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Serotonin enhances the production of type IV collagen by human mesangial cells

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Background. The plasma concentration of 5-hydroxytryptamine (5-HT) in diabetic patients is higher than that in normal subjects. Since recent reports have demonstrated the presence of 5-HT_{2A} receptor in glomerular mesangial cells, it is possible that 5-HT may be involved in the development of diabetic nephropathy through the 5-HT_{2A} receptor in mesangial cells. Because expansion of the glomerular mesangial lesion is a characteristic feature of diabetic nephropathy, we examined the effect of 5-HT on the production of type IV collagen by human mesangial cells.

Methods. Human mesangial cells were incubated with 5-HT with or without 5-HT receptor antagonists, protein kinase C (PKC) inhibitor or transforming growth factor-β (TGF-β) antibody. Type IV collagen mRNA and protein concentration in medium were measured by Northern blot analysis and enzyme-linked immunosorbent assay (ELISA), respectively. TGF-β mRNA and bioactivity in the medium were measured by Northern blot analysis and bioassay using mink lung epithelial cells, respectively.

Results. 5-HT stimulated the production of type IV collagen by human mesangial cells, which was inhibited by ketanserin and sarpogrelate hydrochloride, 5-HT_{2A} receptor antagonists, but not by ondansetron, a 5-HT_3 receptor antagonist. 5-HT increased the bioactivities of both active and total TGF-β. However, the 5-HT-enhanced production of type IV collagen was completely inhibited by an anti-TGF-β antibody. Furthermore, a PKC inhibitor, calphostin C, inhibited the 5-HT-induced increase in type IV collagen secretion, and the activity of membrane PKC was increased by 5-HT. Phorbol ester activated type IV collagen production as well as active and total TGF-β. Calphostin C completely inhibited the 5-HT-enhanced activity of active TGF-β, but did not inhibit exogenous TGF-β-induced increase in type IV collagen secretion.

Conclusions. Our results suggest that 5-HT-enhanced production of type IV collagen by human mesangial cells is mediated by activation of PKC and subsequent increase in active TGF-β activity.

Key words: serotonin, type IV collagen, human mesangial cell, transforming growth factor-β, protein kinase C, diabetic nephropathy.

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Serotonin [5-hydroxytryptamine (5-HT)], a decarboxylation derivative of the amino acid tryptophan, is an active molecule released mainly from activated platelets [1]. Recent reports have emphasized that the proximal renal tubules also produced a significant amount of 5-HT that may modulate renal phosphorus transport in a paracrine fashion [2]. 5-HT is also known to have several biological activities, for example, it acts as a neurotransmitter in the central and sympathetic nervous systems, a potent constrictor of smooth muscle cells, and an activator of platelets [3]. The plasma concentration of 5-HT is particularly high in diabetic patients [4, 5], suggesting that it may be involved in the development of diabetic complications (through arterial contraction and platelet aggregation), although there is no clear clinical evidence for its involvement in the pathogenesis of diabetic nephropathy. However, a recent study by Nebigil et al showed the presence of 5-HT_{2A} receptor subtype on glomerular mesangial cells [6], suggesting that a direct effect of 5-HT on mesangial cells through its receptor might be involved in the development of diabetic nephropathy.

Expansion of the glomerular mesangial lesion is one of the histological features of diabetic nephropathy [7]. The mesangial matrix is normally composed of various macromolecules, including fibronectin, laminin, and collagen [8, 9]. Among these molecules, the metabolism of type IV collagen has been extensively examined in diabetic mesangial lesions. Mesangial cells are thought to play an important role in the metabolism of type IV collagen by modulating its synthesis and degradation [8, 9]. *In vitro* experiments using rat mesangial cells have shown that high glucose concentrations [10] and exogenous transforming growth factor- β (TGF-β) [11, 12] induce the secretion of type IV collagen. However, the mechanism of accumulation of type IV collagen in diabetic mesangial lesions remains to be elucidated.

In the present study, we examined the effect of 5-HT on the production of type IV collagen by human mesangial cells, and demonstrated that 5-HT stimulated type IV

collagen secretion from human mesangial cells by activation of protein kinase C (PKC) with subsequent increase in active TGF-β.

METHODS

Chemicals

Serotonin (5-hydroxytryptamine hydrochloride crystalline), ketanserin (5-HT_{2A} receptor antagonist), and calphostin C (a PKC inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calphostin C was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.1% in the culture medium which did not affect viability and growth of mesangial cells. Sarpogrelate hydrochloride (5-HT_{2A} receptor antagonist) was a generous gift from Tokyo Tanabe Co., Ltd. (Tokyo, Japan). Ondansetron (5-HT_3 receptor antagonist) was a generous gift from Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals were of the best grade available from commercial sources.

Cells

The experimental protocol was approved by the Human Ethics Review Committee of our institution and a signed consent form was obtained from each patient. Human glomeruli were isolated according to the method of Gröne et al [13], with minor modifications [14, 15]. Briefly, the kidneys of patients with ureteral cancer were removed and the cortices of normal sections were cut and pressed through a series of sieves of decreasing pore size (250, 125 and 110 μm). After passing through the finest sieve, the collected tissue was rinsed twice with Hank's balanced salt solution (HBSS; Nissui Pharmaceutical Co., Tokyo, Japan) and centrifuged (3,000 $\times$ *g*) for 10 minutes. The pellet was resuspended in 5 ml of HBSS containing 750 U/ml collagenase (Sigma) and incubated for 30 minutes at 37°C. After incubation, digested glomeruli were washed five times with phosphate buffered saline (PBS) and the glomerular cores were cultured at 37°C in RPMI 1640 (Life Technologies, Inc., Grand Island, NY, USA) with 20% fetal calf serum (FCS), 100 U/ml penicillin, 10 μg/ml streptomycin, 250 μg/ml amphotericin B and ITS™ premix (Becton Dickinson, Bedford, MA, USA) in 75 cm^2 plastic tissue culture flasks in a 5% CO_2 incubator. Cultured human mesangial cells between the fifth and eighth passages formed a uniform cell population as evaluated by the following criteria: *(1)* spindle shape, *(2)* positive immunohistochemistry with antibody for α-actin and vimentin, and negative for cytokeratin and Factor VIII, and *(3)* contraction in response to angiotensin II. Mesangial cells (5×10^4 cells/well) were plated onto 12-well plates and incubated in RPMI 1640 with 20% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin, 250 μg/ml amphotericin B for 24 hours. Then, cells were incubated with 5-HT for 24 hours, 48 hours or 72 hours for determination of type IV collagen secretion and TGF-β bioactivity. 5-HT is known to induce mesangial cell growth [16]. In a series of preliminary studies, mesangial cell growth was induced by 5-HT as confirmed by using [^{3}H] thymidine incorporation and cell counting assays. Thus, the data of type IV collagen secretion and TGF-β bioactivity were expressed on a cellular protein basis.

