Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 156–160, January 1996
Cell Biology

Angiotensin II type 2 receptor mediates programmed cell death

TAKEHIKO YAMADA, MASATSUGU HORIUCHI, AND VICTOR J. DZAU*

Falk Cardiovascular Research Center, Stanford University School of Medicine, Stanford, CA 94305-5246

Communicated by Maurice B. Burg, National Institutes of Health, Bethesda, MD, August 28, 1995 (received for review May 1, 1995)

ABSTRACT **The function of the recently discovered angiotensin II type 2 (AT_2) receptor remains elusive. This receptor is expressed abundantly in fetus, but scantily in adult tissues except brain, adrenal medulla, and atretic ovary. In this study, we demonstrated that this receptor mediates programmed cell death (apoptosis). We observed this effect in PC12W cells (rat pheochromocytoma cell line) and R3T3 cells (mouse fibroblast cell line), which express abundant AT_2 receptor but not AT_1 receptor. The cellular mechanism appears to involve the dephosphorylation of mitogen-activated protein kinase (MAP kinase). Vanadate, a protein-tyrosine-phosphatase inhibitor, attenuated the dephosphorylation of MAP kinases by the AT_2 receptor and restored the apoptotic changes. Antisense oligonucleotide to MAP kinase phosphatase 1 inhibited the AT_2 receptor-mediated MAP kinase dephosphorylation and blocked the AT_2 receptor-mediated apoptosis. These results suggest that protein-tyrosine-phosphatase, including MAP kinase phosphatase 1 activated by the AT_2 receptor, is involved in apoptosis. We hypothesize that this apoptotic function of the AT_2 receptor may play an important role in developmental biology and pathophysiology.**

Angiotensin II (Ang II) exerts via the type 1 (AT_1) receptor diverse actions on its target tissues controlling vascular tone, hormone secretion, tissue growth, and neuronal activities (1, 2). However, little is known about physiological function(s) of the type 2 (AT_2) receptor (3, 4) which is abundantly expressed in fetal tissues and immature brain (5) but present only at low levels in certain adult tissues (6–8). Recently, we and others have reported that the AT_2 receptor mediates anti-growth effects on vascular smooth muscle (9) and endothelial (10) cells. To examine further the mechanism of the anti-growth action of the AT_2 receptor, we studied its effect on programmed cell death (apoptosis). We also elucidated the biochemical signal transduction mechanism of the AT_2 receptor mediating apoptosis.

We first studied PC12W cells, a subline of the PC12 rat pheochromocytoma cell line (11), which express high levels of the AT_2 but not the AT_1 receptor. These cells differentiate into neuronal-like cells in the presence of nerve growth factor (NGF) and provide a good model for investigating programmed neural cell death (12). Second we studied R3T3 cells, a mouse fibroblast cell line whose expression of AT_2 receptor is modulated by the growth state of the cells—i.e., very low in actively growing cells but increasing markedly in the confluent, quiescent state (13). This cell line is a good model for studying the growth-regulatory function of AT_2 receptor.

MATERIALS AND METHODS

Cells and Treatment. PC12W cells were grown and underwent differentiation in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) plus 10% horse serum, 5% fetal bovine serum (FBS), and NGF (10 ng/ml). R3T3 cells were maintained in DMEM plus 10% FBS.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

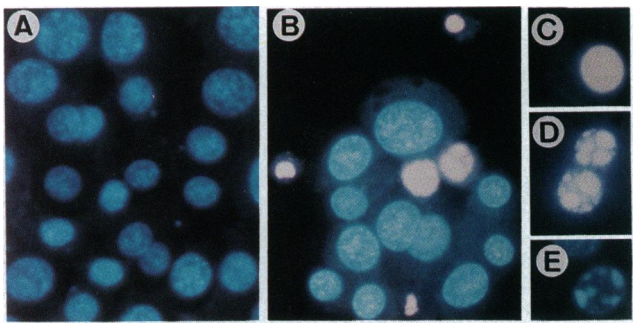

FIG. 1. Ang II-enhanced apoptosis in PC12W cells. Hoechst 33342 (blue) stains chromatin in cells with an intact cell membrane and propidium iodide (pink) stains chromatin in cells without cell membrane integrity. After cells differentiated in medium containing 10% horse serum, 5% FBS, and NGF at 10 ng/ml for 1 week, the medium was exchanged with serum-free medium containing NGF at 1 ng/ml for 2 days. No apoptotic changes were observed in this condition *(A)*. Morphological apoptotic changes appeared 2 days after addition of Ang II (0.1 μM), showing nuclear condensation *(C)*, fragmentation *(D)*, and margination *(E)*. (×400.)

