The mechanisms that cells use to mount protective responses against potentially harmful stresses have been the focus of many recent studies. An emerging theme from these studies is that cellular stress responses can lead to the release of cytokines, which can behave in a paracrine manner to enhance survival, often by inhibiting apoptosis (1–4). In certain organs, such as the heart or the brain, the release of cytoprotective cytokines in response to stress, perhaps even mild stresses, may serve critical cytoprotective roles.

Cytokines related to interleukin-6 (IL-6) compose a family of substances known to play cytoprotective and growth-promoting roles in many different cell types. These cytokines include IL-6, itself, as well as leukemic inhibitory factor, oncostatin M, ciliary neurotrophic factor and, the more recently discovered, cardiotrophin-1 (CT-1). CT-1 is a 21.5-kDa protein that was named after its ability to stimulate the growth of cultured cardiac myocytes (5), and it is expressed in and released from the heart in response to stress (6, 7). However, it is now clear that CT-1 is also expressed in many other tissues and that it exhibits important functional roles in those tissues. For example, in addition to the heart, CT-1 is expressed in the brain, lungs, liver, and kidneys (8). In vivo, CT-1 not only induces growth of the heart but also the liver, kidney, and spleen (9). In vitro, CT-1 has been found to foster the survival of sympathetic and motor neurons by inhibiting apoptosis (10, 11), and the switching of neuronal transmitter phenotype from catecholaminergic to cholinergic (12). CT-1 has also been shown to stimulate the acute phase activation of gene expression in hepatocytes (13, 14) and to induce osteoclast differentiation (13, 14). Thus, CT-1 possesses potentially important growth-promoting and cell survival roles in a number of tissues, thus emphasizing the importance of understanding the signaling mechanisms governing the effects of this cytokine.

The IL-6 family of cytokines bind first to ligand-specific receptors and then couple through a common, gp130 cell-surface receptor to the activation of a variety of intracellular pathways that could contribute to their cell survival effects (15). For example, CT-1 can activate the extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK), and the protein kinase B (or Akt) pathways (16, 17). The ERK and Akt pathways are cytoprotective (16–21). The mechanisms mediating the cytoprotective effects of CT-1 through these or other pathways form the topics of active investigation and of the present study.

One manner in which a variety of signaling pathways mediate protection from apoptosis involves activation of the transcription factor, NF-κB, which in turn induces the expression of proteins that possess cytoprotective roles (1). For example, in cardiac myocytes, activation of the p38 MAPK pathway by a p38-specific upstream MAPK kinase, MKK6, can foster protection from apoptosis, which is in large part mediated by NF-κB (22). It is unclear whether CT-1 can activate p38. However, because CT-1 is known to activate ERK and Akt, and because
these pathways have been shown to activate NF-κB (23–25), it is quite possible that the cytoprotective effects of CT-1 require activation of all three MAPK family members that converge on NF-κB.

Accordingly, the present study was undertaken to test the following three hypotheses: 1) in addition to ERK and Akt, CT-1 activates p38; 2) ERK, Akt, and p38 all contribute to the activation of NF-κB; and 3) NF-κB activation is required for the anti-apoptotic effects of CT-1.

To test these hypotheses, the ability of CT-1 to protect cells from apoptotic death was evaluated using a well characterized, cultured cardiac myocyte model system. This is a particularly appropriate model system for these studies, since it is believed that the CT-1, which is released from the heart in vivo in response to hypoxia, acts in an autocrine or paracrine manner to serve potentially critical cardioprotective roles (26).