Enzyme-linked immunosorbent assay of type IV collagen

Type IV collagen was measured by enzyme-linked immunosorbent assay described by Rennard et al [17] with minor modifications. In brief, 50 μl type IV collagen standards or conditioned media from mesangial cells were incubated with 50 μl of anti-type IV collagen IgG diluted with PBS containing 0.5% bovine serum albumin at 4°C for 16 hours. In the next step, 90 μl of the reaction mixture was transferred to the well of 96 multi-well plate (Falcon; Becton Dickinson and Co., Lincoln Park, NJ, USA) precoated with type IV collagen (1 μg type IV collagen/well in 0.02 mol/liter carbonate buffer, pH 9.6 at 4°C overnight) and the plate was incubated at 37°C for 60 minutes. After rinsing each well three times with a washing buffer (PBS with 0.1% Tween 20), 100 μl anti-rabbit IgG conjugated with peroxidase (Tago, Inc., Burlingame, CA, USA) was added to the well, incubated at 37°C for 60 minutes and rinsed three times. The final reaction mixture (0.04 mg/dl *o*-phenylenediamine dihydrochloride and 0.06% H_2O_2 0.05 mol/liter citrate buffer, pH 5.0) was then added and incubated at room temperature for two minutes. The reaction was stopped by adding 50 μl H_2SO_4 followed by measurement of absorbance at 490 nm.

TGF-β bioassay

The assay used in the present experiments was essentially similar to that described previously by Ikeda, Lioubin and Marquardt [18]. An aliquot of the medium was used to determine the active form of TGF-β by the bioassay described below. To determine the total TGF-β in the medium, the remaining portion was acidified to pH 2 with 1 mol/liter HCl for 30 minutes at room temperature, dialyzed for 24 hours at 4°C against RPMI 1640 and subjected to the bioassay described below.

Mvl Lu mink lung epithelial cells (CCL-64) were purchased from American Type Culture Collection and cultured in RPMI 1640 with 10% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (medium A). Before the bioassay, cells were subcultured for two hours at 3.5×10^3 cells/well in a 96-well tissue culture plate in 50 μl of medium A. In the next step, 50 μl of the conditioned medium to be tested was added to each well and incubated for four days at 37°C. During the last 24 hours of incubation, cells in each well were chased with 10 μl of 10 μCi/ml [^{3}H] thymidine, and the radioactivity incorporated into the cells was counted in a liquid scintillation spectrophotometer. For each assay, a standard curve for TGF-β was constructed by 0.01 to 5 ng/ml of a recombinant human TGF-β [18] with or without

5-HT. [^{3}H] thymidine incorporation into CCL-64 cells was not affected by 5×10^{-5} mol/liter of 5-HT (data not shown), demonstrating that 5-HT in the conditioned medium of mesangial cells did not alter TGF-β bioactivity. Under these assay conditions, the addition of 10 μg/ml of a mouse anti-human TGF-β antibody (Genzyme, Cambridge, MA, USA) [19] could neutralize $>$ 95% of the TGF-β-induced growth inhibition of CCL-64 cells.

Assay of protein kinase C activity

PKC activity in human mesangial cells was assayed by MESACUP Protein Kinase Assay Kit (Medical and Biological Laboratories, Nagoya, Japan). Human mesangial cells (1×10^7 cells/well) in 2 ml of serum-free RPMI 1640 medium were treated with 10^{-6} mol/liter of 5-HT for indicated time intervals. The cells were washed three times in ice-cold PBS and detached from the plates with rubber policeman. Cells were suspended in 1 ml of sample preparation buffer (in mmol/liter, 5 EDTA, 10 EGTA, 50 2-mercaptoethanol, 1 PMSF, 10 benzamidine and 50 Tris-HCl, pH 7.5), and sonicated for 30 seconds at 4°C by Sonifier (Branson Sonic Power Co., USA). Homogenates were centrifuged at 100,000 $\times$ *g* for one hour at 4°C. The supernatant was discarded and the precipitates were resuspended in 1 ml of preparation buffer and used as the membrane fractions. PKC activity in the membrane fractions was measured as follows. Twelve microliters of membrane fractions (5 μg) were added to 108 μl of component buffer (final concentration of reaction mixture was 3 mmol/liter $MgCl_2$, 0.1 mmol/liter ATP, 2 mmol/liter $CaCl_2$, 50 μg/ml phosphatidylserine, 0.5 mmol/liter EDTA, 1 mmol/liter EGTA, 5 mmol/liter 2-mercaptoethanol and 25 mmol/liter Tris-HCl, pH 7.0). In the next step, 100 μl of this mixture was transferred to each well coated with PKC-specific peptide (RFARKGSLRQKNV) using a multichannel pipette. Each well was incubated at 25°C for five minutes and the reaction was terminated by the addition of 100 μl of 20% H_3PO_4, followed by washing five times with 200 μl of PBS. To each well we added 100 μl of biotinylated anti-phosphorylated serine antibody (2B9) and the mixture was incubated at 25°C for 60 minutes, and washed five times with PBS. The mixture was further incubated at 25°C for 60 minutes after the addition of 100 μl of peroxidase conjugated streptavidin to each well, followed by washing five times with PBS. After the addition of 100 μl of H_2O_2 and *o*-phenylenediamine, the mixture was incubated at 25°C for five minutes, followed by the addition of 100 μl of 20% H_3PO_4. Finally, the absorbance was read at 492 nm with a microplate reader. Nonspecific serine/threonine-kinase activities were measured both in the absence of phosphatidyl serine and in the presence of 2 mmol/liter EGTA in component buffer. The enzyme activity in the membrane fraction specific for conventional PKC was quantitated by subtracting the nonspecific serine/threonine-kinase activities from total kinase activity.

