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PROSTAGLANDIN E_2 MODIFIES SMAD2 AND PROMOTES SMAD2-SMAD4 COMPLEX FORMATION

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Abstract

We report that PGE_2 promotes Smad2-Smad4 complex formation and this phenomenon could be blocked by DIDS, an anion transporter inhibitor. Our data suggest that PGE_2 had no effects on Smad2 phosphorylation, suggesting that PGE_2-mediated Smad2-Smad4 complex formation is independent of TGF-β signaling and that PGE_2 induced Smad2 modification which is different from TGF-β-mediated phosphorylation. We demonstrate that in primary human glomerular mesangial cells PGE_2 caused modification of Smad2 as detected by Smad2N antibody, raised against a peptide near the N-terminus of Smad2. We hypothesize that Smad2 protein is post-translationaly modified by PGE_2. Direct evidence of Smad2 modification by PGE_2 was achieved by avidin pulldown assay which showed that endogenous Smad2 and recombinant Smad2 protein were attached by biotin-labeled PGE_2. Taken together, our results provided evidence that post-translational modification of Smad2 could be a mechanism for the action of PGE_2 in the pathogenesis of human pathologies.

Introduction

Prostaglandins (PGs) are a group of lipid mediators derived from cyclooxygenase (COX) metabolites of arachidonic acid [24]. The COX-derived PGs are critical modulators of numerous physiological and pathophysiological conditions including cardiovascular homeostasis, inflammation and immune regulation [29]. Cyclooxygenase-2 (COX-2) is the inducible form of COX which is implicated in progression of multiple types of cancer [6;8;18;30]. MAPK cascades are the convergence point of the diverse stimuli which induce COX-2 expression [23;35]. Often, the biological role of COX-2 is associated with antiapoptotic action of COX-2 derived PGs [5;15].

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PGs actions are likely mediated through their specific receptors. However, some effects of PGs may be non-receptor-mediated. A number of studies implied that PGs exerted their diverse effects through post-translational modification of cellular proteins [17;19;34]. Since PGs possess anionic moieties at physiological pH and diffuse poorly through the lipid bilayer [2;4] the covalent modification of proteins by PGs should be a carrier-mediated transport process. Several PGs carriers have been cloned and characterized [28].

Smad proteins are key signal effectors for TGF-β signaling. When binding to and activating the signaling receptors, TGF-β phosphorylates R-Smads (Smad2 or Smad3) in their C-terminal SXS motif. The activated R-Smads undergo conformational changes and form a complex with Smad4, which then translocates into the nucleus and regulates gene transcription in combination with other transcriptional cofactors [13]. Besides phosphorylation, Smads can be post-translationaly modified through other mechanisms, such as ubiquitination, SUMOylation and acetylation [14;20;21]. Some of these covalent modifications are induced by non-TGF-β signals, thus providing ways for other signals to affect TGF-β signal transduction.

PGE_2, the most abundant PGs detected in the kidney, plays an important role in the development of many inflammatory renal glomerular diseases [11;12;22]. Intracellular signaling pathways initiated by PGE_2 contribute to the manifestation of glomerulonephritis [31]. In this study, we report that the Smad2 protein can be post-translationaly modified by PGE_2. Furthermore, PGE_2 promotes the Smad2-Smad4 complex formation, which is independent of TGF-β stimulation. Meanwhile, we also found that PGE_2-mediated Smad2-Smad4 complex formation can be blocked by DIDS, an anion transporter inhibitor. Therefore, post-translational modification of Smad2 protein by PGE_2 may be a mechanism for the action of PGE_2 in the pathogenesis of renal disease.

Materials and methods

Cell culture

Isolation of primary human renal glomerular mesangial cells (HMC) was done previously [37;38]. For this study cells were taken from previously prepared frozen stock and were maintained in RPMI 1640 containing 16.7% FBS and 100ug/ml penicillin and streptomycin as previously described [37;38]. HMC at passages 5 through 8 were used.

Plasmid constructs and protein purification

Human Smad2 cDNA was subcloned into pGEX-2T. Plasmids encoding Smad2 were transformed into *Escherichia coli* BL21 (DE3) RIL (Stratagene). Protein expression was achieved by culturing *E. coli* at 30 °C for 2h with shaking (250rpm), 1mM IPTG was added, and the incubation was continued for an additional 2h. GST-Smad2 was purified using glutathione-Sepharose 4B affinity chromatography (Amersham Biosciences).

