mackman, 1995; Grilli et al., 1996; Pierce et al., 1996). Triflusal (2-acetoxy-4-trifluoromethylbenzoic acid) is a nonsteroidal anti-inflammatory drug structurally related to the salicylate group of compounds, with 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 h (McNeely and Goa, 1998). Recent studies have shown that triflusal and HTB block the inflammation-related transcription factor NF-κB more effectively than aspirin (Bayon et al., 1999; de Arriba et al., 1999; Acarin et al., 2000; Hernandez et al., 2001). 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 (Purcell et al., 2001; Gupta et al., 2002; Higuchi et al., 2002; Hirotani et al., 2002) 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.

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 GE Healthcare (Little Chalfont, Buckinghamshire, UK). Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories (Belmont, CA) and Alexa fluoro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were from Invitrogen (Carlsbad, CA) MG-132 and ALLN were obtained from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

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 mM) 24 h before treatments. In this study, PE was used to stimulate neonatal rat cardiomyocytes. Animal handling and disposal were performed in accordance with law 5/1995 21 July, of the Generalitat de Catalunya.

The embryonic rat heart-derived H9c2 cells (American Type Culture Collection, Manassas, VA) 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 were treated with 500 μM HTB for 24 h.

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. (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 h. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4°C for 30 min to precipitate the protein. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted by liquid scintillation counting.

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 of 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 h at room temperature. Secondary antibodies, Alexa fluor 488 goat anti-rabbit and Alexa fluor 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 (Fluoview FV500; Olympus, Tokyo, Japan). Photographic images were taken from five random fields.

RNA Preparation and Analysis. Total RNA was isolated by using the Ultraspec reagent (Biotecx Laboratories Inc., Houston, TX). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by reverse transcription-polymerase chain reaction as described previously (Cabrero et al., 2002). The sequences of the sense and antisense primers used for amplification were: ANF, 5′-TCCTCTTCCTGGCCTTTTGGC-3′ and 5′-AGACGGGTTGCTTC-CCCAGTC-3′; α-actinin, 5′-GGCTGTGTTCCCATCCATCGT-3′ and 5′-CCCGGTTAGCTTTGGGGTTCA-3′; IκBα, 5′-TGAAGGGAGAC-CTGGCCTTCC-3′ and 5′-GTGGCCGTTGTAGTTGGTGGTGGC-3′; and adenosyl phosphoribosyl transferase (APRT), 5′-GCCTCTTG-GCCAGTCACCTGA-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 (225–250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1°C). They were fed standard diet (Panlab, Barcelona, Spain) for 5 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% (w/w) triflusal (which resulted in approximately 15 mg/kg/day). The diets were prepared as described previously (Cabrero et al., 2001). Throughout the study, 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 (six 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 −80°C. Animal handling and disposal were performed in

accordance with the law 5/1995 21 July, from the Generalitat de Catalunya.

Immunoblotting. Cell lysates and nuclear extracts from hearts were obtained as described previously (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 Biotechnologies, Inc., Santa Cruz, CA), phospho-IκBα serine 32 (Cell Signaling), and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries Beit Haemek Ltd., Beit Haemek Kibbutz, Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies). Furthermore, 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. Isolation of nuclear extracts was performed as described previously. EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the NF-κB nucleotide (5′AGTT-GAGGGGACTTTCCCAGGC-3′) and Oct-1 (5′-TGTCGAATG-CAAATCACTAGAA-3′). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5× 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 37°C for 1 h. The reaction was stopped by adding 90 μl of Tris/EDTA 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 dithiothreitol, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml bovine serum albumin, 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 4°C. 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 4°C. Protein-DNA complexes were resolved by electrophoresis at 4°C 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 *N*-acetyl-DEVD-*p*-nitroaniline (Calbiochem) as described previously (Jorda et al., 2003). After treatment with 300 μM HTB for 24 h or 0.5 mM H$_2$O$_2$ for 4 h followed by 2-h incubation in H$_2$O$_2$-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 *N*-acetyl-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 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm in microplate reader (Bio-Rad, Hercules, CA). Results are expressed as absolute absorbance measured (mean ± S.D. of four 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, 2% BSA, and 1 mg/ml nonfat milk for 6 h at 4°C and incubated with 4 μg of anti-p65. Immunocomplex was captured by incubating the samples with protein A-agarose suspension overnight at 4°C 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 100°C. 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 human embryonic kidney 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 for 3 h (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 analysis of variance, according to the number of groups compared, using the computer program Instat (ver. 2.03; GraphPad Software Inc., San Diego, CA). Differences were considered significant at $P < 0.05$.