Northern blot analysis

The cDNA probes used were a 0.8 kb *Eco*RI fragment encoding human α1(IV) collagen and a 0.6 kb *Sma*I fragment encoding human TGF-β. Each mRNA was isolated from human mesangial cells using QuickPrep (Pharmacia, Uppsala, Sweden). For Northern blot analysis, 0.4 μg of mRNA from each sample of human mesangial cells was electrophoresed in 1% agarose gel and transferred to nitrocellulose filter (Nitroplus 2000; Micro Separations, Westboro, MA, USA) for 18 hours at room temperature using 10 $\times$ SSC as the transfer solution and cross-linked by UV (FS 1500, Funakoshi, Japan) at 12 J/cm^2. Prehybridization with salmon sperm DNA was performed for three hours followed by hybridization for eight hours using 50% formamide with a ^{32}P-radiolabeled probes (Multiprine; Amersham, Arlington Height, IL, USA). Hybridized filters were washed with 1 $\times$ SSC (0.1% SDS) for 15 minutes, and twice with 0.1 $\times$ SSC (0.1% SDS) for 15 minutes at 60°C. The filters were then exposed to Kodak XAR-5 film using an intensifying screen for two days at -80°C.

Statistical analysis

Data were expressed as mean $\pm$ SD. Differences between groups were evaluated by paired Student's *t*-test. A *P* value less than 5% denoted the presence of a statistically significant difference.

RESULTS

Effect of 5-HT on production of type IV collagen by human mesangial cells

We first examined the *in vitro* effect of 5-HT on the production of type IV collagen by human mesangial cells. When mesangial cells were incubated with the medium alone for 24 hours, the baseline concentration of type IV collagen was 15 μg/mg cell protein, indicating that type IV collagen was constitutively produced by human mesangial cells (Fig. 1A). Incubation of mesangial cells with various concentrations of 5-HT for 24 hours significantly increased the production of type IV collagen in a dose-dependent manner, and the peak concentration was observed at 10^{-6} mol/liter of 5-HT (Fig. 1A). We also examined the kinetics of type IV collagen secretion in the presence or absence of 5-HT. In the control media, the concentration of type IV collagen increased in a time-dependent manner, but its production by mesangial cells was significantly higher, relative to controls, at each time interval when these cells were incubated in the presence of 10^{-6} mol/liter of 5-HT (Fig. 1B). When human mesangial cells were incubated with 10^{-6} mol/liter of 5-HT together with various concentrations of ketanserin or sarpogrelate hydrochloride, 5-HT$_{2A}$ receptor antagonists, 5-HT-induced increase in type IV collagen secretion was completely suppressed by these agents (Fig. 1C). In contrast, the 5-HT$_3$ receptor

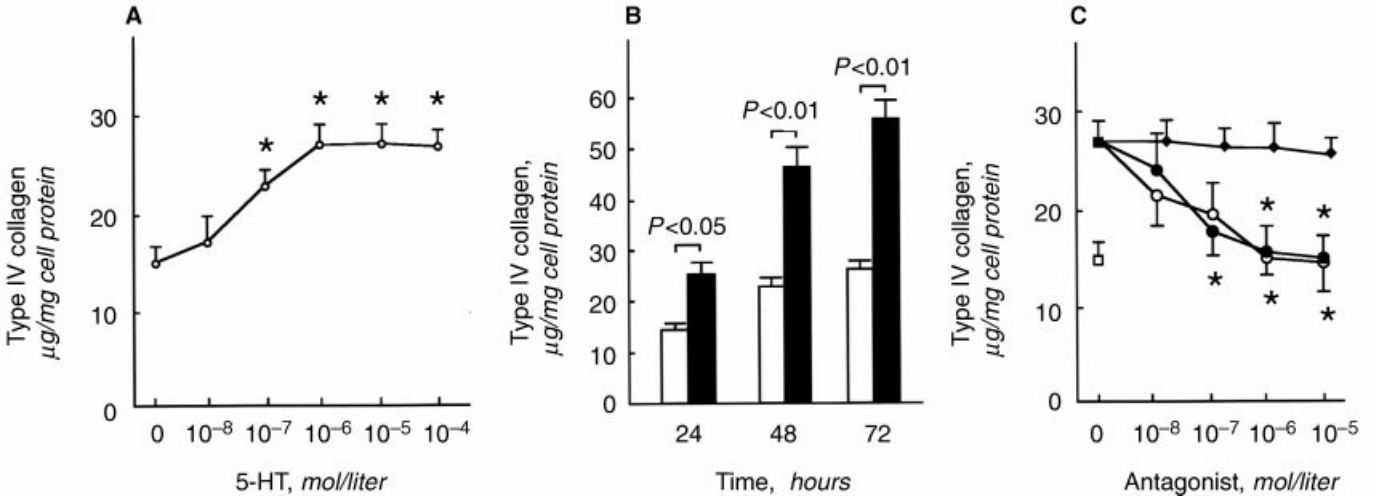

Fig. 1. Effects of 5-hydroxytryptamine (5-HT) and 5-HT receptor antagonists on production of type IV collagen by human mesangial cells. (*A*) Human mesangial cells (5×10^4 cells/well) were incubated with the indicated concentrations of 5-HT. After 24 hours incubation, type IV collagen concentrations in the medium were determined. Data represent the mean of three separate experiments. Error bars indicate SDs. $^*P < 0.05$, compared with control. (*B*) Human mesangial cells (5×10^4 cells/well) were incubated in the absence (□) or the presence (■) of 10^{-6} mol/liter of 5-HT for 24, 48, and 72 hours. After incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. (*C*) Human mesangial cells (5×10^4 cells/well) were incubated with 10^{-6} mol/liter of 5-HT in the absence or presence of indicated concentrations of ketanserin (○), ondansetron (♦), and sarpogrelate hydrochloride (●). As a control, human mesangial cells were incubated with medium alone (□). After 24 hours incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$, compared with cells incubated with 10^{-6}mol/liter of 5-HT alone.

