Angiotensin Type 2 Receptor Dephosphorylates Bcl-2 by Activating Mitogen-activated Protein Kinase Phosphatase-1 and Induces Apoptosis*

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We examined the cellular and signaling mechanism of angiotensin II (Ang II) type 2 (AT2) receptor-induced apoptosis in PC12W (rat pheochromocytoma cell line) cells that express abundant AT2 receptor but not Ang II type 1 receptor. In these cells, nerve growth factor (NGF) inhibited the intracellular DNA fragmenta-
tion induced by serum depletion, whereas Ang II antagonized this NGF cell survival action and induced apoptosis. We studied the mechanism of NGF and AT2 receptor interaction on apoptosis by examining their effects on the survival factor Bcl-2. AT2 receptor activation did affect intracellular Bcl-2 protein levels. Bcl-2 phosphorylation was stimulated by NGF, whereas AT2 receptor activation blocked this NGF effect. Pretreatment with antisense oligonucleotide of mitogen-activated protein (MAP) kinase phosphatase-1 enhanced the effects of NGF on MAP kinase activation and Bcl-2 phos-
phorylation but attenuated the inhibitory effects of AT2 receptor on MAP kinase, Bcl-2 phosphorylation, and apoptosis. Taken together, these results suggest that MAP kinase plays a critical role in inhibiting apoptosis by phosphorylating Bcl-2. The AT2 receptor inhibits MAP kinase activation, resulting in the inactivation of Bcl-2 and the induction of apoptosis.

The processes of cell survival and cell death involve highly regulated signaling pathways that are currently the subject of intense investigation. Apoptosis is a ubiquitous, evolutionally conserved, physiological mechanism of cell death that regulates tissue mass and architecture in many tissues (1). The rat PC12W pheochromocytoma cell line is widely used to examine the molecular and cellular mechanism of apoptosis. Xia et al. (2) demonstrate, using PC12W cells, that signaling through mitogen-activated protein (MAP) kinases plays a critical role in cell survival and death. Extracellular signal-regulated kinases (ERK) (p42 and p44 MAP kinases known as p42MAPK/ERK2 and p44MAPK/ERK1) act as survival signals, whereas c-JUN NH₂-terminal protein kinase and p38 exert cell death signaling. In the presence of nerve growth factor (NGF), the survival signal pathway is activated, whereas the cell death signaling pathway is suppressed. Angiotensin II (Ang II) exerts various actions in its diverse target tissues controlling vascular tone, hormone secretion, tissue growth, and neuronal activities primarily via Ang II type 1 receptor. Recently, a second receptor subtype known as AT2 receptor has been cloned (3, 4). We and others have demonstrated that the AT2 receptor stimulates a tyrosine phospho-
tase (5–8) that inhibits MAP kinase (p42MAPK/ERK2 and p44MAPK/ERK1) activation and induces apoptosis in PC12W cells and confluent R3T3 cells (mouse fibroblast cell line) (7). In this study, we hypothesize that this inactivation of MAP kinase plays a pivotal role in mediating apoptosis via the inactivation of the cell survival factor Bcl-2. Bcl-2 can prevent or delay apoptosis induced by a wide variety of stimuli and insults, suggesting that Bcl-2 controls a distal step in the final common pathway for cell death (9). Recent data support that the post-translational modification of Bcl-2 such as phosphorylation is important for the regulation of Bcl-2 function (10–12). Here, we demonstrated that MAP kinase phosphorylated Bcl-2 and AT2 receptor stimulation activated MAP kinase phosphatase 1 (MKP-1) and inhibited the phosphorylation of Bcl-2 in PC12W cells, resulting in the induction of apoptosis.

EXPERIMENTAL PROCEDURES

Cells and Treatment—PC12W cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% horse serum, 5% fetal bovine serum. Cell number was counted by Coulter counter.

