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Cyclooxygenase inhibition reveals synergistic action of vasoconstrictors on mesangial cell growth

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Abstract

Since endogenous vasoconstrictors promote mesangial cell growth and increase the biosynthesis of antiproliferative prostaglandins, the effects of cyclooxygenase inhibition on mesangial cell proliferation should be strongly dependent on the prevailing levels of neuroendocrine vasoconstrictors. We compared the effects of indomethacin (10^{-6} M), a cyclooxygenase inhibitor, on [^{3}H]thymidine incorporation by cultured rat mesangial cells in the presence of various combinations of angiotensin II (10^{-10} M), [Arg^8]vasopressin (10^{-11} M), (−)-norepinephrine (10^{-8} M) and endothelin-1 (10^{-11} M). Indomethacin did not enhance [^{3}H]thymidine incorporation in cells treated with each individual vasoconstrictor, or in cells treated with two-way combinations with the exception of modestly increased [^{3}H]thymidine incorporation in cells treated with angiotensin II + (−)-norepinephrine or [Arg^8]vasopressin + (−)-norepinephrine. In contrast, in cells treated with any three-way or the four-way combination, indomethacin markedly increased [^{3}H]thymidine incorporation. Importantly, a highly significant interaction ($P < 0.0001$) was observed for thymidine incorporation between the number of vasoconstrictors present and indomethacin treatment, thus demonstrating that cyclooxygenase inhibition reveals a synergistic action of vasoconstrictors on the DNA synthesis in mesangial cells.

Keywords: Neuroendocrine vasoconstrictor; NSAID (nonsteroidal anti-inflammatory drug); Cyclooxygenase inhibition; [^{3}H]Thymidine incorporation; Mesangial cell

1. Introduction

Several clinical conditions characterized by ineffective circulatory blood volume, e.g., advanced liver cirrhosis and congestive heart failure, are well-known to be associated with elevated circulating levels of neuroendocrine vasoconstrictors, i.e., norepinephrine, angiotensin II, arginine-vasopressin and endothelin-1 (Asbert et al., 1993; Seino et al., 1993; Tsai et al., 1995; Parmley, 1995). In such patients, the increased synthesis of renal vasodilatory prostaglandins, i.e., prostaglandin I_2 and prostaglandin E_2, counteracts the renovascular effects of neuroendocrine vasoconstrictors to maintain normal renal function. Thus, in patients with advanced liver cirrhosis or congestive heart failure, cyclooxygenase inhibition by nonsteroidal anti-inflammatory drugs (NSAIDs) reduces renal blood flow and glomerular filtration rate (Arroyo et al., 1986; Cannon, 1986; Zipser, 1986).

Neuroendocrine vasoconstrictors are also well-known to stimulate mesangial cell proliferation and extracellular matrix synthesis in cell culture studies (Simonson et al., 1989; Bakris et al., 1991; Wolf et al., 1992), and may contribute to the progression of glomerular lesions (Marsen et al., 1994; El Nahas, 1995). In contrast, vasodilatory prostaglandins potently inhibit mesangial cell proliferation in cell culture studies (Menè et al., 1990a; Menè and Dunn, 1990b). Thus, in patients with advanced liver cirrhosis or congestive heart failure, endogenous vasodilatory/growth-inhibitory prostaglandins may oppose not only renal vasoconstriction but also the progression of glomerular lesions.

Several clinical studies demonstrated a greater incidence of a wide variety of glomerular lesions in patients with liver cirrhosis, including minor glomerular abnormalities, immunoglobulin A and membranous nephropathy,

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endocapillary, mesangiocapillary and mesangial proliferative glomerulonephritis, and hepatic glomerulosclerosis (Fukuda, 1982; Montoliu et al., 1986; Axelsen et al., 1995). However, in spite of chronic activation of neuroendocrine vasoconstrictors, proliferative changes of mesangial cells, i.e., mesangial hypercellularity and/or the expansion of extracellular matrix, is not always the dominant abnormality of glomerular lesions associated with liver cirrhosis (Fukuda, 1982; Montoliu et al., 1986; Axelsen et al., 1995). Although numerous factors may contribute to glomerular lesions associated with liver cirrhosis (e.g., infections, nephrotoxic agents, acute hemodynamic changes and neoplasms), these clinical findings suggest that endogenous vasodilatory/growth-inhibitory prostaglandins effectively inhibit neuroendocrine vasoconstrictor-induced progression of glomerular lesions in these patients.