**Experimental Procedures**

**Cell Culture**

Primary ventricular myocyte cell cultures were prepared from 1- to 4-day-old Harlan Sprague-Dawley rats as described (27, 28). The apical two-thirds of the ventricle were dissected away from the atria in DMEM/saline. Ventricles were minced and washed twice with air-compatible, HEPES-buffered DMEM. The isolation of cells was accomplished by multiple 10-min rounds of tissue dissociation using 0.0025% trypsin. After each trypsin incubation, the supernatant was added to an equal volume of DMEM/F-12 (1:1) containing 20% fetal bovine serum, and all of the supernatants were combined. Cells were initially suspended in DMEM/F-12 (1:1) containing 10% fetal bovine serum and plated onto culture dishes for 2 h, during which time most of the non-cardiac myocytes attach to the dish, whereas the myocytes remain in suspension. After 2 h, the suspended myocytes were collected by centrifugation and then resuspended in DMEM/F-12 (1:1) containing 10% fetal bovine serum. Cardiac myocytes were plated onto plastic culture wells, which had been pre-treated for at least 1 h with fibronectin at 5 μg/ml DMEM/F-12 (1:1). Alternatively, myocytes were plated onto 2-chamber Lab-Tek™ glass slides (Nalgen Nunc International) which had been pre-treated for at least 1 h with fibronectin at 25 μg/ml DMEM/F-12 (1:1). For TUNEL analysis and for immunocytofluorescence experiments, myocytes were plated at a density of 1 × 10⁶ cells per Lab-Tek™ glass slide chamber. For Western analyses, myocytes were plated at a density of 2 × 10⁶ cells per 25-mm plastic culture dish.

**Transfection**

Following pre-plating, myocytes were resuspended in serum-free DMEM/F-12 (1:1), and 4–8 × 10⁶ cells were combined with 12–45 μg of the indicated plasmids in a total volume of 300 μl. The total quantity of plasmid DNA used in each electroporation was equalized using pCMV6, when necessary. The optimal quantity of each plasmid was determined following transfection to a density of 1 × 10⁶ cells per Lab-Tek™ glass slide chamber. For Western analyses, myocytes were plated at a density of 0.6 × 10⁶ cells per fibronectin-coated, 24-mm plastic culture dish.

**Plasmids**

The following plasmids were used as indicated in this study.

- **β-Galactosidase**—pCH110-LacZ, which codes for a galactosidase reporter driven by the SV40 promoter, was obtained from Amersham Pharmacia Biotech (code 27-4509-01).
- **NF-κB-Luc**—p2X NF-κB, which codes for a luciferase reporter driven by a minimal prolactin promoter with two nearby, upstream NF-κB consensus sites (29), was obtained from M. Karin (University of California, San Diego, La Jolla, CA).
- **Gal4-Luc**—Gal4-p55 (1–551), which encodes the DNA-binding domain of the yeast Gal4 protein fused to the p55 subunit of NF-κB (25), was obtained from G. Haegeman (University of Gent, Belgium).
- **IκkB-M**—pRK5 C-FLAG IKKβ (K44A), which encodes a kinase-inactive human IKKβ (32), was obtained from M. Rothé (Tularik, San Francisco, CA).
- **TAK1-M**—pFLAG-Tak1, which encodes a kinase-inactive human TAK1 K63W (60), was obtained from T. Sugita (Tanabe Seiyaku Co., Ltd., Osaka, Japan).
- **MEK-M**—pEXV3 MAPKK1-Ala-217, which encodes a dominant-negative mutant of MAPKK1/MEK1, was obtained from S. Fuller (Imperial College, London, UK).
- **PI3K-M**—pMTN110, which encodes the first 100 amino acids of bovine p110α subunit of PI3K and acts as a dominant negative by binding to p85, was obtained from T. Franke (Columbia University, New York).

**Reporter Assays**

β-Galactosidase—After the indicated times, cells were lysed in ice-cold lysis buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO₄, 4 mM EDTA, 0.25% Triton X-100) containing 1 mM dithiothreitol. Cell debris was eliminated by centrifugation, and 200–μl samples of each cell lysate were combined with 400 μl of galactosidase buffer (60 mM NaHPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄) containing 1 mg/ml chloroform and 0.1 mg/ml p-nitrophenol red-fibronectin poly(t-galactopyranoside) and 50 mM β-mercaptoethanol. After incubation for 2 h, the absorbance was measured at 570 nm.

Luciferase—After cell lysis and removal of cell debris by centrifugation, as described above, 100–μl samples of cell lysate were combined with 100 μl of luciferase buffer (25 mM Gly-Gly, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 45 mM KPO₄, pH 7.8, 1 mM dithiothreitol, 0.3 mM nuclease-free ATP). An MGM Instruments OptoComp II luminometer was used to measure light emission of each sample for 10 s. Relative luciferase activities were determined by dividing luciferase values by β-galactosidase values.