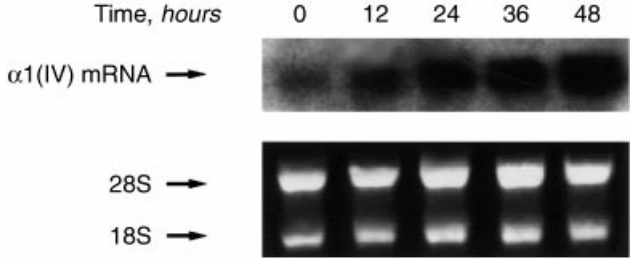

Fig. 2. Effect of 5-HT on mRNA of type IV collagen α1 chain by human mesangial cells. Human mesangial cells were incubated with 10^{-6} mol/liter of 5-HT for indicated time. The mRNA was isolated and 0.4 μg of mRNA were separated by electrophoresis in formaldehyde-containing gel and transferred to a nitrocellulose filter. The filter was hybridized with a heat-denatured ^{32}P-labeled α1(IV) probe. As a control, 18S and 28S were visualized on a UV monitor.

antagonist, ondansetron, did not affect 5-HT-induced increase in type IV collagen production (Fig. 1C). These results suggested that the 5-HT-enhanced production of type IV collagen might be mediated through the 5-HT_{2A} receptor.

Northern blot analysis of type IV collagen α1 showed that 5-HT increased the mRNA level of type IV collagen α1 chain in a time-dependent manner and a significant difference was observed at 24 hours following the addition of 10^{-6} mol/liter of 5-HT (Fig. 2). Figure 3 shows that both constitutive and 5-HT-induced type IV collagen production were completely inhibited by cycloheximide (Fig. 3). These results suggested that 5-HT-enhanced production of type IV collagen is regulated at a transcriptional level.

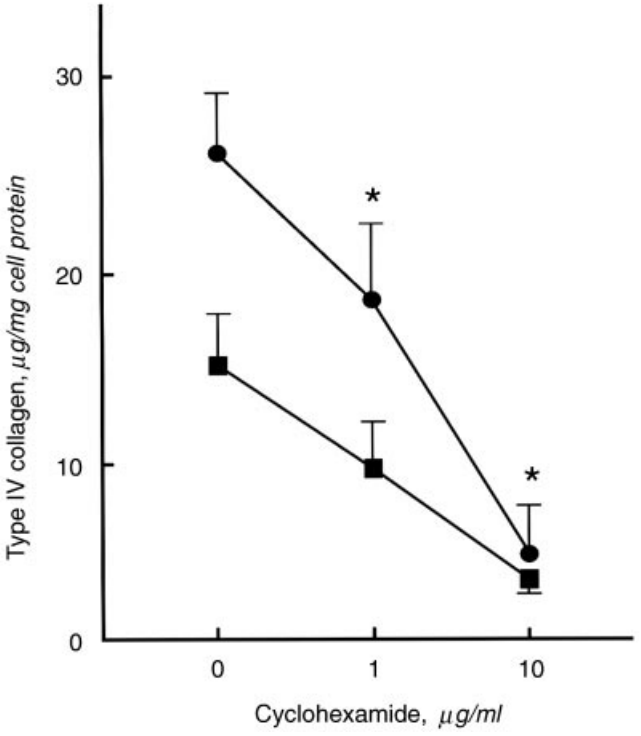

Fig. 3. Effect of cycloheximide on production of type IV collagen by human mesangial cells. Human mesangial cells (5×10^4 cells/well) were incubated with (●) or without (■) 10^{-6} mol/liter of 5-HT in the absence or the presence of indicated concentrations of cycloheximide. After 24 hours of incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$, compared with cells incubated with 10^{-6}mol/liter of 5-HT without cycloheximide.