Staining with Chromatin-Binding Dyes. PC12W cells were seeded into six-well plates (Falcon) at 2.0×10^5 cells per well. The chromatin binding dyes Hoechst 33342 and propidium iodide (Molecular Probes) were added to the serum-free medium at 5 μM and 0.1 μM, respectively, to examine the morphological changes of nuclei. After incubation at 37°C for 1 hr, cells were collected by centrifugation, suspended in phosphate-buffered saline (PBS), and were viewed by UV microscopy.

Internucleosomal DNA Fragmentation (DNA Ladder). Cells were seeded into T-75 flasks (Falcon) at 1.0×10^6 cells per flask. DNA was extracted from these cells for 3′ end labeling, gel electrophoresis, and quantitation of DNA fragmentation (14). The amount of [α-^{32}P]ddATP (Amersham) incorporated into low molecular weight (<20-kb) DNA fractions was quantitated by cutting the respective fraction of DNA from the dried gel for scintillation counting.

Receptor Binding Assay. The assay was performed as described (3).

Mitogen-Activated Protein Kinase (MAP Kinase) Activity. Serum-starved (1 day) PC12W or R3T3 cells were stimulated by NGF (1 ng/ml) or serum (10% FBS), respectively, for 15 min with or without Ang II. The cells were then lysed and centrifuged. The supernatant was assayed for MAP kinase activity with myelin basic protein as substrate (15).

MAP Kinase Dephosphorylation. ^{32}P-labeled MAP kinase (activated form) was prepared from lysates of one day serum-starved (1 day) PC12W cells (16). Then ^{32}P-labeled MAP

Abbreviations: Ang II, angiotensin II; AT_1 receptor, Ang II type 1 receptor; AT_2 receptor, Ang II type 2 receptor; FBS, fetal bovine serum; MAP kinase, mitogen-activated protein kinase; MKP-1, MAP kinase phosphatase 1; NGF, nerve growth factor; PTPase, protein-tyrosine-phosphatase; PTX, pertussis toxin.

*To whom reprint requests should be addressed at: Falk Cardiovascular Research Center, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305-5246.

kinase was immunoprecipitated and incubated in dephosphorylation buffer (40 mM Hepes, pH 7.5/0.01% bovine serum albumin/2 mM dithiothreitol/1 μM okadaic acid) for 1 hr with lysates prepared from Ang II-treated PC12W cells (100 μg of protein). The released $^{32}P_i$ from immunoprecipitated ^{32}P-labeled MAP kinase was quantified by scintillation counting.

MKP-1 Antisense Oligonucleotide Transfection. PC12W cells were transfected with 300 nM/MKP-1 antisense or sense oligonucleotide (phosphorothioate-modified) in Lipofectin (Life Technologies, Grand Island, NY) according to Duff *et al* (17). Oligonucleotide sequences (20 nt) were as follows (17): MKP-1 antisense, 5′-GGAACTCAGTGGAACTCAGG-3′; MKP-1 sense, 5′-CCTGAGTTCCACTGAGTTCC-3′. The cells were maintained in the presence of serum and NGF (10 ng/ml) for 2 days. Then the medium was changed to serum-free medium containing NGF (1 ng/ml) with or without Ang II. Two days later, cells were harvested and DNA fragmentation was studied. MKP-1 antisense-transfected PC12W cells were also used for measurement of MAP kinase activity as described above.

Statistics. Statistical analysis was performed by ANOVA, followed by Scheffe's test. $P < 0.05$ was considered significant. Data are expressed as mean ± SE.

RESULTS AND DISCUSSION

Upon serum or NGF starvation, PC12W cells underwent morphological changes typical of apoptosis, in a manner similar to that reported by Pittman *et al.* (12) for PC12 cells.