Measurement of Bcl-2 Phosphorylation and Immunoblot Assay—PC12W cells were seeded onto a 10-cm dish (Becton Dickinson) at 2 × 10⁶ cells/dish. The cells were first grown in serum-fed Dulbecco’s modified Eagle’s medium and then kept in serum-free medium for 12 h. The cells were washed three times in serum-free and phosphate-free medium and equilibrated with ³²P(orthophosphoric acid (Amersham Life Science, Inc.) at the concentration of 100 μCi/ml in phosphate-free medium for 12 h. The radiolabeled cells were treated with NGF (20 ng/ml), Ang II (10⁻⁸ and 10⁻⁷ M), and/or PD123319 (10⁻⁵ M) at 37 °C for 30 min. The cells were washed with HEPES-buffered saline and lysed in 0.5 ml of radioimmune precipitation buffer containing 1 mM phenyl-
methylsulfonyl fluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotenin. Cell lysates were centrifuged at 8,500 × g for 20 min, and the supernatant was incubated with 10 μg of Bcl-2 antibody (Santa Cruz Biotechnology) at 4 °C for 12 h. After precipitation with protein A/G-
agarose (Santa Cruz Biotechnology), samples were boiled in Laemmli loading buffer for 3 min and resolved by 12% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, dried, and analyzed by autoradiography. The bands corresponding to the Bcl-2 were cut, and their radioactivity was measured.

For immunoblotting, cell lysates (100 μg) were run on 12% SDS-
PAGE, electroblotted onto nitrocellulose membrane, and immuno-
blotted with Bcl-2 antibody or MKP-1 antibody (Santa Cruz Biotechnol-
ogy). Antibodies were detected by horse radish peroxidase-linked...
secondary antibody using ECL (enhanced chemiluminescence) system (Amersham).

For the assay of tyrosine phosphorylation of Bcl-2, the cells were grown in serum-free medium for 12 h and stimulated with NGF (20 ng/ml), Ang II (10^{-7} M), and NGF (20 ng/ml) plus Ang II (10^{-7} M). The cell lysates were immunoprecipitated with Bcl-2 antibody (10 μg), resolved on 12% SDS-PAGE, electroblotted onto nitrocellulose membrane, and immunoblotted with anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.).

**MAP Kinase Activity Determination—**MAP kinase activity was assayed by its ability to phosphorylate myelin basic protein as described previously (13) with a slight modification (6–8).

**MKP-1 Antisense Oligonucleotide Transfection—**PC12W cells were transfected with 300 nM anti-MKP-1 antisense oligonucleotide (phosphorothioate modified) in Lipofectin (Life Technologies) according to the approach of Duff et al. (14). After transfection, the cells were maintained in the presence of 10% horse serum and 5% fetal bovine serum for 1 day. Then the medium was changed to serum-free medium with or without Ang II. Two days later, cells were harvested and subjected to analysis of DNA fragmentation. Anti-MKP-1 antisense transfected PC12W cells were also used for Bcl-2 phosphorylation, and MAP kinase activity determinations as described above. Oligonucleotide sequences (20 base pairs) are as follows: MKP-1 antisense, 5′-GGAACTCAAGTGGAAGTCAGG-3′; MKP-1 sense, 5′-CCTGAGTTCCACCTGAGTTC-3′.

**Interruclidean DNA Fragmentation (DNA Ladder)—**DNA extraction, subsequent 3′ end labeling of DNA, gel electrophoresis, and quantitation of DNA fragmentation were performed as described previously (7, 15, 16).

**Statistical Analysis—**All values are expressed as mean ± S.D. Statistical significance was assessed by ANOVA followed by Scheffe’s test. *p < 0.05 was considered significant.