RNA isolation and RT-PCR

HMC total cellular RNA was isolated using TRIzol (Invitrogen). Amplification of transcripts was performed using 200 ng of total RNA and one-step RT-PCR system (Qiagen)

according to the manufacturing protocol. The PCR primer sequences used in this study were designed using Primer3 software and the forward and reverse primers are presented as follows: EP1 (350bp), 5'-CTTGTCGGTATCATGGTGGTGTC-3' and 5'-GGTTGTGCTTAGAAGTGGCTGAGG-3'; EP2 (111bp), 5'-CTGTTCTGAGACTAATGCGTTCA-3' and 5'-GGTCAGCCTGTTTACTGGCA-3'; EP3 (298bp), 5'-GGGCTGACCATGACTGTTTT-3' and 5'-CAGAGGCGAAGAAAAGGTTG-3'; EP4 (120bp), 5'-AGCTGGGACTCGTCTTTGAA-3' and 5'-GGTCAGAGTTGCCAGCTTTC-3'. The cycling parameters consisted of one cycle of 50°C for 30 min, one cycle of 95°C for 15 min then 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min followed by a single 10 min cycle at 72°C for extension. RT-PCR products were electrophoresed on a 2% agarose gel using DNA markers (Promega) as a standard to determine the molecular size.

Co-immunoprecipitation and immunoblotting

HMC were lysed in immunoprecipitation buffer (20mM Tris-Cl, 150mM NaCl, 1% Triton X-100 and 10% glycerol; pH 7.5) and precipitated with the Smad2 antibody conjugated with agarose for 2 h at 4°C. The beads were then washed, boiled, and subjected to SDS-PAGE. For immunoblotting, the proteins were electrophoretically transferred onto PVDF membranes and blotted with primary antibodies. The primary antibodies were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies and detected by chemiluminescence.

Avidin pulldown assay

HMC grown on 150mm plates were incubated with 1uM of biotin-labeled PGE2 (bio-PGE_2) for 2h at 37°C. The cells were rinsed with PBS and lysed in 1% NP-40 lysis buffer. After centrifugation, supernatants were incubated with 50ul of 50% slurry of immobilized NeutrAvidin agarose for 1h at 4 °C. The avidin agarose was then washed with lysis buffer, boiled, and subjected to SDS-PAGE. In experiments with purified Smad2 protein, 2ug of GST or GST-Smad2 protein was incubated with bio-PGE_2 in 1% NP-40 lysis buffer for 2h at room temperature.

Materials

PGE_2 and AH6809 were purchased from Sigma. Biotin-labeled PGE_2 and GW627368X were purchased from Cayman Chemical. SC51322 was purchased from BIOMOL International. Phospho-Smad2 antibody, Smad2 antibody and Smad4 antibody were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Neutravidin agarose resin was purchased from Thermo Scientific. The Smad2 cDNA was a gift from Dr. Heldin (Ludwig Institute for Cancer Research, Sweden).

Statistics

All data are expressed as means ± s.e.m. from at least three separate experiments. Effect of PGE_2 was compared to a normal level 1 with the one sample t-test. The effect of PGE_2 versus the effect of PGE_2 with tested antagonists was compared with the two sample t-test. Values were considered statistically significant at $P < 0.05$.

Results

In TGF-β signaling, the phosphorylated/activated Smad2 exerts its transcriptional activity through forming a complex with Smad4 [7]. To investigate effect of PGE_2 on Smad2-Smad4 complex formation, we performed Co-IP experiments. Smad2 protein from cell lysates of human mesangial cells (HMC) was immunoprecipitated with Smad2L antibody. Co-IP demonstrated that Smad4 was precipitated along with Smad2 following PGE_2 treatment; DIDS, an anion transporter inhibitor, blocked PGE_2-mediated Smad2-Smad4 complex formation (Fig. 1*A*). Meanwhile, we found that PGE_2 had no effects on Smad2 phosphorylation and TGF-β-mediated Smad2 phosphorylation (Fig. 2*B*, 2*C*). These data suggested that PGE_2 induced Smad2 modification which is different from TGF-β-mediated phosphorylation and that PGE_2-mediated Smad2 post-translational modification is likely to contribute to Smad2-Smad4 complex formation.