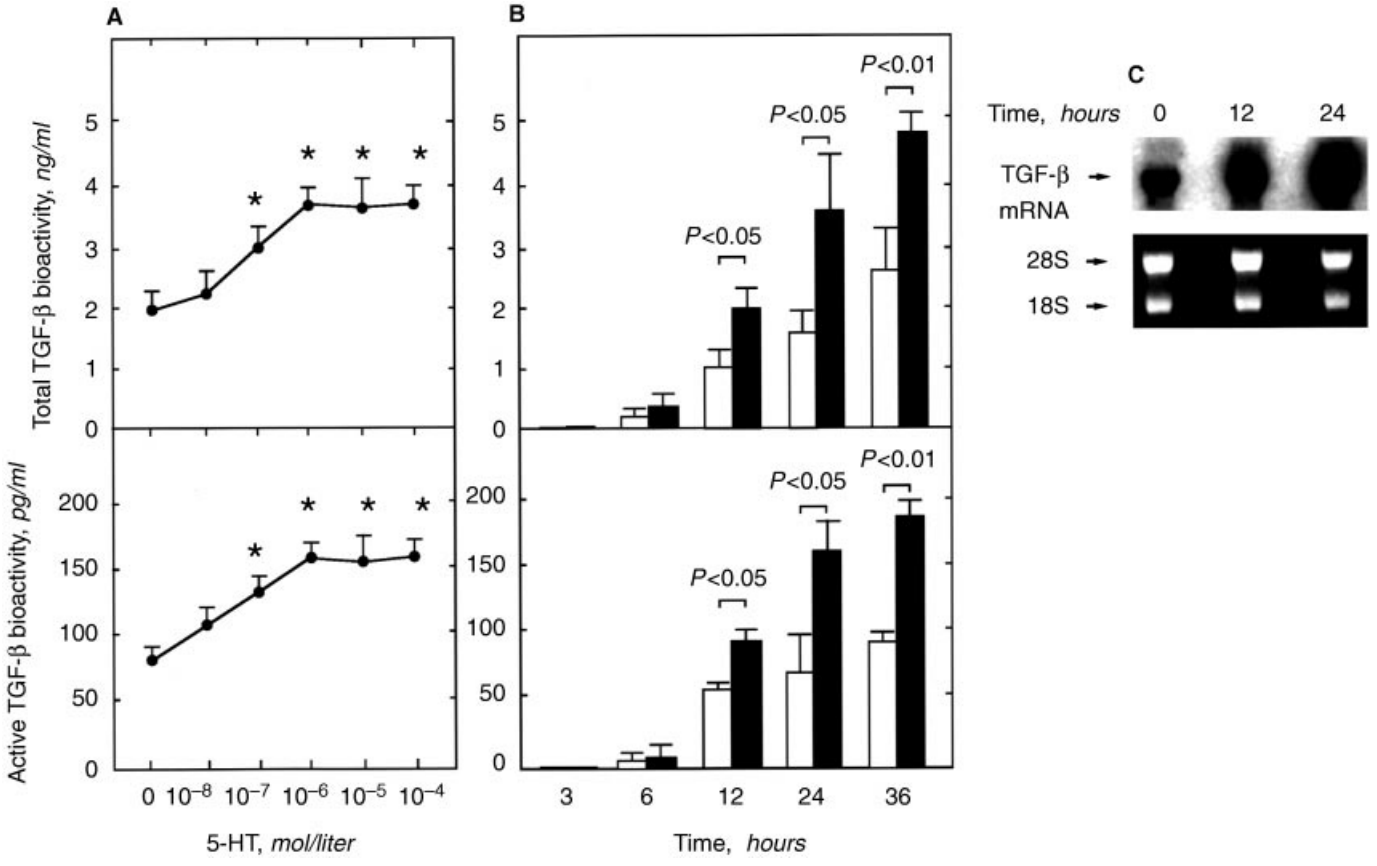

Fig. 4. Effects of 5-HT on total and active TGF-β bioactivities and TGF-β mRNA expression. (*A*) Human mesangial cells (5×10^4 cells/well) were incubated with the indicated concentrations of 5-HT. After 24 hours incubation, total (upper panel) and active (lower panel) TGF-β bioactivities in the medium were determined. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$, compared with the control. (*B*) Human mesangial cells (5×10^4 cells/well) were incubated in the absence (□) or the presence (■) of 10^{-6} mol/liter of 5-HT for indicated times. Total (upper panel) and active (lower panel) TGF-β bioactivities in the medium were determined. Data represent the mean ± SD of three separate experiments. (*C*) Northern blot analysis of TGF-β. Human mesangial cells were incubated with 10^{-6} mol/liter of 5-HT. The mRNA of these cells was harvested at 0, 12, 24 hours. The mRNA (0.4 μg) was separated by electrophoresis in for aldehyde-containing gel and transferred to a nitrocellulose filter. The filter was hybridized with a heat-denatured ^{32}P-labeled TGF-β probe. As a control, 18S and 28S were visualized on a UV monitor.

Involvement of TGF-β in 5-HT-induced increase in type IV collagen production

To elucidate the mechanism of 5-HT-enhanced production of type IV collagen, we examined the involvement of TGF-β in this process in the next series of experiments. Bioactivities of both active and total TGF-β significantly increased following the addition of 5-HT in a dose-dependent manner, and the peak activity of active and total TGF-β occurred in the presence of 10^{-6} mol/liter of 5-HT (Fig. 4A). Interestingly, 5-HT-stimulated bioactivity of active TGF-β (Fig. 4A) paralleled the rise in type IV collagen secretion (Fig. 1A). The time course study of TGF-β bioactivity showed that both active and total TGF-β activities became significant at 12 hours after the addition of 5-HT (Fig. 4B). Figure 4C shows that the level of TGF-β mRNA was increased by 5-HT. These results demonstrated that 5-HT increased both total and active TGF-β bioactivities. Figure 5A shows that non-immune IgG failed to change the effect of 5-HT on type IV collagen production, whereas an anti-TGF-β antibody significantly inhibited 5-HT-induced type IV collagen production. Moreover, anti-TGF-β antibody partially suppressed the constitutive secretion of type IV collagen. The level of anti-TGF-β antibody-induced suppression of type IV collagen production was less than the constitutively produced type IV collagen, suggesting the potential involvement of TGF-β in 5-HT-enhanced production of type IV collagen, as well as, at least in part, in constitutive secretion. To test this conclusion, we determined the effect of exogenous active TGF-β on the production of type IV collagen in the culture medium. Figure 5B shows that the addition of exogenous active TGF-β to these cells markedly increased type IV collagen secretion in a dose-dependent manner (up to 100 pmol/liter). Combined together, these results indicate that TGF-β may play an important role in 5-HT-enhanced production of type IV collagen.

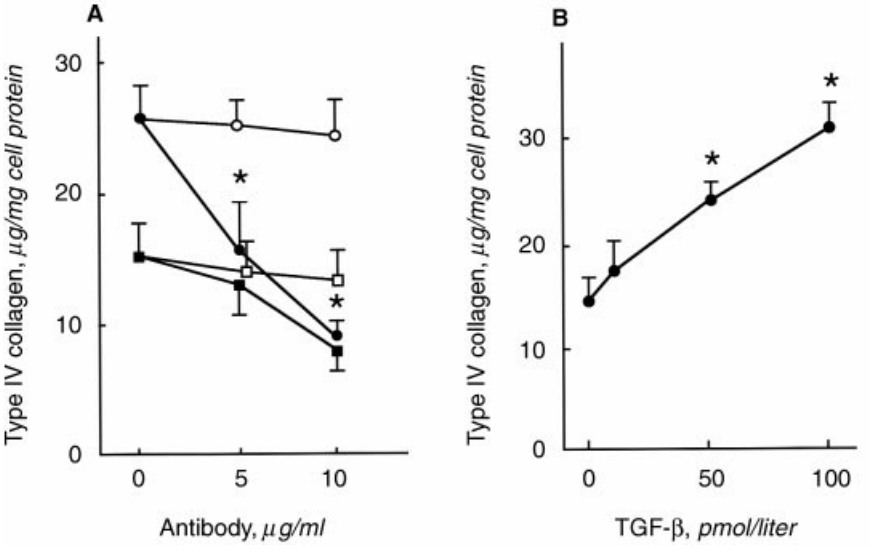

Fig. 5. Inhibitory effect of anti-TGF-β antibody and effect of exogenous TGF-β on type IV collagen production. (*A*) Human mesangial cells (5×10^4 cells/well) were incubated with (○,●) or without (□,■) 10^{-6} mol/liter of 5-HT in the absence or presence of indicated concentrations of anti-TGF-β antibody (●,■) or non-immune IgG (○,□). After 24 hours incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. *$P < 0.05$, compared with non-immune IgG. (*B*) Human mesangial cells (5×10^4 cells/well) were incubated with the indicated concentrations of exogenous TGF-β. After 24 hours incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. *$P < 0.05$, compared with the control.

