

**DHEA Attenuates PDGF-induced Phenotypic Proliferation of Vascular Smooth Muscle A7r5 Cells through Redox Regulation**

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## ABSTRACT

It is known that dehydroepiandrosterone (DHEA) inhibits a phenotypic switch in vascular smooth muscle cells (VSMC) induced by platelet derived growth factor (PDGF)-BB. However, the mechanism behind the effect of DHEA on VSMC is not clear. Previously we reported that low molecular weight-protein tyrosine phosphatase (LMW-PTP) dephosphorylates PDGF receptor (PDGFR)- $\beta$  via a redox-dependent mechanism involving glutathione (GSH)/glutaredoxin (GRX)1. Here we demonstrate that the redox regulation of PDGFR- $\beta$  is involved in the effect of DHEA on VSMC. DHEA suppressed the PDGF-BB-dependent phosphorylation of PDGFR- $\beta$ . As expected, DHEA increased the levels of GSH and GRX1, and the GSH/GRX1 system maintained the redox state of LMW-PTP. Down-regulation of the expression of LMW-PTP using siRNA restored the suppression of PDGFR- $\beta$ -phosphorylation by DHEA. A promoter analysis of GRX1 and  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS), a rate limiting enzyme of GSH synthesis, showed that DHEA upregulated the transcriptional activity at the peroxisome proliferator-activated receptor (PPAR) response element, suggesting PPAR $\alpha$  plays a role in the induction of GRX1 and  $\gamma$ -GCS expression by DHEA. In conclusion, the redox regulation of PDGFR- $\beta$  is involved in the suppressive effect of DHEA on VSMC proliferation

through the upregulation of GSH/GRX system.

**Keywords:**

Dehydroepiandrosterone

Platelet-derived growth factor

Vascular smooth muscle cells

Glutaredoxin 1

Glutathione

Low molecular weight –protein tyrosine phosphatase

## INTRODUCTION

Vascular smooth muscle cells (VSMC), the contractile component of blood vessels, play a critical role in the pathogenesis of atherosclerosis. VSMC express a set of smooth muscle-specific genes, which are characteristic of their contractile, differentiated phenotype [1]. VSMC undergo phenotypic modulation in response to environmental signals. Accelerated migration, proliferation, and production of extracellular matrix components by phenotypically modulated VSMC play a central role in the development of atherosclerotic lesions [1].

Platelet derived growth factors (PDGFs) bind to two cell-surface receptor-tyrosine kinases, PDGF receptor (PDGFR)  $\alpha$  and  $\beta$  [2]. PDGF-dependent activation of receptors causes a mitogenic signal transduction through the phosphorylation of specific tyrosines in the receptors [2-4].

Dehydroepiandrosterone (DHEA) and the sulfated prohormone of DHEA circulate at higher plasma concentrations than any other steroids. Both the occurrence and the clinical manifestation of coronary atherosclerosis have been inversely correlated with plasma levels of DHEA or DHEA sulfate [5]. DHEA has a wide variety of beneficial biological and physiological effects on the prevention of cardiovascular disease [6].

The redox status of sulfhydryl groups in proteins plays an important role in the regulation of cellular functions such as the synthesis and folding of proteins and regulation of the structure and activity of enzymes, receptors, and transcription factors [7]. Glutaredoxin (GRX), a glutathione (GSH)-dependent oxidoreductase, catalyzes the reduction of protein disulfide via a disulfide exchange reaction [8]. Previously, we reported that GRX1 plays an important role in regulating PDGF-BB-dependent signals through down-regulation of the tyrosine phosphorylation of PDGFR- $\beta$  [3]. The GSH/GRX1 system suppresses the PDGF-BB-induced tyrosine phosphorylation of PDGFR- $\beta$ , resulting in suppression of the PDGF-BB-dependent cell proliferation. Furthermore, we found a novel regulatory mechanism for PDGF-BB signaling involving the redox-dependent regulation of low molecular weight protein-tyrosine phosphatase (LMW-PTP) by GRX1 in a GSH-dependent manner [3]. Recently, we also reported that estradiol potentiates GSH/GRX1 redox potential in cardiomyocytes through upregulation of the gene expression of  $\gamma$ -glutamylcysteine synthetase, the rate limiting enzyme of GSH synthesis and GRX1 [9]. Then, we were interested in whether DHEA has any effects on the GSH/GRX1 system in VSMC exposed to PDGF-BB. In the present study, we demonstrate that DHEA attenuates PDGF-BB-induced VSMC proliferation