Our data suggest Smad2 modification caused by PGE_2 involves alteration of Smad2 epitope recognized by Smad2N antibody, which was raised against a peptide near the N-terminus of Smad2 protein. Smad2N antibodies failed to recognize endogenous Smad2 protein in HMC treated with PGE_2. This alteration of epitope recognized by Smad2N antibody occurred as early as 30 min and lasted till 24 h after stimulation (Fig. 2*A*.). Recognition of Smad2 by antibodies raised against other epitope was not affected (Fig.3). PGE_2 exerts its actions by acting on a group of G-protein-coupled receptors, designated as EP_1, EP_2, EP_3, and EP_4 [33]. To investigate the role of EPs subtype receptors in PGE_2-mediated Smad2 modulation, we first examined the expression profile of EPs subtype receptors in HMC by RT-PCR. The mRNAs of EP1, EP2, and EP4 subtype receptors could be detected in HMC, whereas the transcript of EP3 subtype receptor remained undetected (Fig. 2*B*). We next examined whether EP1, EP2 and EP4 subtype receptors were involved in the PGE_2-mediated Smad2 recognition. We found that SC51322 (EP1 antagonist, 10μM), AH6809 (EP2 antagonist, 10μM) and GW627368X (EP4 antagonist, 10μM) failed to block PGE_2-mediated Smad2 protein apparent decline (Fig. 2*C*). In addition, we excluded the possibility that protein degradation contributed to the PGE_2-mediated Smad2 protein recognition by using proteasome inhibitors (UBEI-41, MG-132 and lactacystin), protease inhibitors (chloroquine, leupeptin and NH_4Cl) and QVD-OPH, a pan caspase inhibitor (data not shown). Based on these results, it appears that a new mechanism participates in PGE_2-mediated Smad2 modification which prevents detection of protein with Smad2N antibodies.

PGE_2 contains a long-chain fatty acid portion that could bind covalently to proteins by an ester bond between its carboxyl group and a hydroxyl amino acid of a protein [34]. The peptide used for generation Smad2N antibody includes a hydroxyl amino acid threonine. Thus, it is possible that direct attachment of PGE_2 to the threonine takes place and that alters the epitope of the Smad2 antigen, resulting in the Smad2N antibody failing to detect endogenous Smad2 protein. As shown in Fig. 3, the Smad2L antibody, which recognize the linker region of Smad2, could precipitate equal amount of endogenous Smad2 from HMC cell lysates in both PGE_2-treated and untreated conditions, while the Smad2N antibody failed to detect the precipitated Smad2 in the PGE_2-treated condition by immunoblotting. Correspondingly, the Smad2 protein in total cell lysate (TCL) of HMC could be detected by the Smad2L antibody, but not by the Smad2N antibody following PGE_2 treatment. These

results argue that Smad2 might be post-translationaly modified by PGE_2 through interaction with hydroxyl amino acids, such as threonine located near the N-terminus.

To further investigate Smad2 post-translational modification by PGE_2, we performed avidin pulldown experiments using biotin-labeled PGE_2 (bio-PGE_2). In HMC, after treatment with bio-PGE_2, endogenous Smad2 protein was precipitated by Neutravidin agarose beads, as demonstrated with immunoblotting (Fig. 4*A*). Direct attachment of PGE_2 to Smad2 was further confirmed by avidin pulldown assay using purified recombinant Smad2 protein. As shown in Fig. 4*B*, the recombinant GST-Smad2 protein was precipitated by bio-PGE_2, whereas the GST was not. Smad2 modification by PGE_2 was therefore confirmed.

Discussion and Conclusions

The covalent binding of PGs to proteins has been detected in microsomal cell fractions and in intact platelets [1;9;36]. Takahashi and his colleagues demonstrated that proteins in HL-60 cells were labeled by PGE_2 [34]. PGE_2 possesses a long-chain fatty acid portion that could bind covalently to proteins by an ester bond between its carboxyl group and either a hydroxyl amino acid or a cysteine of a protein. It is possible that PGE_2 attaches the peptide through forming an ester bond between its carboxyl group and the hydroxyl group of the threonine. The PGE_2-modified threonine alters the epitope of the peptide, thus resulting in the Smad2N antibody failing to detect the Smad2 protein. The fact that the Smad2L antibody can recognize Smad2 protein after PGE_2 treatment further confirms our hypothesis and also rules out the possibility that protein degradation mechanisms play a role in the process.