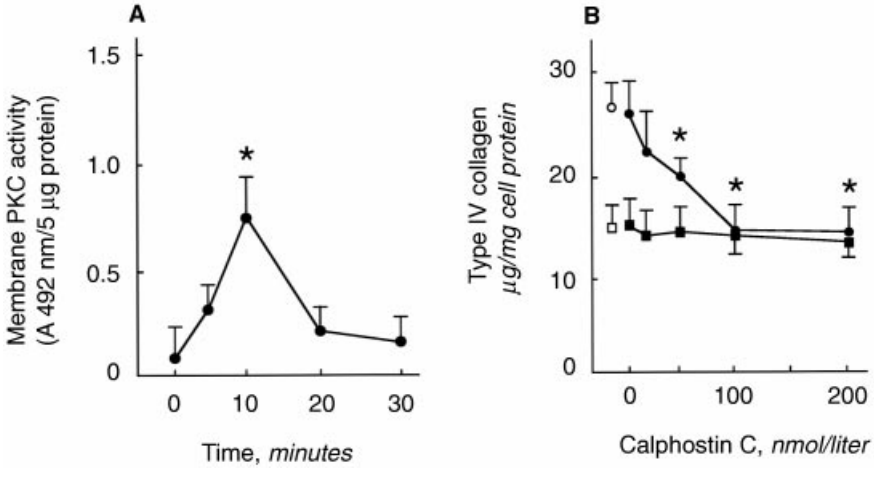

Fig. 6. Involvement of protein kinase C (PKC) in 5-HT-induced production of type IV collagen. (*A*) Human mesangial cells (1×10^7 cells) were incubated with 10^{-6} mol/liter of 5-HT for the indicated time interval. The activity of membrane PKC was determined. Data represent the mean ± SD of three separate experiments. *$P < 0.05$, compared with baseline (0 min). (*B*) Human mesangial cells (5×10^4 cells/well) were incubated with (●) or without (■) 10^{-6} mol/liter of 5-HT in the absence or presence of the indicated concentrations of calphostin C. As control, the cells were incubated with 0.1% DMSO in the presence (○), or absence (□) of 10^{-6} mol/liter 5-HT. After 24 hours of incubation, the concentration of type IV collagen in the medium was determined. Data represent the mean ± SD of three separate experiments. *$P < 0.05$, compared with cells incubated with 10^{-6} mol/liter of 5-HT without calphostin C.

Involvement of protein kinase C in 5-HT-enhanced production of type IV collagen

Figure 6A shows the time course study of membrane PKC activity. The addition of 10^{-6} mol/liter of 5-HT to human mesangial cells resulted in 2.5-fold rise in enzyme activity (relative to basal level) at five minutes and in 7.0-fold increase above the basal level at 10 minutes. However, this was followed by a rapid decline to levels close to the baseline at 20 minutes (Fig. 6A). To elucidate the role of PKC on 5-HT-enhanced production of type IV collagen, we examined the effect of a PKC inhibitor, calphostin C, on this process. Incubation of human mesangial cells with 10^{-6} mol/liter of 5-HT together with various concentrations of calphostin C suppressed 5-HT-induced increase in type IV collagen secretion in a dose-dependent manner, with 50% inhibition at 50 nmol/liter of calphostin C and a maximal inhibition at 100 nmol/liter (Fig. 6B). Interestingly, calphostin C did not affect the constitutive production of type IV collagen in human mesangial cells (Fig. 6B). Under the experimental conditions described above, calphostin C was not cytotoxic to human mesangial cells. This conclusion was based on results of the cell counting assay using trypan blue staining and the release of lactic dehydrogenase from these cells (data not shown). These results suggest that PKC may be involved in the production of type IV collagen. To confirm the involvement of PKC in type IV collagen production, we examined the effect of phorbol ester, a well known PKC activator, on type IV collagen production and TGF-β activity. Figure 7A shows that phorbol ester induced an increase in type IV collagen production as well as activation of active and total TGF- β (Fig. 7B). These results suggested that 5-HT-induced type IV collagen production was mediated by activation of PKC, which in turn increased TGF-β activity in human mesangial cells. To test this notion, we examined the effect of calphostin C on 5-HT-stimulated TGF-β

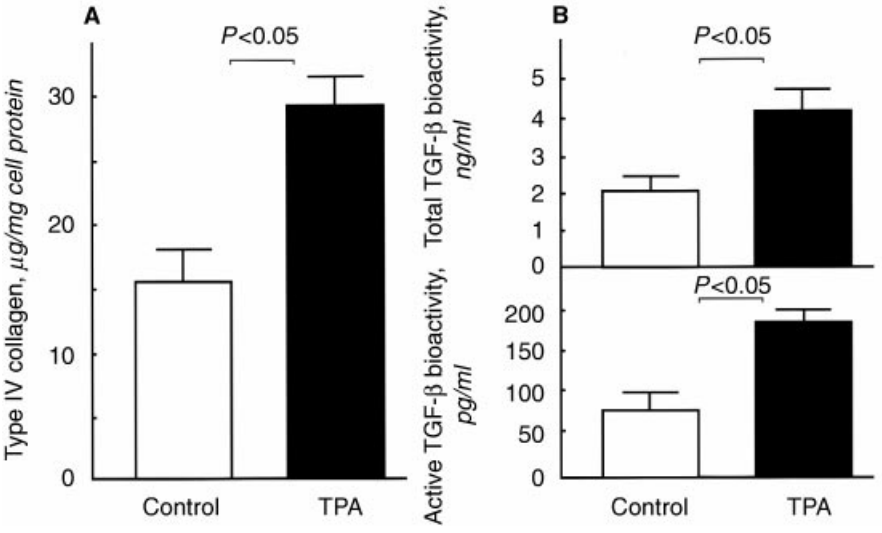

Fig. 7. Effect of phorbol ester on production of type IV collagen and TGF-β activity. (*A*) Human mesangial cells (5×10^4 cells/well) were incubated with or without 10 nmol/liter of phorbol 12-myristate 13-acetate (TPA). After 24 hours incubation, the concentration of type IV collagen in the medium was determined. (*B*) Human mesangial cells (5×10^4 cells/well) were incubated with 10 nmol/liter of TPA. After 24 hours of incubation, total (upper panel) and active (lower panel) TGF-β bioactivities in the medium were determined. Data represent the mean ± SD of three separate experiments.

bioactivity. A complete inhibition of 5-HT-induced increase in active and total TGF-β bioactivities was noted following the addition of 100 nmol/liter of calphostin C (Fig. 8). Moreover, the effect of exogenous TGF-β on the concentration of type IV collagen was not inhibited by calphostin C (data not shown). Thus, our results suggest that 5-HT-induced production of type IV collagen by human mesangial cells is mediated by activation of PKC and the subsequent increase in active TGF-β activity.