Results

HTB, but Not Aspirin, Inhibits PE-Induced Cardiac Hypertrophy in Neonatal Rat Cardiomyocytes. Cardiac hypertrophy is characterized by increased 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 cardiomyocytes, which have been often used for this purpose. Cells were pretreated with either vehicle or drugs for 30 min and subsequently stimulated with 100 μM PE for 24 h. As shown in Fig. 1A, [^{3}H]leucine incorporation was significantly increased by PE (1.5-fold, $P < 0.001$) and this was inhibited by HTB at 300 μM (-74%, $P < 0.01$ compared with PE-induced cells), whereas this drug had no effect at 100 μM. In contrast, 300 μM aspirin (acetylsalicylic acid) did not affect [^{3}H]leucine incorporation. PE-induced cardiac hypertrophy also led to 2-fold induction ($P < 0.05$ versus control) in the mRNA levels of the sarcomere-associated protein α-actinin (Fig. 1B). In contrast, in the presence of 300 μM HTB, PE-induced α-actinin expression was abolished ($P < 0.05$ versus PE-stimulated cardiomyocytes). Immunostaining of cardiac myocytes for α-actinin and the fetal cardiac protein ANF clearly shows an increase in cardiac myocyte size and ANF protein expression after PE stimulation (Fig. 1C). These changes were blocked in the presence of HTB in the culture medium.

Triflusal Treatment Inhibits Pressure Overload-Induced Cardiac Hypertrophy. 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 (Fig. 2A). Treatment with triflusal attenuated the increase in the HW/BW ratio (1.12-fold, $P < 0.01$ versus banded rats). Furthermore, pressure overload enhanced mRNA levels of the cardiac hypertrophy markers ANF and α-actinin, compared with sham-operated rats (Fig. 2, B 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. Because activation of NF-κB is required for hypertrophic
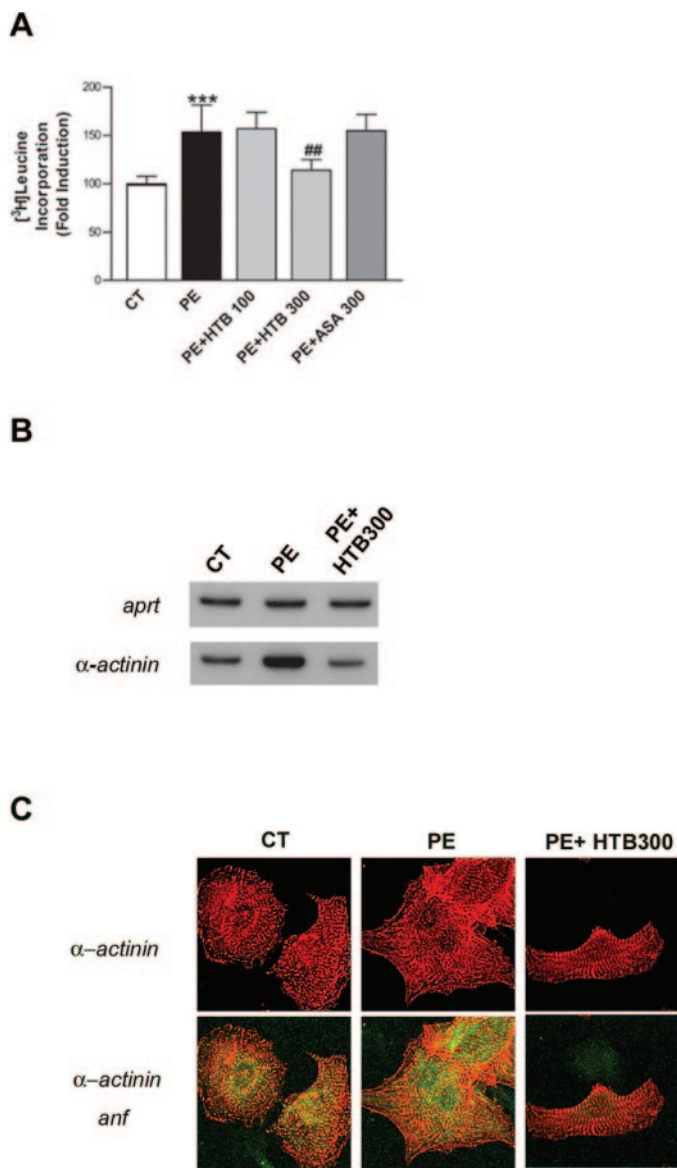