There are four principal bioactive PGs in vivo, including PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$. It has been reported that many effects of PGs are mediated through their specific receptors. However, some effects of PGs may be non-receptor-mediated. Recent studies implied that PGs exert their diverse actions through direct covalent modification of proteins. For example, 15d-PGJ_2, a derivative of PGD_2, has been reported to covalently modify a variety of cellular proteins such as transcription factors NF-κB and AP-1, translational initiation factor eIF4A, and proteins that are regulated by oxidative stress [3;17;27;32]. These 15d-PGJ_2-mediated modifications were reported to be closely associated with the anti-inflammatory effect of 15d-PGJ_2. In this study, we demonstrated that PGE_2 promoted Smad2-Smad4 complex formation. It appeared that it happens as a direct effect of intracellular PGE_2 because PGE_2-mediated Smad2-Smad4 complex formation was blocked by an anion transporter inhibitor, DIDS. It was also demonstrated that PGE_2 had no effects of Smad2 phosphorylation, excluding the possibility that TGF-β signaling played a role in the process. Therefore, it is possible that PGE_2-modified Smad2 mediates Smad2-Smad4 complex formation. The attachment of PGE_2 to Smad2 may alter Smad2 conformation, activate Smad2 and promote Smad2-Smad4 complex formation. The underlining mechanism merits further investigation.

We were able to observe the effect of PGE_2 on Smad2 modification with concentrations of PGE_2 as low as 0.01uM. The transient accumulation of cyclooxygenase-derived prostanoids in kidney tissue could be as high as 10 pg PGE_2 per ug of rat inner medulla tissue [26] with

local concentration of PGE_2 at the cell membrane being significantly higher. PGE_2 concentrations 0.5–1.0 uM are routinely used in cell culture studies [5;10;15].

The covalent modification of proteins by PGs is considered a transporter-mediated process, as PGs diffuses poorly through the lipid bilayer. It is further confirmed by our findings that anion transporters participates in PGE_2-mediated Smad2-Smad4 complex formation. Anion transporters facilitate PGE_2 traversing the plasma membrane, which promotes Smad2-Smad4 complex formation. Recently, specific prostaglandin transporter PGT has been cloned [16]. It has been thought that PGT takes up and oxidizes PGE_2 to the inactive metabolites, thus terminating PGE_2 actions [25]. However, our findings imply that PGE_2 take-up by PGT is the first step in novel intracellular function of PGE_2.

In summary, we examined the hypotheses that Smad2 is post-translationaly modified by PGE_2 and that PGE_2-mediated Smad2 modification might interfere with Smad2-Smad4 complex formation. We found that epitope at the N-terminus of Smad2 is masked by PGE_2. Smad2 modification by PGE_2 was further confirmed in both endogenous Smad2 of HMC and in purified Smad2 protein by bio-PGE_2-avidin pulldown assay. Co-IP demonstrated that PGE_2 promoted the interaction of Smad2 with Smad4 and that DIDS, an anion transporter inhibitor, inhibited PGE_2-mediated Smad2-Smad4 complex formation. In addition, we showed that PGE_2 had no effects on Smad2 phosphorylation. These data suggested that post-translational modification of Smad2 may be a mechanism for the action of PGE_2 and could contribute to PGE_2 action in the pathogenesis of renal disease.