**Immunocytofluorescence**

In order to determine the effects of CT-1 on cellular localization of the p65 subunit of NF-κB, myocardial cells treated ±1 nm CT-1 were fixed in 4% paraformaldehyde. Immunofluorescence was carried out using an anti-polyclonal antibody raised against p65/NF-κB (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by Texas Red conjugated anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR).

**Western Analyses**

Myocardial cells were treated ±1 nm CT-1 for the indicated times. Cells were then lysed in 100 μl of Laemmli sample buffer that had been supplemented with 0.1 mM sodium orthovanadate, 10 μg/ml aprotinin, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM p-nitrophenyl phosphate, and 10 μg/ml leupeptin, boiled for 5 min, and then submitted to 10% SDS-polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane in methanol transfer buffer at 60 V for 5 h or at 30 V for 12–18 h. Membranes were then treated for 30 min at room temperature with 5% nonfat milk dissolved in Tris-buffered saline/Tween (0.01%). Western analyses were carried out using antisera specific for phospho-pS8 (1:200), phospho-Akt (1:500), or phospho-ERK (1:1000). Blots were subsequently stripped with 6:25 mM Tris, 2% SDS, and 100 mM 2-mercaptoethanol for 30 min at 50°C, then washed for 1 h with Tris-buffered saline/Tween (0.01%), and re-probed with antisera specific for either p38 (1:200), Akt (1:500), or ERK (1:1000) for purposes of normalization. IκB Western was carried out using antisera specific for IκB (1:500). All antibodies were purchased from Cell Signaling Technology, Beverly, MA. Densitometric analysis was carried out using Molecular Dynamics Image Quant software.

**Hypoxia/Reoxygenation (H/R)**

Myocardial cells were cultured on fibronectin-coated slides for at least 70 h in DMEM/F-12 with bovine serum albumin (1 mg/ml). The medium was then replaced with glucose-free DMEM, base (Life Technologies, Inc., 23800-022), which had been saturated with N₂/CO₂ (95:5) for 10 min (hypoxia) or O₂/CO₂ (95:5) (control). Cells subjected to hypoxia were placed in a chamber filled with N₂/CO₂ (95:5) for 2 h. Subsequently, the hypoxia and control cells were incubated for 24 h in DMEM/F-12 (1:1) ±1 nm CT-1, 1 μM SB202190, 25 μM U0126, 200 μM NBD peptide, or 200 μM control peptide in an O₂/CO₂ (95:5), normoxic environment. Cell samples were then fixed in 4% paraformaldehyde for 20 min and then stored in 0.4% paraformaldehyde until TUNEL analysis. Preliminary experiments established that 2 h of hypoxia followed by 24 h of reoxygenation resulted in the most consistent induction of 40–60% cell death by apoptosis.
Apoptosis Analyses

TUNEL—H/R-treated cells were subjected to TUNEL analyses (22) according to the manufacturer’s protocol (Roche Molecular Biochemicals). The percentage of TUNEL-positive cells was evaluated by viewing each field at ×40 magnification, as described previously (22). Generally, 10 different microscopic fields containing 10–15 cells each were recorded for each sample. Each experiment was repeated at least three times.

Hoescht—Following H/R treatment, Hoescht bisbenzimide (0.5 μg/ml) was added directly to culture media in the dark. Following 5–10 min of incubation at room temperature, propidium iodide (0.5 μg/ml) was added. Cells were analyzed using a fluorescence microscope equipped with a UV filter cube, using an excitation wavelength of 350 nm. Hoescht-stained nuclei appeared blue and were used for visualization of pyknosis, a characteristic feature of apoptosis. Propidium iodide staining was used to ensure that the nuclear membrane remained intact, as indicated by exclusion of propidium iodide, a feature of cells undergoing apoptosis. Images were analyzed at ×100 magnification and captured using X-Cap Image™ software.