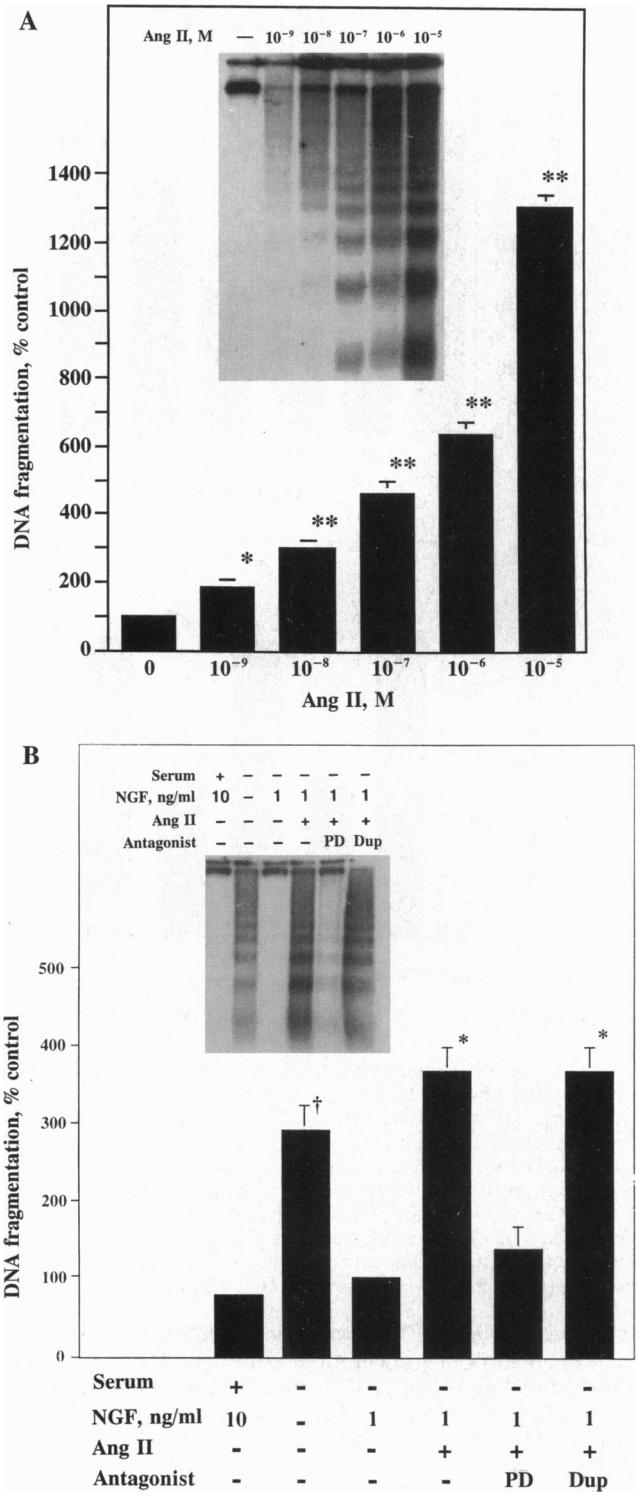

FIG. 2. Quantitative analysis of internucleosomal DNA fragmentation. *(A)* When PC12W cells were cultured without serum but in the presence of NGF at 1 ng/ml, DNA fragmentation appeared in a dose-dependent manner, shown at 48 hr after Ang II addition. * and **, $P < 0.01$ and $P < 0.001$ vs. no Ang II, respectively (mean ± SE, $n = 3$ for each condition). *(B)* Ang II (0.1 μM)-induced DNA fragmentation was completely blocked by PD123319 (PD, 10 μM), an AT_2 receptor antagonist, but not by Dup753 (Dup, 10 μM), an AT_1 receptor antagonist. *, $P < 0.001$ vs. control (NGF at 1 ng/ml); †, $P < 0.01$ vs. (serum plus NGF at 10 ng/ml (mean ± SE, $n = 6$ for each condition). *Insets* show representative autoradiography of DNA laddering.

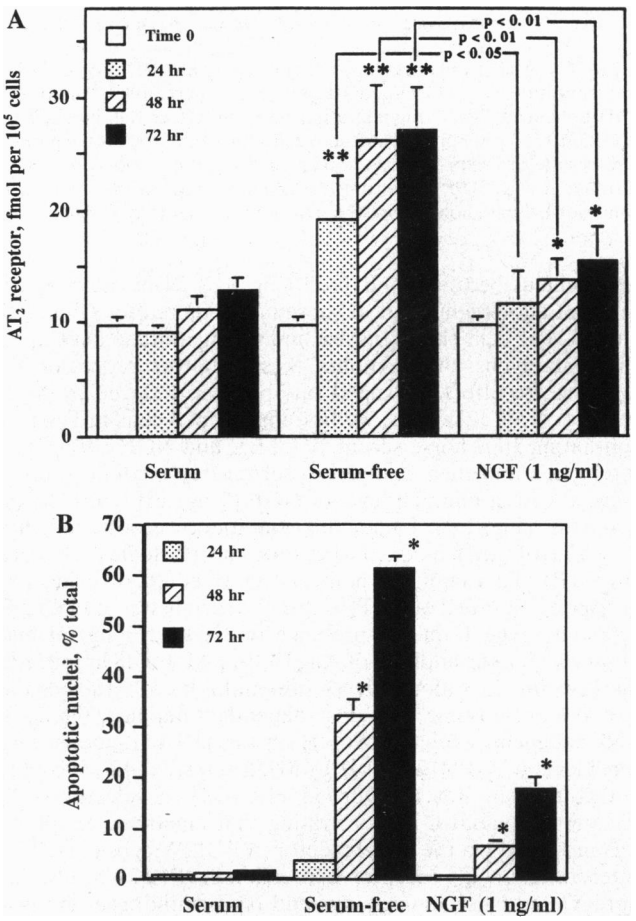

FIG. 3. *(A)* Upregulation of AT_2 receptor levels in PC12W cells when serum was withdrawn. This effect was attenuated when cells were maintained in the presence of NGF at 1 ng/ml. * and **, $P < 0.05$ and $P < 0.001$ vs. time 0, respectively (mean ± SE, $n = 5$ for each condition). *(B)* Time course of percent apoptotic nuclei in PC12W cells after serum withdrawal *, $P < 0.001$ vs. 24 hr (mean ± SE, $n = 6$ for each condition).