Fig. 1. The main metabolite of triflusal, HTB, inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μM PE in the presence or absence of HTB (100 or 300 μM) or aspirin (acetylsalicylic acid, ASA) (300 μM) that were added 30 min before experiments.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

growth of cardiomyocytes (Purcell et al., 2001; Gupta et al., 2002; Higuchi et al., 2002; Hirotani 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; Acarin et al., 2000; Hernandez et al., 2001), 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; Fig. 3A). NF-κB binding activity, especially of complex III and V, increased in banded rats compared with sham-operated rats (Fig. 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 supershift, thus showing that specific NF-κB
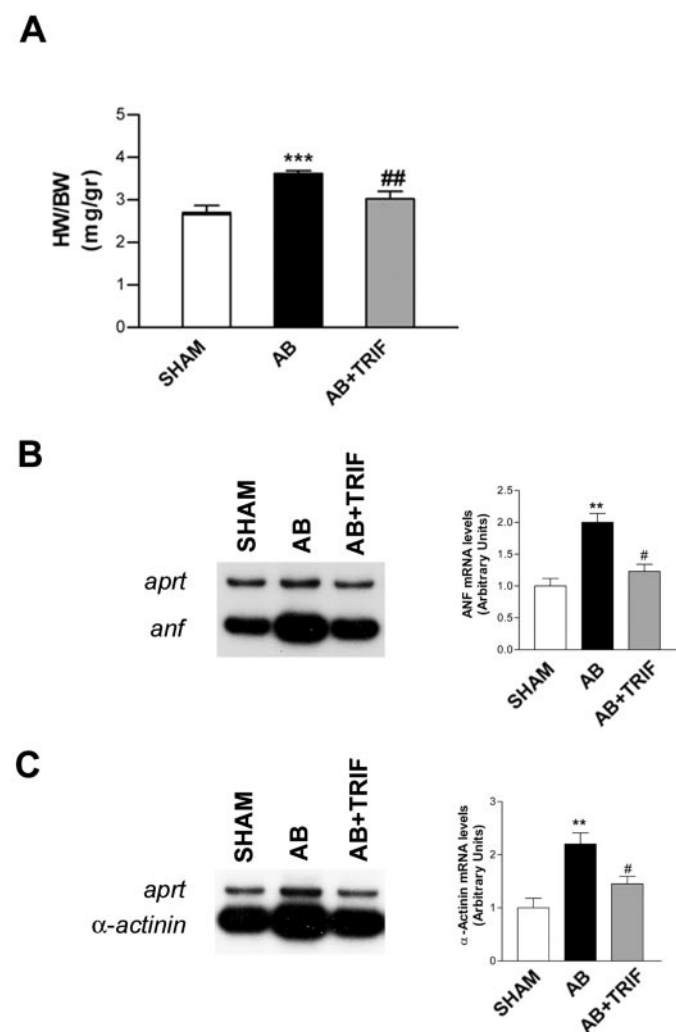

Fig. 2. Triflusal inhibits pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. Treatment with triflusal was initiated 5 days before operation and continued for 15 days as food admixture at a concentration of 0.05% (w/w). At 15 days after 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 six different experiments. **, $P < 0.01$; ***, $P < 0.001$ versus sham-operated rats. #, $P < 0.05$; ##, $P < 0.01$ versus banded rats.

complexes contained p65 (Fig. 3C). No 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 (Fig. 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 IκBa 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 subunits of NF-κB, IκBβ, and IκBα, we observed that triflusal affected only the expression 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 (Fig. 4). Given that enhanced physical interaction between p65 and IκBα has been reported to possibly 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 Fig. 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.