Peptides

A 28-amino acid peptide was synthesized using an Fmoc (N-(9-fluorenylethoxycarbonyl) solid phase synthesis strategy and purified using reverse phase high performance liquid chromatography. The peptide possesses the sequence derived from Antennapedia homeodomain that mediates membrane translocation. The 17 N-terminal and 11 C-terminal amino acids span the NEMO binding domain of IKKβ (underlined), DBQKIQWFQRRMRKWTALDWSWLQTE. This peptide, named NEMO binding domain (NBD) peptide, has been shown previously to inhibit the induction of NF-κB by disrupting the IKKβ-NEMO interaction (34). A peptide with a similar size and chemical composition was synthesized and purified using the same procedure and was used as a non-active control peptide.

Materials

CT-1 was purchased from R & D Systems, Minneapolis, MN, and lipopolysaccharide (LPS) was purchased from Sigma (catalog number L-6529). SB202190 and LY294002 were purchased from Calbiochem. All antibodies and U0126 were purchased from Cell Signaling Technology, Beverly, MA. Culture media were purchased from Life Technologies, Inc. All other reagents were purchased from Sigma.

RESULTS

The Protection of Cardiac Myocytes from Hypoxia-induced Apoptosis by CT-1 Requires p38, ERK, and PI3K/Akt—To evaluate the effects of CT-1 on cell survival, cardiac myocytes were submitted to a well characterized H/R maneuver (35, 36). The maneuver consisted of maintaining the cells in a hypoxic environment for 2 h and then in a normoxic environment for 24 h. TUNEL analysis indicated that, in general, control cells displayed no apparent DNA strand breaks (Fig. 1A) and exhibited intact nuclei, as determined by Hoescht staining (Fig. 1B) coupled with propidium iodine staining (not shown). In contrast to control cells, a greater proportion of H/R-treated cells exhibited pyknotic, TUNEL-positive nuclei (Fig. 1, C and D), which are hallmarks of apoptotic cell death. Treating the cultures with CT-1 during the reoxygenation phase2 effectively abrogated H/R-mediated apoptosis of myocardial cells (Fig. 1, E and F). A quantitative analysis revealed that compared with control cells, myocardial cells subjected to H/R displayed an approximate 30% increase in apoptosis and the addition of 1 nM CT-1 during the reoxygenation phase of H/R resulted in protection from apoptosis (Fig. 1G, experiment 1).

To investigate the potential roles of the p38, Akt, or ERK pathways in CT-1-mediated cytoprotection, myocytes were incubated with various cell-permeable inhibitors of these pathways during the reoxygenation phase of H/R. Under these conditions, SB202190, a specific inhibitor of p38α and p38β (37), compromised the ability of CT-1 to protect the cardiac myocytes, resulting in ~2-fold more apoptosis than observed in cells treated with H/R + CT-1 (Fig. 1G, experiment 2).
Myocardial cells were treated with p38, Akt, and ERK, resulting in diminished cytoprotective effects of CT-1, U0126, a specific inhibitor of MEK1/2, and thus, experiment 3 had no effect on the active forms of either Akt or ERK, as expected (Fig. 3, B and C). These results substantiate the selective effects of the cell-permeable inhibitors, and they support the view that CT-1 can activate each of these signaling pathways independently.

CT-1 Induces NF-κB-mediated Transcription and p65/NF-κB Translocation—NF-κB can protect various cell types, including cardiac myocytes, from apoptosis (1, 22). Accordingly, the effects of CT-1 on the activation of NF-κB were assessed using an NF-κB-dependent reporter gene. CT-1 stimulated NF-κB-dependent reporter production in a dose-dependent manner, resulting in an approximate 5-fold activation over control at 5 nM CT-1 (Fig. 4A).

Under unstimulated conditions, NF-κB is retained in the cytosol by virtue of binding to an anchoring protein called inhibitor of κB (IκB) (41). When cells are treated with agonists known to activate NF-κB, IκB kinase-β (IKKβ) phosphorylates IκB on serine residues 32 and 36 and, in so doing, targets IκB to proteosomal degradation (42). This liberates NF-κB to translocate to the nucleus where it binds to regulatory elements of NF-κB-inducible genes. Accordingly, the effect of CT-1 on the cellular localization of the p65 subunit of NF-κB was assessed by immunocytofluorescence.