Norepinephrine, angiotensin II, [Arg^8]-vasopressin and endothelin-1 are well-known to stimulate the synthesis of prostaglandin E_2 in cultured mesangial cells or glomeruli (Scharschmidt and Dunn, 1983; Matsumura et al., 1986; Fukunaga et al., 1991), and the increased prostaglandin E_2 synthesis could importantly attenuate mesangial cell proliferation stimulated by these neuroendocrine vasoconstrictors in an autocrine and paracrine manner. Thus, it is possible that the effect of cyclooxygenase inhibition by NSAIDs on mesangial cell proliferation is strongly augmented by the milieu of neuroendocrine vasoconstrictors in patients with advanced liver cirrhosis or congestive heart failure. However, despite the fact that NSAIDs are one of the most popular non-prescription drugs, this possibility has not been tested. Therefore, the goal of the present study was to determine whether the ability of indomethacin, a potent NSAID, to promote mesangial cell proliferation is strongly dependent on the neuroendocrine environment. In this regard, we compared the ability of indomethacin to increase [^{3}H]thymidine incorporation (a measure of DNA synthesis) in cultured rat mesangial cells in the presence of various combinations of pathophysiologically relevant concentrations of angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium nutrient mixture F-12; 1:1 mixture (DMEM/F12), Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate-buffered saline (D-PBS), RPMI1640 medium, 1 M HEPES buffer solution, 7.5% sodium bicarbonate solution, penicillin–streptomycin solution and 0.25% trypsin–EDTA solution were purchased from Gibco (Grand Island, NY). Fetal calf serum was purchased from HyClone Laboratories, (Logan, UT). All tissue culture ware were purchased from Fisher Scientific (Pittsburgh, PA). Angiotensin II acetate salt, (−)-norepinephrine bitartrate salt, [Arg^8]vasopressin acetate salt, endothelin-1, indomethacin and type IV collagenase were purchased from Sigma (St. Louis, MO). [Methyl-^{3}H]thymidine (20.0 Ci/mmol) was purchased from NEN Life Science (Boston, MA).

2.2. Rat glomerular mesangial cell culture

Kidneys were obtained from 70 to 100 g male Sprague–Dawley rats (Charles River, Wilmington, MA) that had been anesthetized with ether, and glomeruli were isolated from renal cortical tissue using a previously described sieving method (Kreisberg and Karnovsky, 1983) with stainless steel screens of three different pore sizes. The isolated glomeruli were incubated in D-PBS containing 0.5 mg/ml collagenase at 37°C for 20 min. After washing several times in D-PBS, the glomeruli were resuspended in 10 ml RPMI1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), $NaHCO_3$ (2200 mg/l), HEPES (25 mM) and 20% fetal calf serum, plated in 75 cm^2 tissue culture flasks, and incubated under standard tissue culture conditions (37°C, 5% CO_2/95% air and 98% humidity). After 2 to 3 days, glomerular epithelial cells started to grow and were numerically dominant for another 5 to 7 days. After this period the epithelial cells deteriorated for several days and mesangial cells became the prevailing cell type and grew to confluence within 4 to 5 weeks. The identification of subcultured mesangial cells was determined by morphology (stellate or spindle shaped cells that grew in a swirl-like fashion) using phase-contrast microscopy and by the presence of myosin and desmin filaments by immunofluorescent staining. Subcultured mesangial cells were used for concentration–response (7th and 8th passages) and combination (7th passage) experiments.