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 μM HTB for 24 h resulted in a modest increase in the levels of IκBα mRNA (similar results were obtained when cells were treated with 300 μM HTB for 24 h; data not shown) (Fig. 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. Because 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
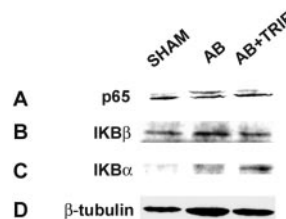
Fig. 4. Triflusal treatment increases the protein levels of IκBα. Cardiac protein extracts from sham-operated rats, banded rats (aortic banding, AB), and banded rats treated with triflusal (AB+TRIF) were assayed for western-blot analysis with p65 (A), IκBβ (B), IκBα (C), and β-tubulin (D) antibodies. All blot data are representative of at least three separate experiments.
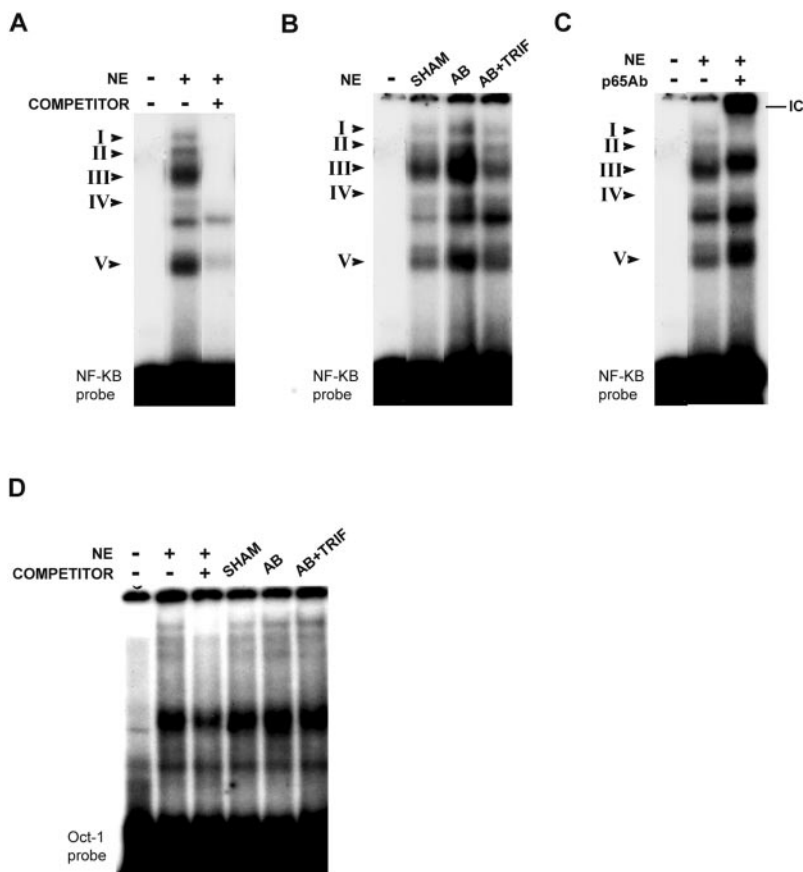
Fig. 3. Treatment with triflusal prevents NF-κB activation in pressure overload-induced cardiac hypertrophy. A, autoradiograph of EMSA performed with a ³²P-labeled NF-κB nucleotide and nuclear protein extract (NE) shows four specific complexes (I–V), based on competition with a molar excess of unlabeled probe. B, autoradiograph of EMSA performed with a ³²P-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 ³²P-labeled Oct-1 nucleotide. All blot data are representative of at least three separate experiments.