As expected, under control conditions, p65/NF-κB staining was distributed in a punctate pattern, consistent with a localization to the cytoplasm (Fig. 4B). Following treatment with
CT-1 for 5 min, a small increase in nuclear p65/NF-κB was detected (Fig. 4C), and after 15 min of cytokine treatment, nuclear localization of p65/NF-κB was even more evident (Fig. 4D). After 60 min of CT-1 treatment there was a striking increase in the accumulation of immunoreactive p65/NF-κB in the nucleus (Fig. 4E). These results are the first to show that CT-1 can induce p65/NF-κB translocation and that it can increase the transcription of an NF-κB-dependent reporter gene. Moreover, the time that is required for CT-1 nuclear translocation is consistent with roles for CT-1-activated p38, Akt, and ERK in mediating this effect.

**FIG. 4.** Effects of CT-1 on NF-κB-mediated transcription and p65/NF-κB cellular localization. **A**, NF-κB-mediated transcription. Myocardial cells were co-transfected with an NF-κB/luciferase reporter (p2X NF-κB; 12 μg) and a β-galactosidase reporter (pCH110; 20 μg) as described under “Experimental Procedures.” Following 24 h in serum-free medium, the cells were treated for another 24 h with serum-free medium ± CT-1 at the concentrations indicated. Cultures were then extracted and assayed for luciferase and β-galactosidase activities as described under “Experimental Procedures.” The average relative luciferase (Rel Luc) values (luciferase/β-galactosidase) for each treatment ± S.E. are shown. Each bar represents the mean of the results obtained from three identically treated cultures. This experiment is representative of three similar experiments.

**B**, CT-1 at the concentrations indicated. Cultures were then extracted and submitted to SDS-polyacrylamide gel electrophoresis followed by Western analysis to determine the relative quantities of IκB. Densitometry and image analyses were carried out as described in the legend to Fig. 2. This experiment is representative of three similar experiments.

**C**, p65-Gal4. Following 24 h in serum-free medium, the cells were maintained for another 6 h in serum-free medium ± 1 μM CT-1 ± 1 μg LPS/ml (Sigma L-6529), as indicated. The average relative luciferase (Rel Luc) values (luciferase/β-galactosidase) for each treatment ± S.E. are shown. Each bar represents the mean of the results obtained from three identically treated cultures. This experiment is representative of three similar experiments.

**FIG. 5.** Effects of CT-1 on IκB degradation and p65/NF-κB transactivation. **A**, IκB degradation. Myocardial cells were treated ±1 mM CT-1 for the indicated times and then extracted and submitted to SDS-polyacrylamide gel electrophoresis followed by Western analysis to determine the relative quantities of IκB. Densitometry and image analyses were carried out as described in the legend to Fig. 2. This experiment is representative of three similar experiments.

**B**, p65/NF-κB transactivation. Myocardial cells were co-transfected with a Gal4/luciferase reporter (pG5E1b/luciferase; 20 μg), a β-galactosidase reporter (pCH110; 20 μg), and either 12 μg of a construct encoding a Gal4-p65 (51-551) chimera or 12 μg of pCMV6. Following 24 h in serum-free medium, the cells were maintained for another 6 h in serum-free medium ± 1 nM CT-1 ± 1 μg LPS/ml (Sigma L-6529), as indicated. The average relative luciferase (Rel Luc) values (luciferase/β-galactosidase) for each treatment ± S.E. are shown. Each bar represents the mean of the results obtained from three identically treated cultures. This experiment is representative of three similar experiments.

**CT-1 Stimulates IκB Degradation but Not the Transcriptional Function of p65/NF-κB**—If CT-1-mediated stimulation of NF-κB involves the activation of IKK, then the cytokine should be able to induce IκBα degradation (42). Accordingly, the quantities of IκB remaining after various times of CT-1 treatment were determined by Western blotting. The levels of IκB were similar to control after 1 and 15 min of CT-1 treatment; however, after 30 or 60 min of treatment, IκB levels were dramatically reduced to ~25% of control values (Fig. 5A). This time frame for IκB degradation was consistent with the translocation of p65 to the nucleus after 60 min of CT-1 treatment (Fig. 4E). Following 90 min of CT-1 treatment, the quantity of IκB returned to control levels (Fig. 5A), an expected finding that is likely due to activation of IκBα gene transcription soon after IκBα degradation is initiated (43). These results indicate that IKK is activated in response to CT-1 treatment and that this leads to the degradation of IκBα and the subsequent translocation of NF-κB to the nucleus.