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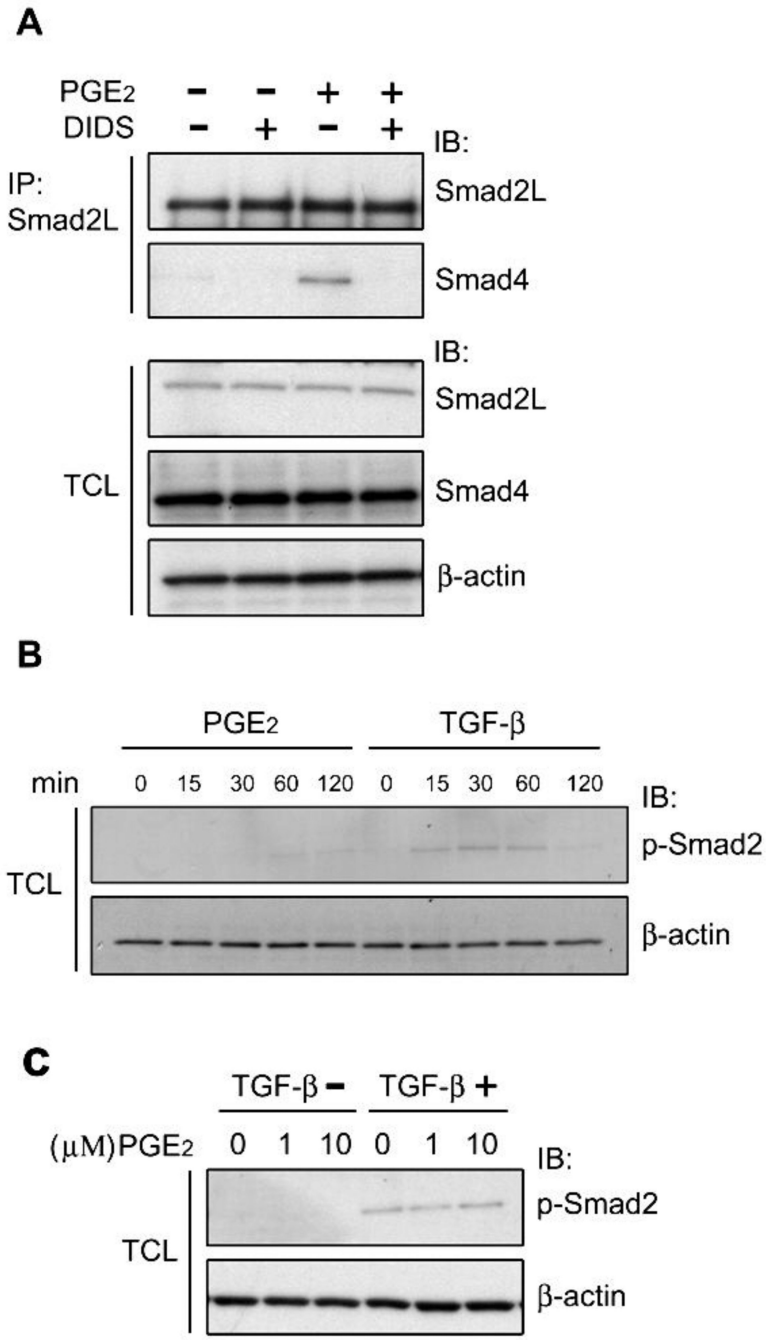

Fig. 1.

Effects of PGE_2 treatment upon Smad2-Smad4 complex formation. *A*, Serum-starved HMC were pretreated with DIDS for 30min, followed by stimulation with 1μM PGE_2 for 2h at 37°C. Cell lysates were collected and immunoprecipitated with the Smad2L antibody. Precipitated Smad2 and Smad4 were analyzed by immunoblotting. Expression of endogenous proteins was detected by immunoblotting. β-actin was used as a loading control. *B*, HMC were treated with 1 μM of PGE_2 or 10ng/ml TGF-β for the indicated time. TCL were collected and phosphorylated Smad2 was examined by immunoblotting. *C*, HMC were

pretreated with 1 or 10 μμ of PGE_2 for 45min, followed by stimulation with 10ng/ml TGF-β for another 30min. Cell lysates were collected and phosphorylated Smad2 was examined by immunoblotting. β-actin was used as a loading control.