leads to enhancement of phospho-IκBα protein levels (Ganesh et al., 2003). A large increase in phosphorylation of IκBα on serine 32 was detected (4.4-fold increase, $P < 0.001$) in cells exposed to HTB, suggesting that it inhibited the proteosome-mediated dependent degradation of this protein (Fig. 6B). We then examined whether similar changes occurred in neonatal rat cardiomyocytes. In cells exposed to PE, the protein levels of IκBα were reduced (20%, $P < 0.05$) (Fig. 7A), but this reduction was not observed when cells were coincubated with HTB. It is noteworthy that 300 μM HTB treatment did not enhance apoptosis in rat neonatal cardiomyocytes, because no changes were observed in either the percentage of death cells (sub-G_1 fraction analysis by flow cytometry of propidium iodide staining; data not shown) or 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. ∗∗∗, $P < 0.001$ versus control cells) compared with control cells, whereas a 45% increase was observed in cardiomyocytes exposed to hydrogen peroxide. Furthermore, 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 with control cells (Fig. 7B). To obtain direct evidence for inhibition of proteosome 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).

Discussion

Although several signaling cascades have been implicated in the development of cardiac hypertrophy (Hunter and Chien, 1999), relatively little is known about the intrinsic
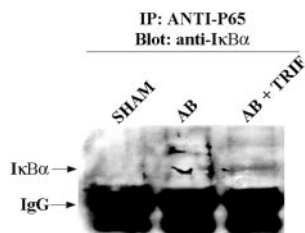
Fig. 5. Triflusal enhances protein-protein interaction between the p65 subunit of NF-κB and IκBα. Nuclear cardiac protein extracts (equalized by protein concentrations) from sham-operated rats, banded rats (aortic banding, AB), and banded rats treated with triflusal (AB+TRIF) were subjected to immunoprecipitation 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.
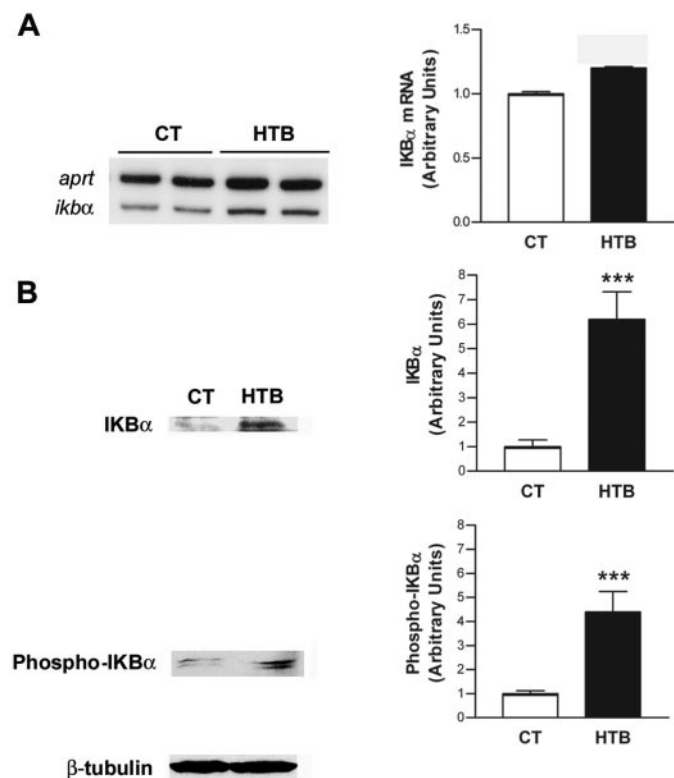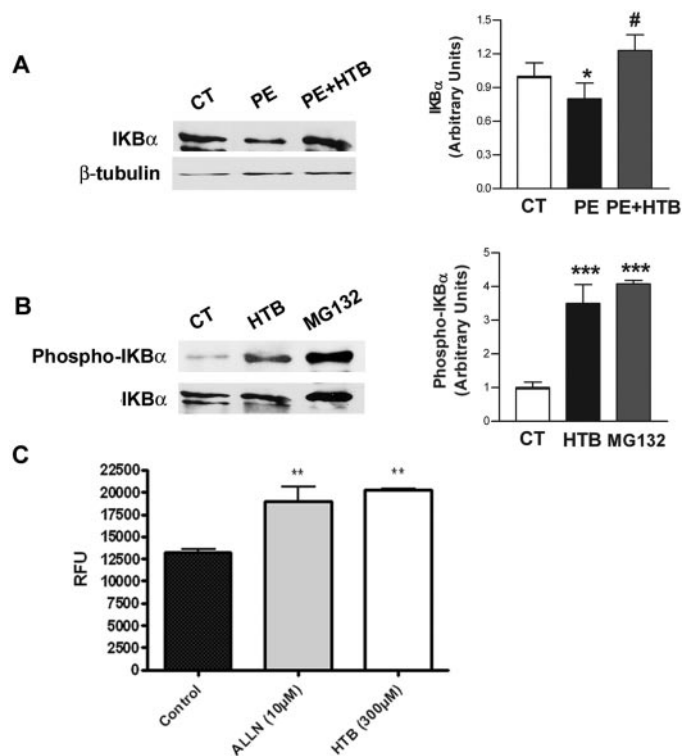
Fig. 6. The active metabolite of triflusal, HTB, induces the expression of IκBα in the embryonic rat heart-derived H9c2 cells. Analysis of the mRNA levels (A) and protein levels (B) of IκBα and phospho-IκBα in H9c2 myocytes in the presence or absence of 500 μM HTB for 24 h. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six different experiments.