The translocation of NF-κB to the nucleus is a first, obligate step in the activation of NF-κB-dependent gene expression. As such, nuclear NF-κB can bind to and increase the expression of genes with NF-κB elements. An additional mechanism that augments the activity of NF-κB-dependent gene expression...
beyond that afforded by localizing NF-κB to the nucleus is the phosphorylation of p65/NF-κB in a manner that increases its ability to drive transcription (44, 45). To evaluate this transactivation as a possible mechanism by which CT-1 might contribute to NF-κB gene induction, a “one-hybrid” technique was used. This system requires the co-transfection of a plasmid encoding a chimera composed of the test protein (e.g. p65/NF-κB) fused to a Gal4-DNA binding domain and a plasmid composed of Gal4-binding sites driving luciferase expression. In this assay, the Gal4/p65 fusion protein is localized to the nucleus by virtue of a nuclear localization signal residing in the Gal4-DNA binding domain; given this system, the transcriptional activation potential of p65/NF-κB can be assessed (25). By using this assay, we found that CT-1 was unable to stimulate the transactivation of p65/NF-κB (Fig. 5B). However, LPS3, a well known stimulator of p65/NF-κB transcriptional activation was clearly positive. This indicates that whereas CT-1 induces the nuclear localization of NF-κB, thus providing a certain degree of induction of the NF-κB luciferase reporter, it does not lead to any apparent increase in p65/NF-κB transactivation.

CT-1 Activation of NF-κB Is Mediated by the p38, PI3K, and ERK Pathways—Since Akt and ERK have been shown to contribute to the activation of NF-κB (1, 23–25, 46–48), and since p38 has been shown to enhance NF-κB activation, the roles of all three pathways in CT-1-mediated NF-κB activation were assessed. The activation of NF-κB-dependent transcriptions by CT-1 was reduced by ~35, 50, or 55% by SB202190, LY294002 or U0126, respectively (Fig. 6A). When the cells were treated with the three inhibitors together, the activation of NF-κB by CT-1 was completely inhibited.

Further experiments were carried out using dominant negative signaling proteins to inhibit the IKK, p38, ERK, or PI3K pathways in order to substantiate the roles of these kinases in CT-1-mediated NF-κB activation. A form of IκBo harboring a double mutation of S32A and S36A, IκBo-M, cannot be phosphorylated by IκB kinase. Accordingly, IκBα-M serves as a constitutive cytosolic anchor for of NF-κB, which inhibits its ability to translocate to the nucleus. Transfection of cells with either IκBα-M or a dominant negative mutant of IκBα (IκB-M) decreased CT-1-mediated NF-κB activation by 65 and 70%, respectively (Fig. 6B).

TGFB-β-activated protein kinase (TAK) is an MAPKKK that lies directly upstream of MKK6 in the p38 pathway (49, 50). A dominant negative form of TAK (TAK1-M) has been shown to interrupt agonist-activated p38 in cardiac myocytes (1). In the present study, TAK1-M effectively reduced CT-1-mediated NF-κB activation by about 68% (Fig. 6B), thus confirming the importance of the TAK1/MKK6/p38 pathway in the mechanism of CT-1 action. Dominant negative forms of MEK1 (MEK-M) and of the catalytic subunit of PI3K (p110-M) also reduced CT-1-mediated NF-κB activation by about 64 and 60%, respectively (Fig. 6B), further confirming the involvement of the MEK/ERK and PI3K/Akt pathways, respectively.