**RESULTS**

**Bcl-2 Phosphorylation by NGF and Its Inhibition by AT2 Receptor—**We recently demonstrated (7) that AT2 receptor antagonizes the anti-apoptotic effect of NGF and induces apoptosis in PC12W cells, which express abundant AT2 receptor and very low levels of Ang II type 1 receptor (17). In this study, we examined the possibility that the AT2 receptor regulates apoptosis via its influence on Bcl-2 (Fig. 1). We first stimulated these cells with NGF and then studied the effect of Ang II on Bcl-2 protein levels. We observed that the Bcl-2 level was not affected with Ang II (Fig. 1B). Since phosphorylation of Bcl-2 is essential for its physiological function (10–13), we then focused on the effect of AT2 receptor on the Bcl-2 phosphorylation.

PC12W cells were equilibrated with [32P]orthophosphoric acid in phosphate-free medium, and the radiolabeled cells were treated with NGF (20 ng/ml), Ang II (10^{-8} and 10^{-7} M), and PD123319 (10^{-5} M) at 37 °C for 30 min. Cell lysates were immunoprecipitated with Bcl-2 antibody, analyzed by SDS-PAGE, and autoradiographed. As shown in Fig. 1, A and C, we observed that NGF stimulated the phosphorylation of Bcl-2, whereas Ang II inhibited the NGF-induced Bcl-2 phosphorylation (Fig. 1B). Inhibition of the NGF-mediated phosphorylation of Bcl-2 by Ang II was restored by PD123319, a specific AT2 receptor antagonist, suggesting that this Ang II effect is exerted specifically via the AT2 receptor.

**Regulation of Bcl-2 Phosphorylation by MAP Kinase and MKP-1—**Since the signaling pathway through MAP kinases appears to play a critical role in the survival of PC12W cells (2), we postulated that MAP kinase enhances Bcl-2 phosphorylation and that the AT2 receptor inhibits this. MAP kinase activity is regulated by a dual-specificity phosphatase known as MKP-1 (18), leading us to hypothesize that the AT2 receptor activates MKP-1, inhibits NGF-mediated MAP kinase activation, and results in the inhibition of Bcl-2 phosphorylation.

We applied antisense oligonucleotide to block basal MKP-1 expression in PC12W cells. Due to the short half-life of MKP-1 mRNA and protein, MKP-1 is an ideal target molecule for studies with antisense inhibition. PC12W cells were transfected with 300 nM anti-MKP-1 antisense oligonucleotide (phosphorothioate modified) in Lipofectin (Life Technologies) according to the approach of Duff et al. (14). After transfection, the cells were maintained in the presence of 10% horse serum and 5% fetal bovine serum for 1 day. Then the medium was changed to serum-free medium with or without Ang II. Two days later, cells were harvested and subjected to analysis of DNA fragmentation. Anti-MKP-1 antisense transfected PC12W cells were also used for Bcl-2 phosphorylation, and MAP kinase activity determinations as described above. Oligonucleotide sequences (20 base pairs) are as follows: MKP-1 antisense, 5′-GGAACTCAAGTGGAAGTCAGG-3′; MKP-1 sense, 5′-CCTGAGTTCCACCTGAGTTC-3′.

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observed that MKP-1 antisense oligonucleotide pretreatment blocked AT2 receptor-mediated apoptosis (Fig. 5, A and B). As shown in Fig. 5C, serum deprivation alone induces DNA fragmentation in MKP-1 antisense oligonucleotide treated cells.

**DISCUSSION**

The highly abundant expression of AT2 receptor during embryonic and neonatal growth, the rapid disappearance after birth (19–21), and the up-regulation of AT2 receptor in some diseased states such as in myocardial infarction (22), cardiac hypertrophy (23), and skin wounds (24) suggest that this receptor is closely involved with growth, development, and/or differentiation. Indeed, we have demonstrated that the AT2 receptor is closely involved with growth, development, and/or hypertrophy (23), and skin wounds (24) suggest that this receptor plays an important role in fetal development and in the pathogenesis of some diseases in which apoptosis is involved. However, the molecular and cellular mechanism of AT2 receptor-mediated apoptosis has not been defined.