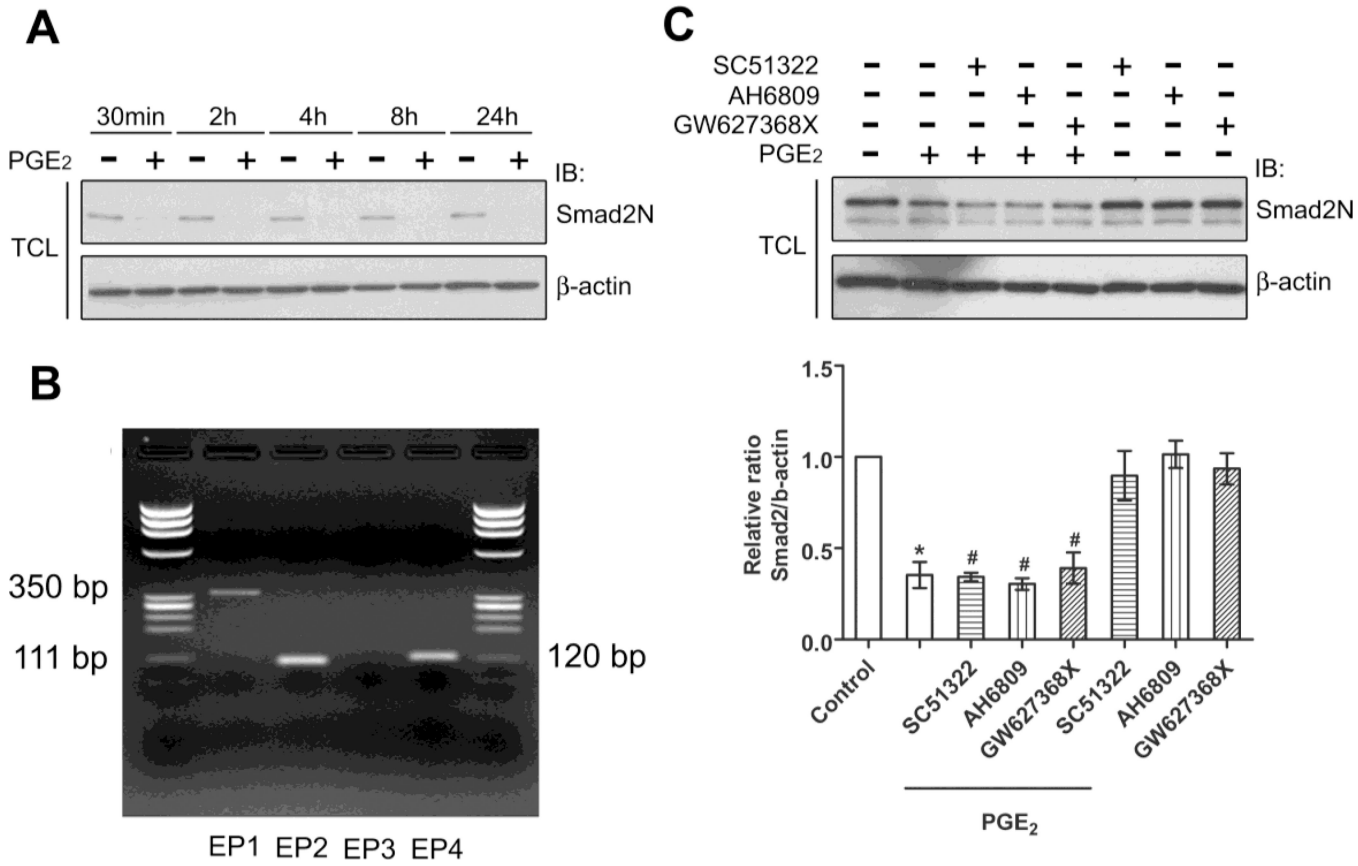

Fig. 2.

The epitope of endogenous Smad2 protein is changed after PGE_2 stimulation. *A*, Serum-starved HMC were stimulated with 1μM of PGE_2 for the indicated time. The total cell lysates (TCL) were collected and endogenous Smad2 protein was examined by immunoblotting with the Smad2N antibody, raised against a peptide near the N-terminus of Smad2 protein. *B*, Total RNA was isolated from HMC, and subjected to RT-PCR for EP1, EP2, EP3, and EP4 mRNAs using specific primers. *C*, Serum-starved HMC were pretreated with selective antagonists of EPs, SC51322 (EP1), AH6809 (EP2), or GW627368X (EP4) for 30min followed by stimulation with PGE_2 for 60min, and endogenous Smad2 was examined by immunoblotting with the Smad2N antibody. The lower panel shows the results of three independent experiments (means± s.e.m). * , p <0.001 versus control; #, p> 0.05 versus PGE_2-treated group.