2.3. Concentration–response study

Mesangial cells were plated at a density of 4×10^3 cells/well in 24-well tissue culture plates and allowed to grow for 24 h in complete culture medium (DMEM/F12 supplemented with $NaHCO_3$ [2200 mg/l] and HEPES [25 mM]) containing 10% fetal calf serum under standard tissue culture conditions. The subconfluent cells were washed twice with D-PBS and growth-arrested by incubating with thymidine-free culture medium (DMEM supplemented with $NaHCO_3$ [3700 mg/l] and HEPES [25 mM]) containing 0.25% fetal calf serum for 48 h. The growth-arrested cells were treated for 20 h with 500 μl of the thymidine-free culture medium containing 0.5% fetal calf serum and either angiotensin II (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M), [Arg^8]vasopressin (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M), (−)-norepinephrine (10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M) or endothelin-1 (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M). Four wells were used for each concentration of each neuroendocrine vasoconstrictor and for control. After

20 h of incubation, cells were pulsed with [methyl-^{3}H]thymidine (1 μCi/ml) for an additional 4 h. After this, the cells were washed twice with 1 ml of ice-cold D-PBS to terminate the experiment and treated with 500 μl of 20% trichloroacetic acid at 4°C for 60 min. After washing once with 1 ml of D-PBS, the precipitates were dissolved at 80°C in 500 μl of 0.3 N NaOH and 0.1% sodium dodecyl sulfate solution. All wells were counted using a liquid scintillation counter. Each experiment was repeated four times.

2.4. Combination study

Growth-arrested cells in 24-well tissue culture plates were obtained as described for the concentration–response study (see above). The growth-arrested cells were treated for 20 h in 500 μl of thymidine-free culture medium containing 0.5% fetal calf serum with all possible one-, two-, three- and four-way combinations of 10^{-10} M angiotensin II, 10^{-11} M [Arg^8]vasopressin, 10^{-8} M (−)-norepinephrine and 10^{-11} M endothelin-1, in the absence and presence of 10^{-6} M indomethacin. Four wells were used for each combination in the absence and presence of indomethacin. The concentrations of neuroendocrine vasoconstrictors used in this combination study were selected based on the results of the concentration–response study (see above) and plasma concentrations in patients with liver cirrhosis or congestive heart failure reported previously (Asbert et al., 1993; Seino et al., 1993; Tsai et al., 1995; Parmley, 1995). In this regard, for each neuroendocrine vasoconstrictor, the lowest concentration that significantly ($P < 0.01$) increased [^{3}H]thymidine incorporation was employed. After 20 h of the incubation, [^{3}H]thymidine incorporation was determined as described for the concentration–response study (see above). The combination experiment was repeated six times.

2.5. Analysis of data

Data are summarized as mean ± S.E.M. In the concentration–response study, the values of [^{3}H]thymidine incorporation in treatment wells were compared with those in control wells using a one-factor analysis of variance (ANOVA) followed by a Fisher's Protected Least Significant Difference test (Fisher's PLSD). In the combination study, a Student's *t*-test was used to analyze the effect of indomethacin on [^{3}H]thymidine incorporation at each combination of factors, and a Kruskal–Wallis *H*-test, followed by Dunn's test, was used to determine whether neuroendocrine vasoconstrictors exerted a different effect on [^{3}H]thymidine incorporation depending on the total number of vasoconstrictors present. A two-factor ANOVA was used to analyze the interaction between the indomethacin treatment and the numbers of neuroendocrine vasoconstrictors present. Statistical significance was defined as $P < 0.05$.