MAP kinase mediates multiple cellular pathways. In neuronal cells, MAP kinase activity mediates the action of growth factors like epidermal growth factor, which stimulates cellular proliferation, as well as factors like NGF, which maintains neuronal survival and differentiation (28–30). Activation of the p42 and p44 isoforms of MAP kinase requires dual phosphorylation on Thr-183 and Tyr-185 residues (31). It has been suggested that the inactivation of MAP kinase is a critical event regulating the physiological response for cell growth (18). This inactivation is mediated by dephosphorylation of Thr-183 and Tyr-185 residues by a “dual specificity” phosphatase known as MKP-1, which is encoded by the mitogen-inducible gene 3CH134 (18). Recently, an isoform of MKP-1 was isolated from PC12W cells named MKP-2 (32). Cellular response to various stimuli may regulate MAP kinase activity by coordinating the action of the MAP kinase activation cascade (33) and MKPs, resulting in the cell differentiation and survival or cell death. Using MKP-1 antisense strategy, we demonstrated that...
MKP-1 is involved in the inactivation of MAP kinase by the AT2 receptor.

MKP-1 was discovered as an immediate early gene whose rapid transcription and subsequent translation have been suggested to provide a feedback loop to terminate growth factor signaling (18, 33). However, the mechanism of activation of this phosphatase is not known. We previously reported that the dephosphorylation of MAP kinase was observed within 5 min after AT2 receptor stimulation in PC12W cells and that this effect was blocked by sodium vanadate and pertussis toxin (7). In contrast, Duff et al. (34) report that Ang II type 1 receptor stimulation rapidly induced MKP-1 mRNA (30 min maximum) in rat vascular smooth muscle cells. Therefore, we examined MKP-1 mRNA expression in PC12W cells after AT2 receptor stimulation and observed that AT2 receptor stimulation did not increase the MKP-1 mRNA in this cell line (data not shown). Taken together, these results suggest that AT2 receptor stimulation activates MKP-1 phosphatase activity without the apparent induction of MKP-1 expression.

Apoptosis is controlled in part by a family of cytoplasmic proteins, the Bcl-2 family. Bcl-2 can prevent or delay apoptosis induced by a wide variety of stimuli and insults (9). It has been reported that the Bcl-2 protein requires post-translational modification, specifically phosphorylation, to be functionally active (35, 36). Recent data support the notion that phosphorylation of Bcl-2 is important for the regulation of Bcl-2 function and thereby apoptosis (10–12). In this study, we demonstrated that NGF enhanced the phosphorylation of Bcl-2 and that the AT2 receptor inhibited the NGF-mediated Bcl-2 phosphorylation and induced apoptosis.

Our results suggest that NGF-mediated MAP kinase activation is closely linked to increases in the phosphorylation of Bcl-2, resulting in the cell survival signal. MAP kinase is a serine/threonine kinase, and the minimal consensus sequence of substrate specificity of this kinase is the (Ser/Thr)-Pro (37) and Ser/Pro sequence, conserved in murine and human Bcl-2 at the positions 70 and 71 (38, 39). Indeed, the phosphorylation of the serine residue in Bcl-2 has been reported (10–12). These results suggest that MAP kinase phosphorylates the serine residue of Bcl-2. Moreover, we examined the possibility that MKP-1 could directly dephosphorylate the tyrosine residue of Bcl-2, since MKP-1 exerts tyrosine phosphatase activity (18). However, in PC12W cells, we did not observe any effect of NGF and AT2 receptor on tyrosine phosphorylation of Bcl-2.

We also observed that inhibition of MKP-1 expression blocked the AT2 receptor-mediated DNA fragmentation. Based on these results, we propose that MAP kinase plays a critical role in suppressing apoptosis in PC12W cells by phosphorylating and activating Bcl-2. The AT2 receptor inhibits MAP kinase activation by activating MKP-1 and subsequently dephosphorylates Bcl-2, resulting in the development of apoptosis.

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