and phenotypic modulation. Importantly, we show that DHEA increases the expression of GRX and GSH synthesis. This increase in GSH/GRX1 redox potential stimulates the LMW-PTP to down-regulate the activity for tyrosine phosphorylation of PDGFR- $\beta$ .

## **Materials and methods**

### ***Reagents***

Rabbit antibodies against PDGFR- $\beta$  and phospho (Tyr-751)-PDGFR- $\beta$  were obtained from Cell Signaling Technology. PDGF-BB, GSH, GSSG, NADPH, 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), flutamide and p-nitrophenyl phosphate were from Sigma Chemical Co.. DHEA, hydrogen peroxide, and dithiothreitol (DTT) were from Wako Pure Chemicals (Osaka, Japan). Normal goat, rabbit and mouse IgG were from Sigma Chemical Co. 4-Acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid (AMS) was purchased from Molecular Probes. ICI182,780 was from Tocris (Ballwin, MO).

### ***Cell culture and proliferation.***

Rat embryonic thoracic aorta smooth muscle-derived A7r5 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured as described [10]. Briefly, cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. After the attainment of confluency (70-80%), the cells were incubated in serum-free DMEM containing 0.2% bovine serum albumin for 20-24 h. The proliferation of cultured cells was evaluated by

measuring attached live cells photometrically after staining with crystal violet. A7r5 cells incubated in the presence or absence of DHEA were placed in 100  $\mu$ l of medium/well in 96-well plates and cultured in medium containing 0.2 % BSA with or without 0.5 nM PDGF-BB for specific periods. Then the cells were fixed with 4 % paraformaldehyde in phosphate-buffered saline (PBS, pH 7.5), washed, and stained with 0.01 % crystal violet at room temperature for 20 min. Each well was extensively washed with water and dried. The stained cells were lysed by adding 100  $\mu$ l of lysis buffer A (10 % SDS and 0.1 N HCl), and the cell number was then estimated photometrically by measuring the absorbance at 570 nm using a microplate reader.

***Purification of recombinant LMW-PTP and generation of antibody against LMW-PTP***

LMW-PTP was purified with the glutathione S-transferase (GST) gene fusion system (Amersham Biosciences) according to the manufacturer's instructions. In brief, E.coli strain BL21 cells were transformed with pGEX6p-LMW-PTP, and protein expression was induced by isopropyl  $\beta$  -D-1-thiogalactopyranoside (IPTG). GST-fused LMW-PTP (GST-LMW-PTP) was affinity purified from cell lysates using glutathione-Sepharose 4B (Amersham Biosciences), and then digested with

PreScission protease. The cleaved GST was removed from the glutathione-Sepharose 4B, and LMW-PTP was purified. The LMW-PTP was used to immunize rabbits to generate anti-LMW-PTP antibodies, and also used for experiments concerning the redox regulation of LMW-PTP as described below.

### ***Immunoblot analysis***

Cultured cells were harvested and lysed for 20 min at 4°C in lysis buffer. The supernatants obtained by centrifugation of the lysates at 8000 x g for 15 min were used in subsequent experiments. Protein concentrations were determined using a BCA assay kit (Pierce). Protein samples were electrophoresed on 10, 12.5 or 15% SDS-polyacrylamide gels under reducing conditions, except for thiol-modified protein samples. The proteins in the gels were transferred onto nitrocellulose membranes, and were incubated with horseradish peroxidase-conjugated anti-IgG antibodies. Proteins in the membranes were visualized using the enhanced chemiluminescence detection kit (Amersham Biosciences) according to the manufacturer's instructions.