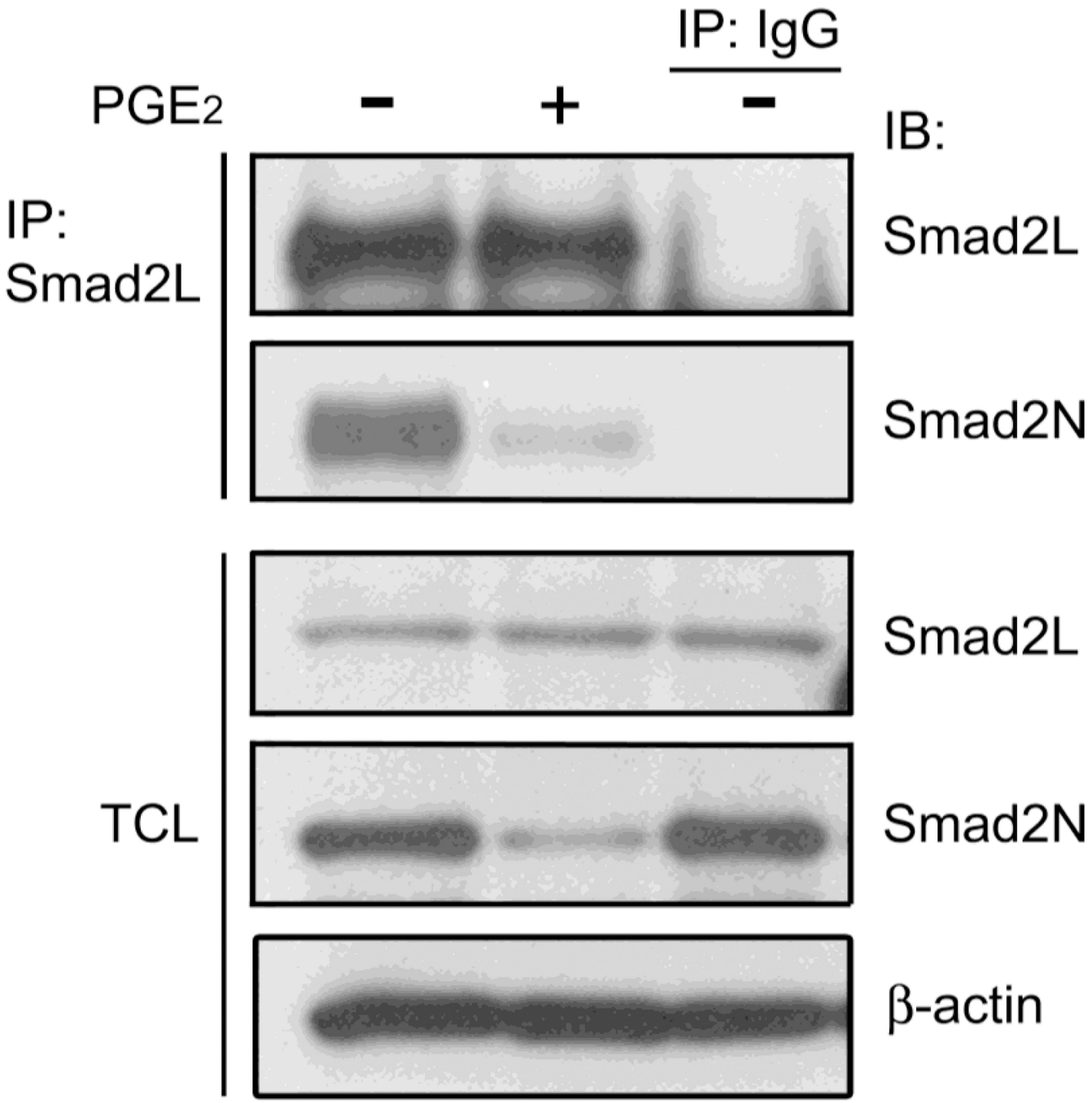

Fig. 3.

The level of Smad2 protein is not affected by PGE_2. Serum-starved HMC were stimulated with PGE_2 for 2h. The TCL were collected and endogenous Smad2 protein was pulled down with by the Smad2L antibody, raised against a peptide near the link region of Smad2 protein. The precipitated Smad2 protein and Smad2 protein in TCL were examined by the Smad2L and Smad2N antibodies, respectively. β-actin was used as a loading control.