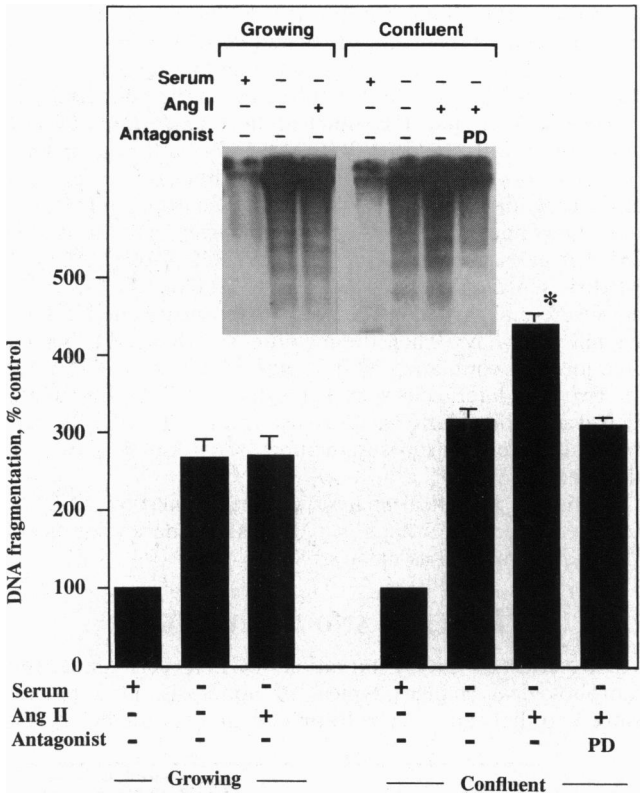

FIG. 4. Ang II-enhanced apoptosis in confluent R3T3 cells. DNA fragmentation was determined 3 days after serum removal. Ang II (0.1 μM) enhanced DNA fragmentation and this effect was blocked by PD123319 (10 μM) in the confluent cells, but not in the growing cells. The results are expressed as percent control (i.e., cells exposed to serum). *, $P < 0.05$ vs. serum-free (mean ± SE, $n = 4$ for each condition). *Inset* shows representative autoradiography of DNA laddering.

The nucleus became condensed (Fig. 1*C*) 24 hr after serum removal and fragmented or marginated thereafter (Fig. 1 *D* and *E*). The cells shrank in size and the membrane developed ruffle and blebs. NGF inhibited these apoptotic processes. To evaluate the effect of Ang II on apoptosis induced by NGF removal in PC12W cells, we first maintained cells in DMEM containing 10% horse serum, 5% FBS, and NGF (10 ng/ml) for 1 week and then changed to serum-free medium supplemented with a minimal level of NGF (1 ng/ml) that blocked apoptosis (Fig. 1*A*). Under this condition, we observed that Ang II (0.1 μM) induced apoptotic morphological changes (Fig. 1*B*). To examine whether Ang II could override the antiapoptotic effect of NGF, we treated serum-starved PC12W cells with Ang II in the presence of NGF at 1 ng/ml and observed that the addition of Ang II (0.1 μM) for 48 hr induced marked fragmentation of DNA into multiples of ≈180 bp (the size of a nucleosome) in a dose-dependent manner (Fig. 2*A*). This enhancing effect of Ang II on the DNA fragmentation was blocked by PD123319 (10 μM), a selective AT_2 receptor antagonist but not by Dup753 (10 μM), a selective AT_1 receptor antagonist, demonstrating that enhanced apoptosis was mediated via the AT_2 receptor in PC12W cells (Fig. 2*B*). Interestingly, AT_2 receptor expression in PC12W cells was upregulated by serum removal and NGF withdrawal but was downregulated by the addition of NGF (Fig. 3*A*), suggesting that AT_2 receptor expression was closely related to the apoptotic events. The fraction of cells undergoing apoptosis is shown in Fig. 3*B*.