DISCUSSION

The major findings of the present study were that 5-HT enhanced the production of type IV collagen by human mesangial cells in the kidney, and that this effect was mediated by activation of PKC with a subsequent increase in active TGF-β bioactivity. These conclusions were supported by the following findings: *(1)* production of type IV collagen by human mesangial cells increased following the addition of 5-HT to the medium (Fig. 1 A, B), which was inhibited by 5-HT_{2A} receptor antagonists (Fig. 1C); *(2)* the bioactivities of both active and total TGF-β were increased by 5-HT (Fig. 4); *(3)* 5-HT-enhanced production of type IV collagen was completely inhibited by an anti-TGF-β antibody (Fig. 5A); *(4)* the activity of membrane PKC was increased by 5-HT (Fig. 6A); *(5)* inhibition of PKC by calphostin C suppressed the stimulatory effect of 5-HT on type IV collagen production (Fig. 6B); *(6)* phorbol ester enhanced activation of active TGF-β and production of type IV collagen (Fig. 7); *(7)* increased bioactivity of TGF-β by 5-HT was completely inhibited by calphostin C (Fig. 8); and *(8)* the exogenous TGF-β-induced increase in type IV collagen secretion (Fig. 5B) was not inhibited by calphostin C (data not shown).

The 5-HT_{2A} subreceptor type is present in high concentrations in the brain, platelets, vascular smooth muscle cells and glomerular mesangial cells [3, 6, 20–23]. Ketanserin is a well known 5-HT_{2A} receptor antagonist [3, 24]. Sarpogrelate hydrochloride is also a selective 5-HT_{2A} receptor antagonist that inhibits 5-HT-induced platelet aggregation [25] and constriction of vascular smooth muscle cells [25]. In the present study, the 5-HT-induced increase in type IV collagen secretion by human mesangial cells was completely suppressed by these 5-HT_{2A} receptor antagonists but not by the 5-HT_3 receptor antagonist, ondansetron (Fig. 1C) [26], suggesting that the effect of 5-HT was, at least in part, mediated through 5-HT_{2A} subreceptor type present on human mesangial cells.

Our results also showed that calphostin C inhibited the stimulatory effect of 5-HT on the secretion of type IV collagen (Fig. 6B), while the activity of membrane PKC was increased by 5-HT (Fig. 6A). Moreover, phorbol ester, a well known PKC activator, increased the production of type IV collagen (Fig. 7A). These results strongly suggest that the effect of 5-HT on type IV collagen secretion is mediated by activation of PKC in mesangial cells. PKC is involved in agonist-induced cellular responses in various cell types [27–29]. The enzyme is activated by high concentrations of diacylglycerol (DAG) and calcium in the cell membrane, which are derived from agonist-induced hydrolysis of inositol phospholipid [27]. In cultured rat glomerular mesangial cells, 5-HT stimulates the proliferation of these cells and prostaglandin synthesis by the same cells [16, 30], which are mediated by stimulation of phospholipase C [16, 30]. This pathway of cellular signals induces a transient rise in cytosolic calcium followed by activation of PKC [16]. Thus, it is possible that 5-HT-enhanced production of type IV collagen by human mesangial cells is mediated by activation of phospholipase C, and subsequently by a rise in DAG and calcium, and then further by activation of PKC. Recently, Koya et al reported that PKC beta was activated in the glomeruli of diabetic rats [31]. Moreover, an oral PKC beta inhibitor ameliorated renal

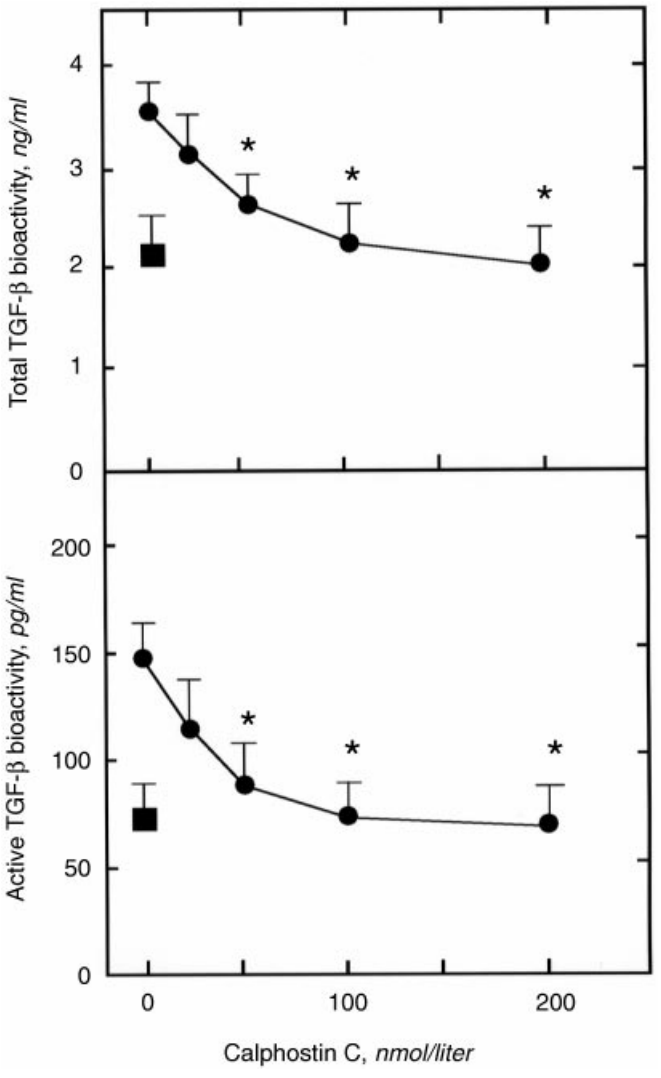

Fig. 8. Inhibitory effect of calphostin C on 5-HT-enhanced bioactivities of total and active TGF-β in human mesangial cells. Human mesangial cells (5×10^4 cells/well) were incubated with (●) or without (■) 10^{-6} mol/liter of 5-HT in the absence or presence of the indicated concentrations of calphostin C. After 24 hours of incubation, total (upper panel) and active (lower panel) TGF-β bioactivities in the medium were determined. Data represent the mean ± SD of three separate experiments. $^*P < 0.05$, compared with cells incubated with 10^{-6} mol/liter of 5-HT without calphostin C.