The Cardioprotective Effect of CT-1 Is Dependent on the NF-κB Pathway—Although maximal CT-1 activation of NF-κB was shown to require p38, Akt, and ERK, and inhibitors of those pathways blocked the cytoprotective effects of CT-1, it remained important to demonstrate that NF-κB activation is required for CT-1-mediated cytoprotection against H/R. Accordingly, a method for inhibiting NF-κB activation during H/R was identified. Since the cells needed to be cultured for several days before the H/R maneuver, a specific inhibitor of NF-κB activation that could be added to the cultures at the appropriate time was sought. Maximal activation of NF-κB requires an IKK complex that is composed of IκB kinases (IKKα, IKKβ, and NEMO (NF-κB essential modifier)) (51, 52). A recent study has shown that in cultured cells treated with a cell-permeable peptide composed of a 13-amino acid region of IκBα (IκBα-644–756),
The binding of NEMO to the IKKβ-IKKα complex is disrupted, leading to the inhibition of IKKβ-mediated NF-κB activation (34).

Accordingly, during the reoxygenation phase of the H/R maneuver, myocardial cells were treated with CT-1 and either the NEMO binding domain (NBD) peptide or an unrelated peptide of similar length and chemical characteristics. Although the control peptide had no effect (Fig. 7B and C), the NBD peptide inhibited CT-1-mediated nuclear localization of the p65 subunit of NF-κB (Fig. 7D). When the effects of the control or NBD peptide on H/R-mediated apoptosis were assessed, it was apparent that the control peptide had no effect, whereas the NBD peptide completely blocked any cytoprotective effects of CT-1 (Fig. 7E). Taken together, these results indicate that NF-κB induction plays a crucial role in CT-1-mediated protection from apoptosis during H/R.

**Discussion**

To the best of our knowledge, the present study is the first to show that CT-1 can stimulate NF-κB nuclear translocation and the transcriptional activation of NF-κB-dependent gene expression. Moreover, we also believe that results from the present study are the first to demonstrate that NF-κB activation is required for the protective effects of CT-1 on cardiac myocytes that have been challenged with hypoxia/reoxygenation, a stress that usually leads to apoptotic cell death (53). These results underscore the importance of understanding the signaling mechanisms through which CT-1 mediates its effects on NF-κB.

The molecular events by which CT-1 activates NF-κB remain to be determined. However, the effects of CT-1 and/or other gp130-coupled cytokines on several other signaling pathways have been well characterized and thus may provide clues as to how gp130-coupled cytokines affect the NF-κB pathway. It is thought that after binding to the leukemic inhibitory factor receptor, CT-1 couples through gp130 to stimulate the Janus kinase/signal transduction activator of transcription pathway (7). Interestingly, Janus kinase can phosphorylate gp130 in a manner that fosters the binding of SH2 domain-containing signaling molecules, such as signal transduction activator of transcription and Sht (54, 55), the latter of which is a critical initial event in Ras activation (Fig. 8). PI3K can be activated by directly binding to some cytokine receptors through the SH2 domain on the p85 subunit of PI3K (56). Alternatively, Ras, which is well known to activate Raf, MEK, and ERK, can foster the activation of PI3K, PDK1, and Akt (57–59). Akt, which is cytoprotective in many cell types, including cardiac myocytes (16, 17), is believed to augment NF-κB signaling by phosphorylating and activating IKK (24, 47, 60). Akt is also thought to enhance the phosphorylation and trans-activation of p65/NF-κB (44, 45, 61); however, results from the present study were not consistent with this view, suggesting that p65/NF-κB trans-activation may occur in a cell-specific manner. Even though ERK activates NF-κB, the mechanisms underlying this effect are poorly understood. To our knowledge, only one study is directly relevant; that study suggested that ERK can enhance the trans-activation function of p65/NF-κB and that activation of the ERK pathway can lead to the phosphorylation of p65/NF-κB (25), although there is no solid evidence that ERK itself phosphorylates p65/NF-κB in a functionally significant manner.