To examine whether this effect could be observed in other cells, we studied the mouse fibroblast cell line R3T3, which expresses the AT_2 receptor in the confluent stage, but not in

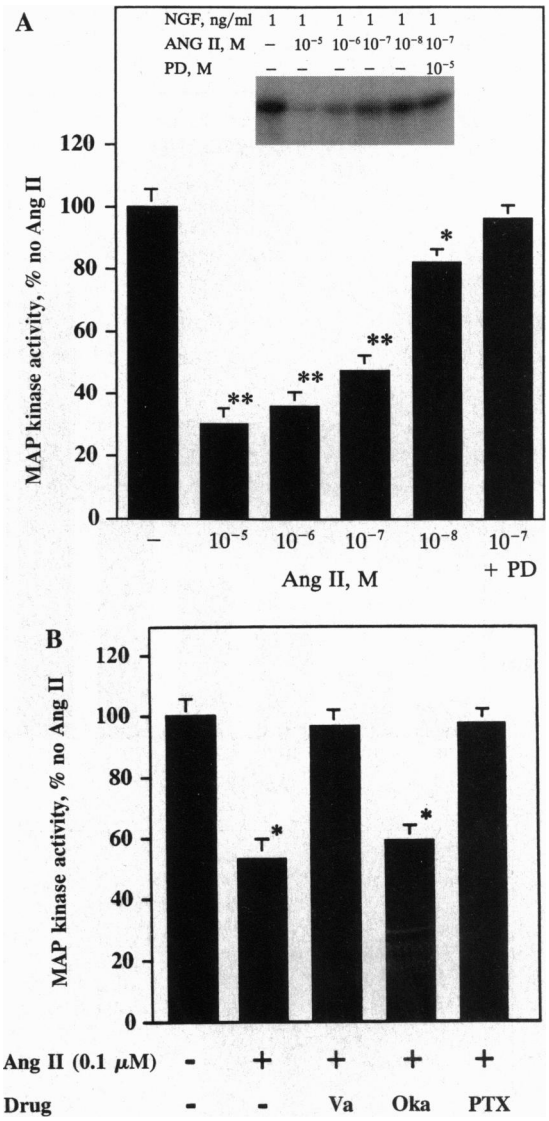

FIG. 5. *(A)* NGF-stimulated MAP kinase activity in PC12W cells was attenuated by Ang II in a dose-dependent manner (15 min after treatment) and this effect of Ang II (0.1 μM) was abolished by pretreatment of cells with PD123319 (PD, 0.1 μM). * and **, $P < 0.05$ and $P < 0.01$ vs. no drug, respectively (mean ± SE, $n = 4$ for each condition). *Inset* shows representative autoradiography of phosphorylated MBP. *(B)* Vanadate (Va, 0.1 μM) and pertussis toxin (PTX, 200 ng/ml) also blocked the AT_2 receptor-mediated inhibition of MAP kinase activity, but okadaic acid (Oka, 0.1 μM) did not. *, $P < 0.01$ vs. no drug (mean ± SE, $n = 4$ for each condition).

the growing phase (13). Serum starvation caused DNA fragmentation in both growing and confluent R3T3 cells. The addition of Ang II (0.1 μM) further enhanced DNA fragmentation in the confluent R3T3 cells, but not in the growing cells (Fig. 4). This effect was blocked by PD123319 (10 μM). Serum depletion induced AT_2 receptor expression in confluent R3T3 cells but not in growing R3T3 cells (data not shown).

The intracellular signal transduction mechanism that follows the activation of AT_2 receptor is not well defined. Activation of the Ras and MAP kinase pathway has been reported to be necessary and sufficient for cell differentiation or proliferation in PC12 cells in response to NGF (18). We focused on the effect of AT_2 receptor on NGF-stimulated MAP kinase activity. NGF stimulated the MAP kinase activity of PC12W cells in a dose-dependent fashion. This was attenuated by Ang II in a dose-dependent manner and this effect of Ang II (0.1 μM) was abolished by PD123319 (10 μM) (Fig. 5*A*). Vanadate, [a