Fig. 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 pretreated with HTB (300 μM) for 24 h and then stimulated with 100 μM PE for 30 min. Blot data are representative of three separate experiments. Data are expressed as mean ± S.D. of three different experiments. B, analysis of total and phospho-IκBα protein levels in neonatal cardiomyocytes treated with HTB (300 μM) or MG 132 (10 μM) for 24 h. Blot data are representative of three separate experiments. Data are expressed as mean ± S.D. of three 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 eight determinations. Proteosome activity is expressed in relative fluorescence units (RFU). ∗∗∗, $P < 0.001$; ∗∗, $P < 0.01$; ∗, $P < 0.05$ versus control (CT). #, $P < 0.05$ versus PE-stimulated cells.

mechanisms with the potential to inhibit or even reverse hypertrophy. In the present study, we demonstrated that triflusal, which is currently used as an antiplatelet agent, and its main metabolite, HTB, inhibited PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes and in the pressure overload animal model of cardiac hypertrophy. Furthermore, our findings indicated that the antihypertrophic effect of these salicylate-derived compounds reduced 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 (Purcell et al., 2001; Gupta et al., 2002; Higuchi et al., 2002; Hirotani et al., 2002). Furthermore, increased NF-κB activation was also observed in the myocardia of patients with congestive heart failure (Wong et al., 1998; Grabellus et al., 2002). This association between NF-κB and myocyte hypertrophy is interesting given the hypothesis that inflammatory cytokines are involved in cardiomyopathic disease states and that NF-κB itself is regulated by several of these cytokines. In this study, we demonstrated that triflusal and its main metabolite inhibited NF-κB activation in cardiac cells and that this mechanism might be responsible for their antihypertrophic effect. Our findings are consistent with those from several studies reporting that prevention of NF-κB activation leads to inhibition of cardiac hypertrophy. Thus, both peroxisome proliferator-activated receptor γ activators (Yamamoto et al., 2001) and antioxidants (Nakamura et al., 1998; Hirotani et al., 2002) 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 (Lanas et al., 2003; Matias-Guiu et al., 2003). The concentrations found to inhibit cardiac hypertrophy in this work (300 μM) 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 because 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. It is noteworthy that 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 demonstrated that triflusal and HTB increased the expression of IκBα. Therefore, increased expression of this inhibitor of κB resulted 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, this inhibitor has also been re-

ported to be 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 increased phospho-IκBα protein levels, suggesting that this drug inhibits proteosome-dependent degradation of IκBα. It remains to be studied 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 because 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 (Bayon et al., 1999; de Arriba et al., 1999; Acarin et al., 2000; 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 showed that triflusal and HTB inhibited cardiac hypertrophy in vitro and in vivo through a mechanism that might involve inhibition of the NF-κB signaling pathway, an important intracellular mediator of this process. Therefore, these findings suggest that triflusal might become a therapeutic option to reduce cardiac hypertrophy.

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Address correspondence to: Dr. Manuel Vázquez-Carrera, Unitat de Farmacologia, Facultat de Farmàcia, Diagonal 643, E-08028 Barcelona, Spain. E-mail: mvazquezcarrera@ub.edu