In addition to Akt and ERK, it was shown in the present study that p38 is involved in CT-1-activated NF-κB and cytoprotection. Consistent with this view were our observations that CT-1 was able to activate p38 and that inhibiting p38 partially blocked CT-1-activated NF-κB, as well as CT-1-mediated protection from apoptosis. This result is consistent with a previous study which demonstrates that in cardiac myocytes tumor necrosis factor-α activates NF-κB in a manner that requires both IKK and p38, and it protects cultured neonatal rat cardiac myocytes from apoptosis (1). Furthermore, in that same study it was shown that MKK6, an upstream activator of p38, can bind directly to IKKβ and enhance its activity, thus forming a possible link between the p38 and IKK signaling pathways (Fig. 8). Another recent report has demonstrated cross-talk between IKK and p38 in 3T3 fibroblasts; in that
protection from apoptosis

FIG. 8. Simplified diagram of the signaling mechanisms by which CT-1 may activate p38, Akt, ERK, and NF-κB. Shown is a simplified diagram designed after Wollert and Chien (71) and Winston and Hunter (70), and the results from the present study, which depicts a possible mechanism by which CT-1 could mediate the activation of Akt, ERK, and p38 and how these pathways could converge on the activation of NF-κB. Also depicted is the central role NF-κB plays in cytoprotection, as determined in the present study. Not shown are the many NF-κB-independent mechanisms by which Akt, ERK, and p38 contribute to cytoprotection. See “Discussion” for details.

study, IL-1β-activated Akt was shown to stimulate NF-κB in a manner that requires both IKK and p38 (62). Other studies have suggested that like Akt, and perhaps ERK, activation of p38 can lead to enhanced phosphorylation and trans-activation of p65/NF-κB, although p38 itself cannot phosphorylate p65 (63, 64). However, as with Akt, this view was not supported by results in the present study, which showed that CT-1 does not increase p65/NF-κB trans-activation (present study and Ref. 1). Nonetheless, the requirement of p38 for the maximal CT-1-mediated p65/NF-κB nuclear translocation, reporter induction, and cytoprotection is consistent with previous studies demonstrating that in cardiac myocytes, p38 can foster protection from apoptosis in an NF-κB-dependent manner (1, 22).

In addition to demonstrating that CT-1 can activate NF-κB, another novel aspect of the present study was the finding that CT-1 activated p38, an event that was required for optimal CT-1-activated NF-κB and cytoprotection. It is not known how CT-1 leads to the activation of p38. However, since gp130 ligation leads to Shc/Grb2/Sos activation, CT-1 most likely activates Ras. It is therefore conceivable that Ras could then activate MAPKKs, which are capable of stimulating the p38 and/or NF-κB pathways. In support of this view is a recent study demonstrating that Ras can activate the NF-κB pathway through a tumor necrosis factor-α receptor scaffold protein called tumor necrosis factor receptor-associated factor (TRAF6) (65). Interestingly, TRAF6 is known to lead to the activation of TAK1 (66), a MAPKK, which can activate both IKK and MKK6 (49). Thus, through TRAF6, Ras activation of TAK1 would establish a mechanism by which cytokines like CT-1 could foster the parallel activation of the NF-κB and p38 pathways (Fig. 8). Another MAPKK that lies downstream of Ras, which could also lead to activation of p38, is MEKK1 (67). MEKK1 is also believed to exist as part of a macromolecular complex, the signalosome (68), which includes IKK (69) and other NF-κB-related signaling proteins. Moreover, MEKK1 is well known as a strong activator of NF-κB-dependent gene induction (68). Accordingly, TAK1 and MEKK1 are both potential activators of NF-κB and p38, serving roles as mediators of cytokine signaling to these important cytoprotective pathways.

In summary, the results from the present study have demonstrated a central function for NF-κB in the mechanism by which CT-1 protects cardiac myocytes against H/R-induced apoptosis. Moreover, results from the present study have also established important roles for p38 in CT-1-activated NF-κB. In conjunction with other recent results regarding the function of another gp130-coupled cytokine, IL-6, on myocardial cell protection (1), these findings establish important autocrine or paracrine roles for signaling substances known to be released from cardiac myocytes or non-myocyte cell types during times of ischemic stress. And, since CT-1 and other gp130-coupled cytokines have such a widespread tissue distribution, these results have broad implications. Future studies aimed at further delineating the mechanisms by which gp130-coupled cytokines activate p38, as well as studies oriented toward understanding the downstream events by which p38, Akt, and ERK foster cytoprotection, will be critical to our understanding of how the heart and other tissues respond to stress.

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