### ***Determination of redox states.***

The redox states of proteins were assessed by modifying free thiol with AMS [11]. Briefly, after incubation with or without PDGF-BB, cell lysates or proteins

were treated with trichloroacetic acid at a final concentration of 7.5% to denature and precipitate the proteins as well as to avoid any subsequent redox reactions. The protein precipitates were collected by centrifugation at 12000 x g for 10 min at 4°C. The pellets were rinsed in acetone and centrifuged twice, then, dissolved in a buffer containing 50 mM Tris-HCl (pH7.4), 1% SDS and 15 mM AMS. Proteins were then separated by 10% SDS-PAGE without using any reducing agents and blotted to nitrocellulose membranes. Proteins in the membranes were treated with 5% (w/v) nonfat dry milk and 0.1% Tween 20 in TBS solution for 1 h at room temperature, then further kept overnight at 4°C for visualization by immunoblotting as described above.

#### ***Determination of cellular glutathione levels.***

GSH and glutathione disulfide (GSSG) levels were measured as described previously [9] using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc, MD) according to the manufacturer's directions.

#### ***Generation of luciferase reporter constructs.***

A 2.0-kb fragment of the human GRX1 gene promoter (-2023 to -22) was amplified by PCR using *Pfu* turbo DNA polymerase (Stratagene). The primers used were /5'-GGA CTG AGT GAG AGG CAG ACA ATA GTC TCC -3'/ as a forward

primer, and /5'-CGG GAA GAA TCC TCA GTT GCA GGT ATT GCT TGG -3'/ as a reverse primer. The PCR product was subcloned into pUC18 to obtain pUC18-pro-GRX. PUC18-pro-GRX was digested with HindIII, and the resulting fragment containing the promoter region from -2023 to -22 was inserted into the HindIII site of the reporter vector pGL3-Basic (Stratogene) to give pGL3-pro-GRX. To generate a deleted form of the luciferase reporter construct (pGL3-pro-GRX-del), pGL3-pro-GRX was digested with KpnI and PvuII (Takara Biomedicals). Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro-GRX (-2023 to -22) as a template by using a QuickChange site-directed mutagenesis kit (Stratogene). The oligonucleotides used were; peroxisome proliferator-activated receptor (PPAR) response element (PPRE)-like 1 forward (5'-GGT CAG GAT ACC TAG CTA AAT ttT CAT TTG GTG AXA TAG AGG CCA TG -3'), and PPRE-like 1 reverse (5'-CAT GGC CTC TAT GTC ACC AAA TGA aaA TTT AGC TAG GTA TCC TGA CC -3'). The nucleotide sequence was confirmed by sequencing with an ALFexpress II system (Amersham Biosciences). We constructed a 50-bp chimeric promoter with two copies of synthesized fragment of the human  $\gamma$ -GCS heavy subunit gene promoter (-10625bp~-10613bp, Genebank Accession No. AL033397) containing a PPRE-like domain; AGATCACAGGTCA. It was annealed using a

forward sequence (5'-gac ggt acc AGA TCA CAG GTC ATT GAT AAG ATC ACA GGT Cag tgg agc tc-3') and a reverse sequence (5'-gag ctc cac TGA CCT GTG ATC TTA TCA ATG ACC TGT GAT CTg gta ccg tc-3'). The annealed product was subcloned into pUC18 to obtain pUC18-pro- $\gamma$ -GCS. pUC18-pro- $\gamma$ -GCS was digested with Kpn I and Sac I. The resulting fragment containing the promoter region (-10625 ~-10613) was inserted into the HindIII site of the reporter vector pGL3-Basic to give pGL3-pro- $\gamma$ -GCS. To generate a deleted version of the luciferase reporter construct (pGL3-pro- $\gamma$ -GCS-del), pGL3-pro- $\gamma$ -GCS was digested with KpnI and PvuII. Site-directed mutagenesis for luciferase vectors was performed with pGL3-pro- $\gamma$ -GCS as a template.

#### ***Luciferase activity assay.***

Each vector was introduced into A7r5 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The luciferase activity was assayed with cellular extracts by using a luciferase reporter assay system (Promega).