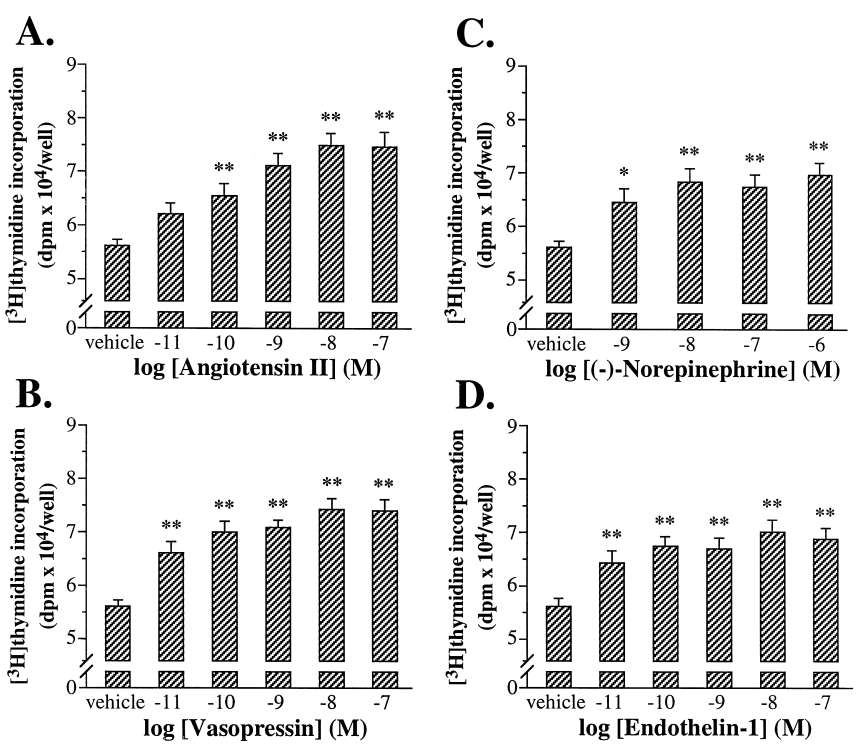

Fig. 1. [^{3}H]thymidine incorporation into mesangial cells after 24 h of incubation with angiotensin II (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M) (A), [Arg^8]vasopressin (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M) (B), (−)-norepinephrine (10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M) (C) or endothelin-1 (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} or 10^{-7} M) (D). Data are mean ± S.E.M. for 16 wells in four experiments. * $P < 0.05$, ** $P < 0.01$ compared with control (Fisher's PLSD).

3. Results

3.1. Concentration–response study

Angiotensin II caused a concentration-dependent increase in [^{3}H]thymidine incorporation (Fig. 1A), and [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1 showed significant increases at all concentrations (Fig. 1B–D). The lowest concentrations that significantly ($P < 0.01$) increased [^{3}H]thymidine incorporation were 10^{-10} M for angiotensin II, 10^{-11} M for [Arg^8]vasopressin, 10^{-8} M for (−)-norepinephrine and 10^{-11} M for endothelin-1, and these concentrations were employed in the combination study.

3.2. Combination study

As shown in Fig. 2A, indomethacin did not affect [^{3}H]thymidine incorporation in mesangial cells treated with a low concentration of either angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine or endothelin-1. Indomethacin significantly ($P < 0.01$), albeit modestly, increased [^{3}H]thymidine incorporation when mesangial cells were incubated with angiotensin II + (−)-norepinephrine (5952 dpm/well) and [Arg^8]vasopressin + (−)-norepinephrine (5294 dpm/well) (Fig. 2B). However, indomethacin did not significantly increase [^{3}H]thymidine incorporation when mesangial cells were incubated with angiotensin II + [Arg^8]vasopressin, angiotensin II + endothelin-1, [Arg^8]vasopressin + endothelin-1 or (−)-norepinephrine + endothelin-1 (Fig. 2B). In contrast, indomethacin caused significant ($P < 0.01$) increases in [^{3}H]thymidine incorporation in the presence of all four possible three-way combinations of the four neuroendocrine vasoconstrictors and in the simultaneous presence of all four neuroendocrine vasoconstrictors (Fig. 2C). The mean indomethacin-induced change in [^{3}H]thymidine incorporation was 21,350 dpm/well in the four-way combination, and ranged—depending on the specific combination—from 11,298 to 16,532 dpm/well in the three-way combinations.