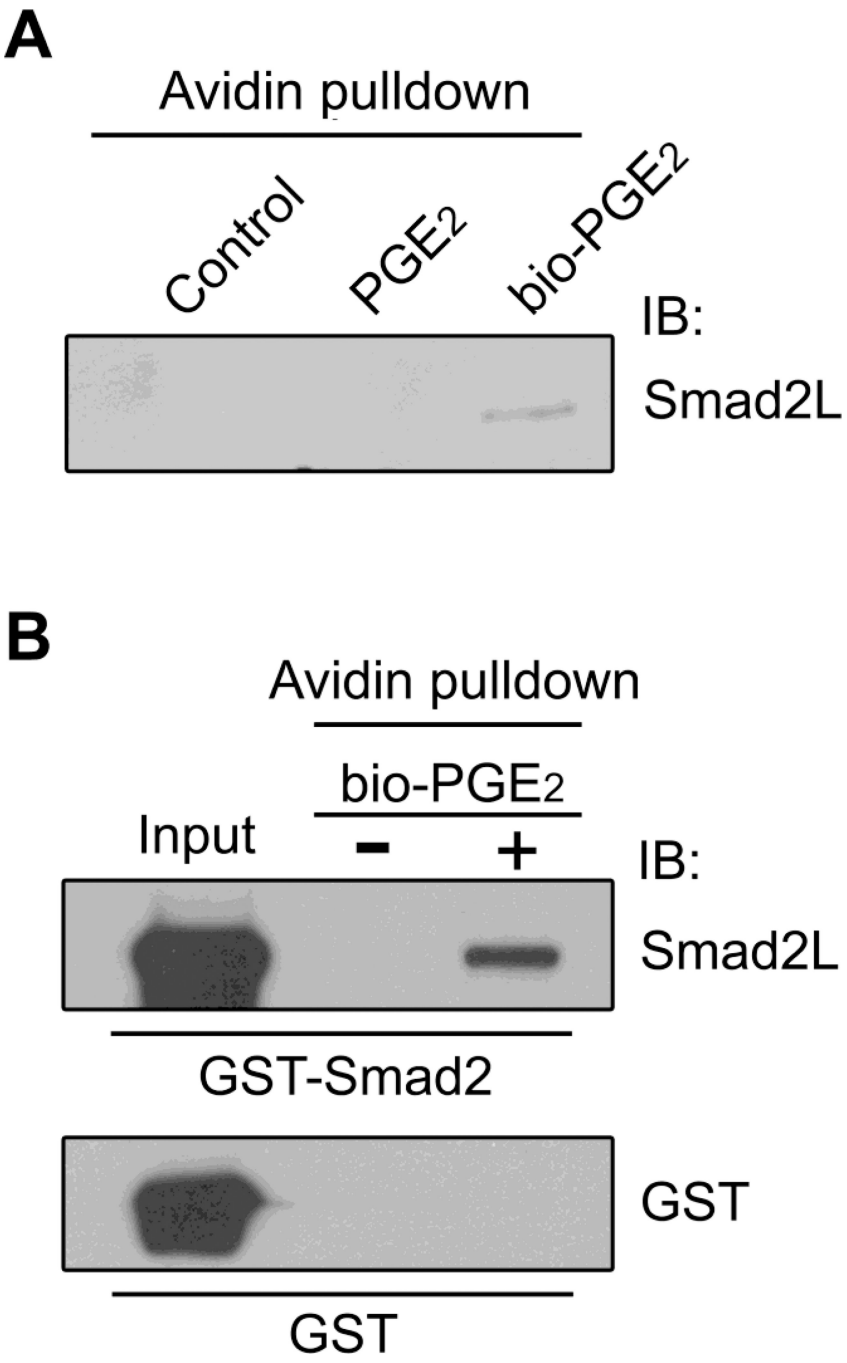

Fig. 4.

Smad2 protein post-translational modification by PGE_2. *A*, HMC were treated with 1μM of PGE_2 or bio-PGE_2 for 2h at 37°C. NeutrAvidin pulldown was then performed as described in experimental procedures. Resin-bound proteins were analyzed by immunoblotting with the Smad2L antibody. *B*, Purified 2 μg of GST or GST-Smad2 protein was incubated with 1μM bio-PGE_2 for 2h at room temperature and then precipitated by NeutrAvidin-agarose.

Resin-bound proteins were analyzed by immunoblotting with GST antibody and the Smad2L antibody, respectively.