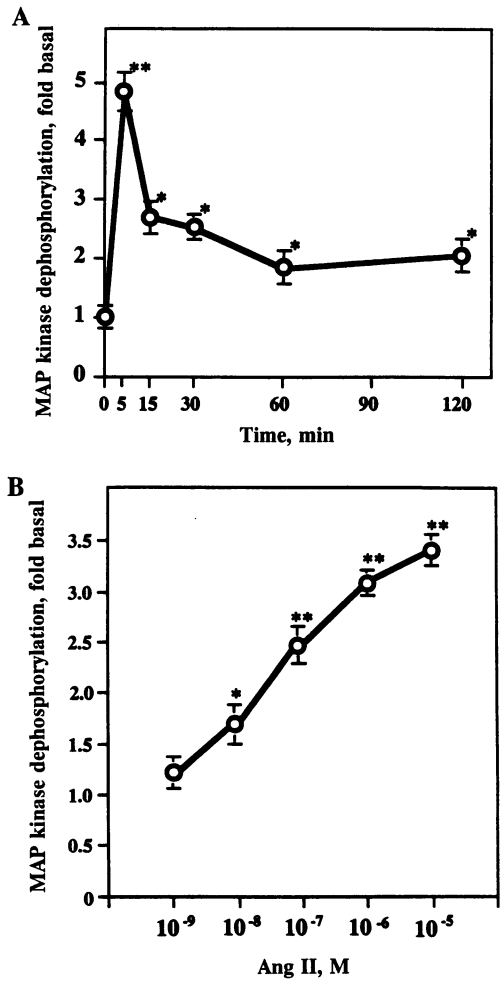

FIG. 6. Dephosphorylation of NGF-stimulated MAP kinase in PC12W cells. Ang II induced dephosphorylation of MAP kinase in a time *(A)* and dose *(B)*-dependent manner (mean ± SE, $n = 3$ for each condition).

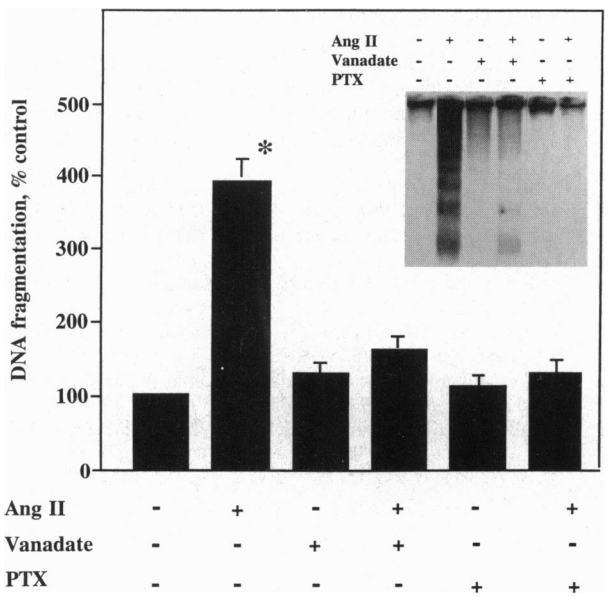

FIG. 7. Effects of vanadate and PTX on AT_2 receptor-mediated apoptosis in PC12W cells. Results are expressed as percent control (i.e. cells exposed to no drugs and no Ang II). Both vanadate and PTX inhibited AT_2 receptor-mediated apoptosis in PC12W cells *, $P < 0.005$ vs. control (mean ± SE, $n = 3$ for each condition). *Inset* shows representative autoradiography of DNA laddering.