Fig. 3 is an alternative view of the interaction between indomethacin and various combinations of neuroendocrine

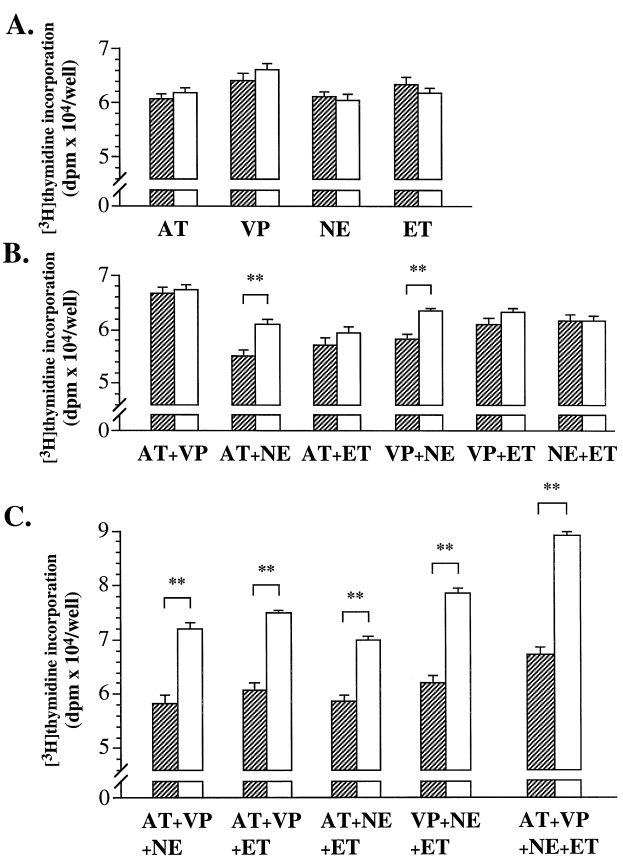

Fig. 2. [^{3}H]thymidine incorporation into mesangial cells after 24 h of incubation with each vasoconstrictor (A) and all possible two-way (B) and three-way and four-way (C) combinations of 10^{-10} M angiotensin II (AT), 10^{-11} M [Arg^8]vasopressin (VP), 10^{-8} M (−)-norepinephrine (NE) or 10^{-11} M endothelin-1 (ET) in the absence (hatched bar) and presence (open bar) of 10^{-6} M indomethacin. Data are mean ± S.E.M. for 24 wells in six experiments. ∗∗ $P < 0.01$, absence versus presence of indomethacin (Student's *t*-test).

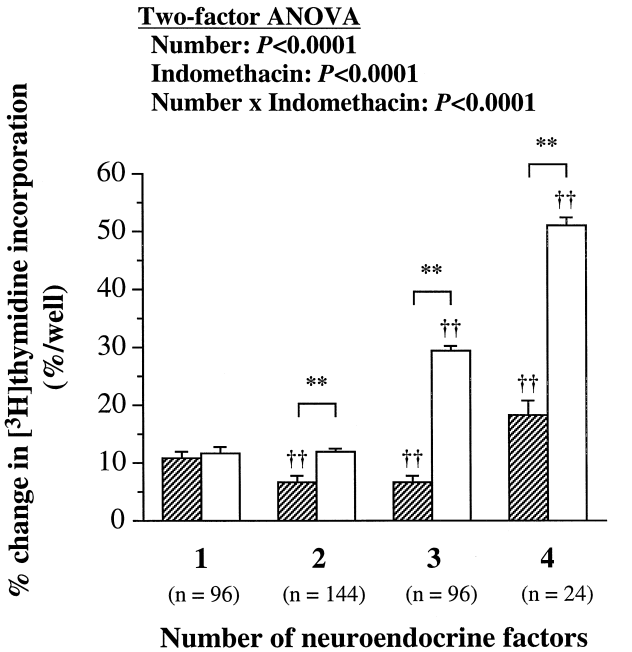

Fig. 3. Mean percentage change from control in [^{3}H]thymidine incorporation induced by the same number of neuroendocrine vasoconstrictors in the absence (hatched bar) and presence (open bar) of 10^{-6} M indomethacin. Data are mean ± S.E.M. (n = number of culture wells). ††$P < 0.01$ compared with respective single neuroendocrine vasoconstrictor group in the absence and presence of indomethacin (Kruskal–Wallis *H*-test followed by Dunn's test). Above the graph, the results of two-factor ANOVA between the number of neuroendocrine vasoconstrictors and indomethacin treatment is presented.