dysfunction of diabetic rats [32]. These studies suggested that PKC beta might play an important role in the development of diabetic nephropathy. However, the PKC family consists of at least 11 different subspecies of serine/threonine protein kinases [33], such as conventional PKC (α, β1, β2 and γ) and novel PKC (δ, ϵ, θ and η). Recent studies have also reported the presence of atypical subspecies of PKC [33]. Therefore, further studies are necessary to determine the exact PKC isozyme(s) involved in 5-HT-enhanced production of type IV collagen by human mesangial cells.

5-HT enhanced the production of type IV collagen from mesangial cells (Fig. 1 A, B) and increased the activity of active TGF-β (Fig. 4). Moreover, an anti-TGF-β antibody induced a complete suppression of type IV collagen production (Fig. 5A). Furthermore, exogenous active TGF-β enhanced the type IV collagen production (Fig. 5B). These results suggest the possible involvement of TGF-β in 5-HT-enhanced production of type IV collagen by mesangial cells. Does the effect of 5-HT on the activity of active TGF-β represent a direct effect or indirect effect through activation of PKC? Our results showed that calphostin C inhibited the effect of 5-HT on collagen secretion (Fig. 6B), suppressed the effects of 5-HT on TGF-β (Fig. 8), but did not affect exogenous active TGF-β-induced type IV collagen production (data not shown). Moreover, the time course studies demonstrated that PKC activity showed peak activity at 10 minutes (Fig. 6A), TGF-β activity become significant at 12 hours (Fig. 4B), and collagen production become significant at 24 hours following the addition of 5-HT (Fig. 1B). These results suggest that the effect of 5-HT on TGF-β are probably mediated by activation of PKC. This conclusion is further supported by results of previous studies from other laboratories. For example, Akhurst, Fee and Balmain reported that phorbol esters stimulated the expression of TGF-β mRNA in mouse epidermal cells [34]. Moreover, angiotensin II stimulated the induction of TGF-β mRNA via PKC activation in rat smooth muscle cells [35]. Furthermore, phorbol esters induced both PKC activation and TGF-β mRNA in rat mesangial cells [36]. In mesangial cells, several activators of PKC have been shown to increase TGF-β mRNA and bioactivity. These activators include high concentrations of glucose in mice [37], angiotensin II in rats [38], thromboxane in rats [39], and low density lipoproteins in rats [40], all of which also increase mesangial cell matrix protein synthesis by mesangial cells.

TGF-β is usually secreted as a biologically inactive form, latent TGF-β [41]. There are three components of the latent TGF-β complex: *(1)* active TGF-β, *(2)* TGF-β latency-associated peptide (LAP), and *(3)* latent TGF-β binding protein (LTAP) [41]. Since TGF-β is secreted in a latent form, the dissociation of active TGF-β from the latent complex is essential for the expression of its biological activities post secretion. Latent TGF-β is activated by proteolysis of the latent complex with plasmin and cathepsin D, or by alteration of carbohydrate structures within LAP. Plasmin cleaves LAP and releases active TGF-β from latent TGF-β. Tissue-type plasminogen activator (tPA) converts plasminogen to the proteolytic active plasmin. Activation of PKC has been shown to induce tPA in HeLa, HepG2, and Bowes melanoma cells [42]. In fact, human mesangial cells also secrete tPA [43]. Accordingly, it is possible that 5-HT-stimulated PKC activation increases tPA activity, thereby enhancing the activation of latent TGF-β via an increased level of plasmin.

The signal pathway downstream TGF-β activation to the

secretion of type IV collagen in human mesangial cells remains unknown. However, previous studies using cultured mouse or rat mesangial cells showed that exogenous TGF-β increases the production of collagen, fibronectin and proteoglycan on a protein level [11, 44]. TGF-β also increases mRNA expression of types I and IV collagen in rat mesangial cells [12]. Moreover, TGF-β is reported to promote the deposition of extracellular matrix by stimulating the synthesis of matrix proteins, increase the activity of tissue inhibitor of matrix-degrading metalloproteinases, and/or inhibit the expression of stromelysin, which are enzymes involved in the metabolism of extracellular matrix [45, 46].

In the present study, 5-HT-enhanced production of type IV collagen was observed at concentrations from 10^{-7} mol/liter (Fig. 1A). Interestingly, the concentrations of plasma 5-HT in diabetic patients ranged from 10^{-8} to 10^{-7} mol/liter [4, 5]. Although the exact concentration and sources of 5-HT in mesangial lesions in diabetic patients are still unknown, it is possible that the high plasma concentrations of 5-HT in diabetic patients may enhance the production of type IV collagen by glomerular mesangial cells, thereby enhancing the expansion of mesangial lesions. Further studies are needed in order to elucidate this issue.

In conclusion, the present *in vitro* experiments demonstrated that 5-HT enhanced the production of type IV collagen by human mesangial cells, which was mediated by activation of PKC and subsequent increase in the activity of active TGF-β.

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APPENDIX

Abbreviations used in this article are: 2B9, anti-phosphorylated serine antibody; CCL-64, Mvl Lu mink lung epithelial cells; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; 5-HT, 5-hydroxytryptamine; LAP, latency associated protein; LTAP, latent transforming growth factor-β binding protein; PBS, phosphate buffered saline; PKC, protein kinase C; TGF-β, transforming growth factor-β; tPA, tissue-type plasminogen activator.

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