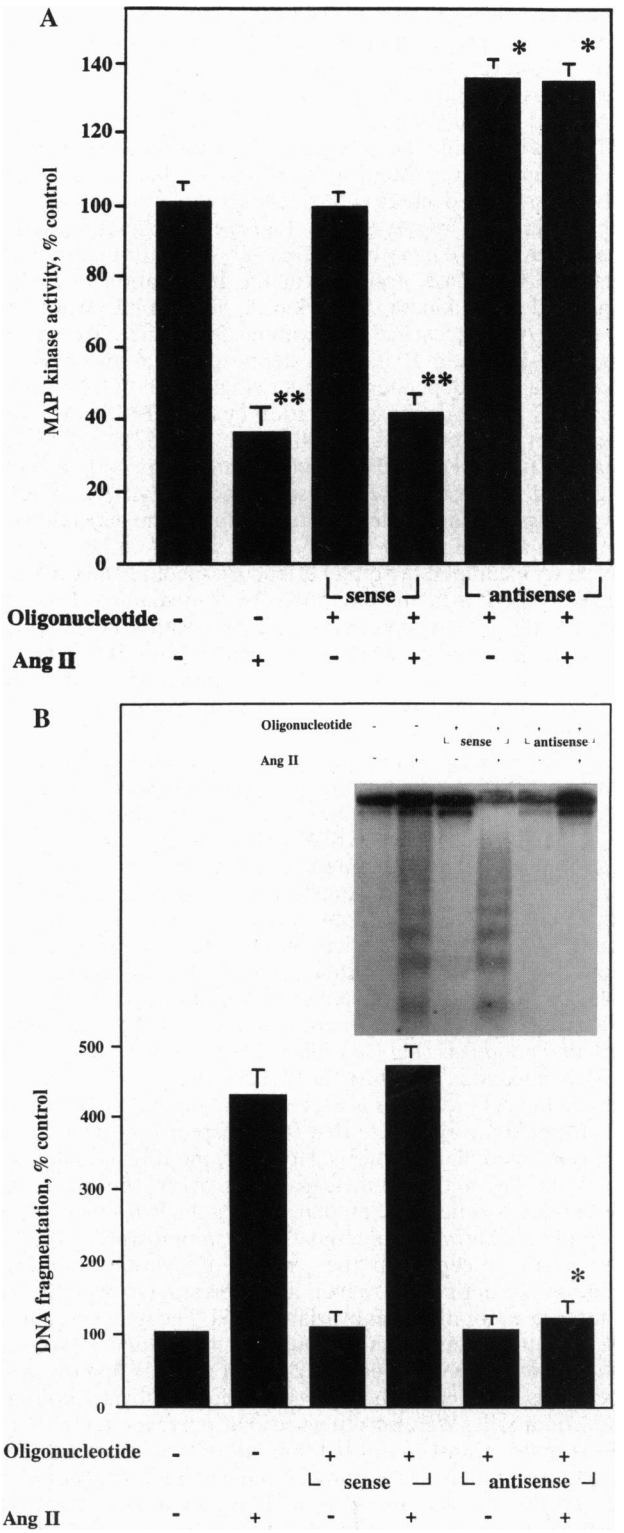

FIG. 8. Effect of MKP-1 antisense oligonucleotide transfection on MAP kinase activity *(A)* and apoptosis *(B)* in PC12W cells. *(A)* MKP-1 antisense oligonucleotide transfection increased the NGF-stimulated MAP kinase activity and blocked the inactivation by Ang II (0.1 μM) in PC12W cells. * and **, $P < 0.05$ and $P < 0.01$, respectively, vs. control (no oligonucleotide transfection and no Ang II treatment) (mean ± SE, $n = 3$ for each condition). *(B)* MKP-1 antisense oligonucleotide-transfected cells did not show any enhancement of apoptosis by Ang II. Results are expressed as percent control (i.e., cells exposed to no oligonucleotide and no Ang II). *, $P < 0.001$ vs. sense oligonucleotide-transfected and Ang II-treated cells (mean ± SE, $n = 3$ for each condition). *Inset* shows representative autoradiography of DNA laddering.

protein-tyrosine-phosphatase (PTPase) inhibitor, 10 μM] and PTX (a G_i/G_o protein inhibitor, 200 ng/ml) also abolished this effect. However, okadaic acid (a protein-serine/threonine-phosphatase inhibitor, 0.1 μM) did not (Fig. 5*B*). Ang II also attenuated serum-stimulated MAP kinase in the confluent R3T3 cells but not in the growing R3T3 cells (data not shown).

MAP kinases are protein-serine/threonine kinases and their activity is rapidly decreased by dephosphorylation of Tyr-185, emphasizing the importance of PTPase in regulating MAP kinase activity. We hypothesize that AT_2 receptor dephosphorylates MAP kinase, resulting in the inactivation of NGF-stimulated MAP kinase. MAP kinase dephosphorylation induced by Ang II reached a maximum 5 min after treatment (Fig. 6*A*) and Ang II induced dephosphorylation of MAP kinase in a dose-dependent manner (Fig. 6*B*) in PC12W cells. The MAP kinase dephosphorylation by Ang II (0.1 μM) was blocked by pretreatment of cells with PD123319 (10 μM), vanadate (10 μM), or PTX (200 ng/ml) (data not shown), suggesting that this MAP kinase dephosphorylation is AT_2 receptor-specific and at least partially due to the activation of G protein and PTPase.

Next we examined the direct effect of vanadate and PTX on the AT_2 receptor-mediated apoptosis. Vanadate or PTX attenuated the AT_2 receptor-mediated apoptotic changes (Fig. 7). However, okadaic acid did not show any effect. These results demonstrate that a G-protein coupling mechanism participates in the induction of AT_2 receptor-mediated apoptosis in PC12W cells.

A PTPase, MAP kinase phosphatase 1 (MKP-1), has been shown to selectively dephosphorylate tyrosine-phosphorylated MAP kinase (19) and is known to be involved in cell growth (17, 19). We transfected PC12W cells with antisense oligonucleotide against MKP-1 mRNA according to Duff *et al.* (17) and observed that this transfection increased the NGF-stimulated MAP kinase activity and blocked the Ang II-induced MAP kinase inactivation (Fig. 8*A*). Sense oligonucleotide-transfected or vehicle-transfected PC12W cells exhibited marked apoptosis in response to Ang II (0.1 μM), whereas antisense oligonucleotide treatment blocked AT_2 receptor-mediated apoptosis (Fig. 8*B*). Thus, MKP-1 is involved in AT_2 receptor-mediated apoptosis in PC12W cells.