vasoconstrictors. This figure illustrates the mean percentage change in [^{3}H]thymidine incorporation from control (untreated) cells induced by neuroendocrine vasoconstrictors, graphed against the total number of neuroendocrine vasoconstrictors, in the absence and presence of indomethacin. In cells not treated with indomethacin, the mean effects of two-way combinations and three-way combinations of neuroendocrine vasoconstrictors on [^{3}H]thymidine incorporation were actually less than the mean effects of the single neuroendocrine vasoconstrictors (Dunn's test; $P < 0.01$), and the effect of the four-way combination of neuroendocrine vasoconstrictors was only slightly greater than the mean effects of the single neuroendocrine vasoconstrictors. In stark contrast, in the presence of indomethacin, there was a significant and steep relationship between the number of neuroendocrine vasoconstrictors and the percentage increase in [^{3}H]thymidine incorporation (Kruskal–Wallis *H*-test; $P < 0.0001$). A significant synergistic interaction between indomethacin treatment and the number of neuroendocrine vasoconstrictors was obtained by two-factor ANOVA ($P < 0.0001$) indicating that the effect of indomethacin on DNA synthesis was highly influenced by the number of neuroendocrine vasoconstrictors.

4. Discussion

There are two major findings of this study. First, these experiments demonstrate that pathophysiologically relevant concentrations of angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1 stimulate [^{3}H]thymidine incorporation, a marker of DNA synthesis and cell proliferation, in cultured mesangial cells. Second, this study reveals that the ability of cyclooxygenase inhibition to enhance [^{3}H]thymidine incorporation in cultured mesangial cell is highly dependent on the neuroendocrine milieu. In this regard, in the simultaneous presence of three or four neuroendocrine vasoconstrictors, cyclooxygenase inhibition increases [^{3}H]thymidine incorporation much more than in the absence of vasoconstrictors or in the presence of only one or two vasoconstrictors. The major implication of this study is that cyclooxygenase inhibition by NSAIDs may stimulate mesangial cell proliferation in patients with chronic and simultaneous activation of the major neuroendocrine vasoconstrictors, e.g., patients with advanced liver cirrhosis or heart failure, thus predisposing this patient group to disease-associated glomerulopathies.

Our first study, i.e., the concentration–response study, provides detailed concentration response information regarding the stimulatory effects of the low levels of each of the four neuroendocrine vasoconstrictors on [^{3}H]thymidine incorporation in mesangial cells in our assay. Importantly, the results of this study indicate that pathophysiological levels (Asbert et al., 1993; Seino et al., 1993; Tsai et al., 1995; Parmley, 1995) of angiotensin II (10^{-10} M), [Arg^8]vasopressin (10^{-11} M), (−)-norepinephrine (10^{-8} M) and endothelin-1 (10^{-11} M) significantly ($P < 0.01$) stimulate thymidine incorporation in mesangial cells. In several studies, angiotensin II did not increase [^{3}H]thymidine incorporation in mesangial cells, even in early passage cells (Ganz et al., 1990; Essig et al., 1997). However, in the present study, similar to the study by Wolf et al. (1992), angiotensin II increased [^{3}H]thymidine incorporation in a concentration-dependent manner. These disparate findings may depend on differences in the condition of the cells, for example the extent of confluence at the time of exposure to [^{3}H]thymidine. In the two studies in which angiotensin II did not stimulate proliferation (Ganz et al., 1990; Essig et al., 1997), confluent cells were employed in the [^{3}H]thymidine incorporation studies, whereas, we used subconfluent cells.

Our second study accomplishes the objective of determining whether the effect of cyclooxygenase inhibition on [^{3}H]thymidine incorporation is influenced by the simultaneous exposure of mesangial cells to several neuroendocrine vasoconstrictors. The rationale for this study is two-fold. First, rarely is a pathophysiological situation associated with the activation of only a single neuroendocrine vasoconstrictor. Indeed, most hemodynamic abnormalities, such as heart failure and advanced liver cirrhosis, are associated with the simultaneous activation of multiple neuroendocrine factors (Asbert et al., 1993; Seino et al., 1993; Tsai et al., 1995; Parmley, 1995). Second, since angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1 individually stimulate the production of vasodilatory prostaglandins in mesangial cells or glomeruli (Scharschmidt and Dunn, 1983; Matsumura et al., 1986; Fukunaga et al., 1991) and since vasodilatory prostaglandins inhibit mesangial cell proliferation (Menè et al., 1990a; Menè and Dunn, 1990b), the effect of cyclooxygenase inhibition on mesangial cell proliferation might be strongly dependent on the number of these vasoconstrictors in the environment of the mesangial cells.

As illustrated in Fig. 2A, cyclooxygenase inhibition does not facilitate [^{3}H]thymidine incorporation when mesangial cells are exposed to a single neuroendocrine vasoconstrictor. Moreover, even in the simultaneous presence of two neuroendocrine vasoconstrictors, the effects of cyclooxygenase inhibition on [^{3}H]thymidine incorporation are small to non-existent (Fig. 2B). On the other hand, as shown in Fig. 2C, cyclooxygenase inhibition markedly enhances [^{3}H]thymidine incorporation in mesangial cells simultaneously exposed to three or four neuroendocrine vasoconstrictors.

A unique and important finding in the present study, as summarized in Fig. 3, is the synergistic interaction between the neuroendocrine environment and cyclooxygenase inhibition on [^{3}H]thymidine incorporation in mesangial cells. This result strongly suggests that the

modulation of mesangial cell proliferation by prostaglandins may be augmented in the presence of multiple neuroendocrine vasoconstrictors. To our knowledge this is the first demonstration of this important interaction. This phenomena may possibly depend on an increase in the synthesis of growth-inhibitory prostaglandins by the mesangial cells in the presence of multiple neuroendocrine vasoconstrictors. An increase in cytosolic free Ca^{2+} concentration and an activation of protein kinase C by neuroendocrine vasoconstrictors activate phospholipase A_2 and increase the release of arachidonic acid, resulting in the induction of prostaglandin E_2 synthesis in mesangial cells (Bonventre and Swidler, 1988). An increase in prostaglandin E_2 synthesis via cyclooxygenase induction has also been reported in mesangial cells exposed to the cytokines, interleukin-1 and tumor necrosis factor (Coyne et al., 1992), and endothelin-1 (Hughes et al., 1995). Thus, the synthesis of growth-inhibitory prostaglandins via phospholipase A_2 activation and cyclooxygenase induction may be enhanced in the simultaneous presence of angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1, and this is a likely explanation for the results obtained in our study. Alternatively several reports indicate that, in addition to prostanoids, lipoxygenase and cytochrome P-450 metabolites of arachidonic acid could modulate the proliferation of mesangial cells (Sellmayer et al., 1991; Nakahama et al., 1994; Zuckerman et al., 1994). Thus the balance of growth-stimulatory and growth-inhibitory eicosanoids produced by these three metabolic pathways could contribute in part to the effects of cyclooxygenase inhibition observed in our study.

In summary, this study demonstrates that angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1, at pathophysiologically relevant concentrations, have enhancing effects on [^{3}H]thymidine incorporation in cultured rat mesangial cells. Moreover, the stimulation of [^{3}H]thymidine incorporation in mesangial cells by cyclooxygenase inhibition is strongly dependent on the simultaneous presence of neuroendocrine vasoconstrictors, such that a highly significant synergistic interaction exists between the number of major neuroendocrine vasoconstrictors impinging on the mesangial cells and cyclooxygenase inhibition. Said differently, our findings clearly indicate that cyclooxygenase inhibition reveals a synergistic growth promoting action of angiotensin II, [Arg^8]vasopressin, (−)-norepinephrine and endothelin-1 on mesangial cells. Thus, it is possible that the anti-proliferative effect of cyclooxygenase activity in mesangial cells importantly counterbalances the proliferative effects of the major neuroendocrine vasoconstrictors whenever these vasoconstrictors are simultaneously activated as in patients with advanced liver cirrhosis or heart failure. In these clinical conditions, the impairment of cyclooxygenase activity in mesangial cells by NSAIDs may influence the pathophysiology of glomerulopathies by promoting mesangial cell growth.

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