Since the AT_2 receptor is expressed in adrenal medulla (6), it is intriguing to speculate that this receptor may play a role in adrenal medullar apoptosis. However, the role of apoptosis in adrenal physiology is unclear. In the ovary, the granulosa cells of the atretic follicles that express high levels of AT_2 receptors are known to undergo marked apoptosis (8, 14). The role of AT_2 receptor in the process of ovarian apoptosis remains to be defined. However, it is interesting to hypothesize that this receptor mediates ovarian atresia. The reexpression of AT_2 receptor after vascular injury or myocardial infarction (20) and in skin wound healing (21) also suggests that the AT_2 receptor is involved in the pathophysiology of these proliferative conditions. We also observed that overexpression of an AT_2 receptor transgene in balloon-injured rat carotid artery reduced neointimal formation (9). Indeed, a recent study also suggests that the AT_2 receptor mediates an antigrowth effect in cultured endothelial cells (10). Together, these data demonstrate that the AT_2 receptor exerts a growth-inhibitory effect and that one of the antigrowth mechanisms is apoptosis.

We thank M. de Gasparo for providing CGP42112A (CIBA–Geigy), D. Taylor for PD123319 (Parke–Davis), A. Chiu for Dup753 (DuPont), K. H. Kim and R. C. Speth for PC12W cells (Washington State University), and D. Dudley for R3T3 cells (Parke–Davis). We thank S. F. Hsu, P. Tsao, M. Pollman, and G. H. Gibbons for comments and technical advice and M. Hing for secretarial assistance. This work was supported by National Institutes of Health Grants HL46631, HL35252, HL35610, HL48638, and HL07708, the American Heart Association Bugher Foundation Center Grant for Molecular Biology in the Cardiovascular System, and a grant from CIBA–Geigy (Basel, Switzerland). V.J.D. is the recipient of National Institutes of Health Merit Award HL35610.

1. Dzau, V. J., Gibbons, G. H. & Pratt, R. E. (1991) *Hypertension* **18,** Suppl. II, II-100–II-105.
2. Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J., Hasegawa, M., Matsuda, Y. & Inagami, T. (1991) *Nature (London)* **351,** 230–233.
3. Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R. E. & Dzau, V. J. (1993) *J. Biol. Chem.* **268,** 24539–24542.
4. Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T. & Inagami, T. (1993) *J. Biol. Chem.* **268,** 24543–24546.
5. Grady, E. F., Sechi, L. A., Griffin, C. A., Schambelan, M. & Kalinyak, J. E. (1991) *J. Clin. Invest.* **88,** 921–933.
6. Chiu, A. T., Herblin, W. F., McCall, D. E., Ardecky, R. J., Carini, D. J., Duncia, J. V., Pease, L. J., Wong, P. C., Wexler, R. R., Johnson, A. L. & Timmermans, P. B. (1989) *Biochem. Biophys. Res. Commun.* **165,** 196–203.
7. Millan, M. A., Jacobowitz, D. M., Aguilera G. & Catt, K. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11440–11444.
8. Pucell, A. G., Hodges, J. C., Sen, I., Bumpus, F. M. & Husain, A. (1991) *Endocrinology* **128,** 1947–1959.
9. Nakajima, M., Hutchinson, H. G., Fujinaga, M., Hayashida, W., Morishita, R., Zhang, L., Horiuchi, M., Pratt, R. E. & Dzau, V. J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10663–10667.
10. Stoll, M., Steckelings, U. M., Paul, M., Bottari, S. P., Metzger, R. & Unger, T. (1995) *J. Clin. Invest.* **95,** 651–657.
11. Speth, R. C. & Kim, K. H. (1990) *Biochem. Biophys. Res. Commun.* **169,** 997–1006.
12. Pittman, R. N., Wang, S., DiBenedetto, A. J. & Mills, J. C. (1993) *J. Neurosci.* **13,** 3669–3680.
13. Dudley, D. T. & Summerfelt, R. M. (1993) *Regul. Pept.* **44,** 199–206.
14. Tilly, J. L. & Hsueh, A. J. W. (1993) *J. Cell Physiol.* **154,** 519–526.
15. Tobe, K., Kadowaki, T., Tamemoto, H., Ueki, K., Hara, K., Koshio, O., Momomura, K., Gotoh, Y., Nishida, E., Akanuma, Y., Yazaki, Y. & Kasuga, M. (1991) *J. Biol. Chem.* **266,** 24793–24803.
16. Shibuya, E. K., Polverino, A. J., Chang, E., Wigler, M. & Ruderman, J V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9831–9835.
17. Duff, J. L., Monia, B. P. & Berk, B. C. (1995) *J. Biol. Chem.* **270,** 7161–7166.
18. Cowley, S., Paterson, H., Kemp, P. & Marshall, C. J. (1994) *Cell* **77,** 841–852.
19. Charles, C. H., Sun, H., Lau, L. F. & Tonks, N. K. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5292–5296.
20. Nio, Y., Matsubara, H., Murasawa, S., Kanasaki, M. & Inada, M. (1995) *J. Clin. Invest.* **95,** 651–657.
21. Viswanathan, M. & Saavedra, J. M. (1992) *Peptides* **13,** 783–786.