#### ***Quantitative RT-PCR.***

Quantitative RT-PCR was performed using the One Step SYBR® RT-PCR kit (Perfect Real Time, TAKARA BIO INC. Japan) according to the manufacturer's

directions. After the RT-PCR using Mx3000P (STRATAGENE), the products were analyzed using SMxPro™ Software version 3.00 (STRATAGENE). A 764-base-pair (bp) DNA  $\gamma$ -GCS heavy subunit cDNA was obtained by digesting a fragment (bp 865-1628) with PstI. The 330-bp oligonucleotides for GRX1 (rat GRX sequence, accession No. AF167981) were obtained using as a forward primer, 5'- GCA TGG CTC AGG AGT TTG TGA ACT GCA AGA TTC AG -3', and as a reverse primer, 5'- CCT TTC ATA ACT GCA GAG CTC CAA TCT GCT TCA GC -3'. The 547-bp oligonucleotides for  $\gamma$ -GCS (rat  $\gamma$ -GCS sequence, BC081702) were obtained using 5'-CCT CTG GAG ACC AGA GTAT GGG AGT TAC-3', and 5'-GCA GAT AGT GGC CAA CTG GTC ATA AAG G-3'. The 410-bp oligonucleotides for rat  $\beta$ -actin (BC063166) were obtained using, 5'-GAG CTA TGA GCT GCC TGA CG-3', and 5'-AGC ATT TGC GGT GCA CGA TG-3'.

#### ***RNA interference and transfections.***

Double-stranded small interfering RNAs (siRNAs) corresponding to rat GRX1 DNA sequences (GenBank accession No. NM-022278) (5'-ACU GCA AGA UUC AGU CUG GdTdT-3' [siRNA-GRX-1] and 5'-AAC GUG GUC UCC UGG AAU UdTdT-3' [siRNA-GRX-2]), and to rat LMW-PTP DNA sequences (NM-021262) (5'-CAC AUU GCA CGG CAG AUU AdTdT-3' [siRNA-LMW-PTP-1] and 5'-UGA

GAG AUC UGA AUA GAA AdTdT -3'[siRNA- LMW-PTP -2]) were synthesized and annealed by Samchully Pharm Co., Ltd., Korea. siRNAs were transfected into the cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 100 nM.

### ***Statistical analysis***

Data were presented as the mean  $\pm$  SD. Differences were examined by using ANOVA (StatView software). A value of  $P < 0.05$  was considered significant.

## **RESULTS AND DISCUSSION**

### ***DHEA suppresses PDGF-induced proliferation of A7r5 cells***

The effect of DHEA on PDGF-induced cell proliferation was examined in A7r5 cells. Serum-starved A7r5 cells were cultured with or without 100 nM DHEA for 18 h, then in medium containing 0.2% FBS with or without 2 nM PDGF-BB. As shown in Figure 1A, cell proliferation induced by PDGF-BB was suppressed by pretreatment with 100 nM DHEA. DHEA at 100 and 200 nM suppressed the PDGF-BB-induced proliferation in a dose dependent manner (Figure 1B). The data indicate that DHEA suppresses the PDGF-induced proliferation of A7r5 cells, consistent with the report by Williams, M.R. *et al.* [12], in which the precise mechanism of the effect of DHEA on the PDGF-BB-induced proliferation of VSMC was not clear.

### ***Suppression of VSMC marker genes by PDGF-BB is restored by DHEA***

Next, the effect of DHEA on the expression of VSMC marker genes was examined. As VSMC marker genes, SM $\alpha$ -actin and SM22 $\alpha$  were estimated by the quantitative RT-PCR method. After incubation with or without 100 nM DHEA for 18 h, A7r5 cells were treated with 2 nM PDGF-BB for 24 h. As shown in Figure

1C, the expression of SM22 $\alpha$  and SM $\alpha$ -actin was suppressed by treatment with PDGF-BB, whereas the suppressive effect of PDGF-BB on the VSMC phenotype was restored by DHEA. Taken together, DHEA attenuates the PDGF-induced proliferation and phenotypic switch in VSMC, suggesting that DHEA has some effects on the PDGF-BB-dependent cell signaling. Then, PDGF-BB-induced phosphorylation of PDGFR- $\beta$  was examined.

***DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR- $\beta$***

PDGFR- $\beta$  -mediated signals are particularly important for vascular remodeling and neointima formation [13]. As shown in Figure 2A and 2B, A7r5 cells expressed abundant PDGFR- $\beta$ . Then, phosphorylation of PDGFR- $\beta$  was estimated by immunoblot analysis using rabbit antibodies against PDGFR- $\beta$  and phosphor-(Tyr-751)-PDGFR- $\beta$ . PDGF-BB-induced phosphorylation of PDGFR- $\beta$  was observed at 5 - 30 min, with a peak at 5 - 10 min (lanes 2-3). Pretreatment with 100 nM DHEA suppressed the phosphorylation of PDGFR- $\beta$  (lanes 6-7). The suppression of the phosphorylation of PDGFR- $\beta$  by DHEA was dose-dependent (25-250 nM, data not shown). Then, the possible suppression of PDGFR- $\beta$ -mediated signaling was examined.

***DHEA induces the expression of GRX1 and  $\gamma$ -GCS mRNA and increases the level of GSH***

PDGFR-mediated signaling is regulated by many factors. These factors are involved in the generation of reactive oxygen species and redox regulation [3]. We were interested in the role of the redox regulation of PDGFR- $\beta$  by DHEA. The effect of DHEA on the mRNA expression of redox-related proteins, and the levels of GSH and oxidized glutathione (GSSG) were analyzed. A7r5 cells incubated with or without 100 nM DHEA for 18 h were treated with 2 nM PDGF-BB for 12 h. The expression was expressed as the relative intensity compared to the control. Treatment with DHEA increased GRX1 mRNA and  $\gamma$ -GCS, a rate-limiting enzyme for GSH synthesis (Figure 2C). The level of GSH was increased by DHEA, while the level of GSSG was not changed by DHEA, resulting in the high GSH/GSSG ratio (Figure 2D). These results indicate that DHEA increases the expression of GRX1 and  $\gamma$ -GCS to elevate the GSH/GRX1 redox potential as well as the GSH/GSSG ratio.

***DHEA-dependent promoter activity of GRX1 and  $\gamma$ -GCS gene is regulated by PPAR $\alpha$***

DHEA upregulates the transcriptional activity mediated by PPAR $\alpha$  [14,15]. This mechanism involves upregulation of the expression of PPAR $\alpha$  mRNA by DHEA [15]. To investigate the transcriptional regulation of GRX1 and  $\gamma$ -GCS by DHEA via the PPAR $\alpha$ -binding domain, a luciferase vector containing PPRE-like domain was constructed and introduced into A7r5 cells. The luciferase activity of the cells treated with DHEA for 18 h showed a 1.8-fold increase, but was almost lost when the PPRE-like site was deleted or mutated (Figure 3A). Deletion of EpRE-like 2 or SP1 had no apparent effect on the DHEA-induced up-regulation of the luciferase activity (data not shown). Similarly, the promoter region of the  $\gamma$ -GCS heavy subunit containing a PPRE-like site was inserted into a luciferase vector. The luciferase activity of the cells treated with DHEA was stimulated by 1.6 fold, but was lost when the PPRE-like site was mutated (Figure 3B). The results suggest that PPAR $\alpha$  plays a role in the DHEA-induced up-regulation of the expression of GRX1 and  $\gamma$ -GCS.

### ***DHEA maintained the redox state of LMW-PTP***

PDGFR is a receptor-type tyrosine kinase, the activation of which is regulated by PDGF-dependent autophosphorylation and dephosphorylation by protein tyrosine phosphatases (PTPs). A number of tyrosine residues are phosphorylated in the cytosolic domain of PDGFR, leading to a site-specific recruitment of signal transduction molecules [4]. PTPs such as LMW-PTP are implicated in the control of PDGFR phosphorylation [16]. LMW-PTP is an 18-kDa enzyme that is widely expressed [17]. We focused on LMW-PTP because its redox-dependent regulation and the role in PDGF-BB/PDGFR signaling have been studied extensively [4]. Oxidative stress generated by PDGF-BB/PDGFR- $\beta$ -mediated signaling causes the oxidization of LMW-PTP to form dithiothreitol-reducible high molecular weight oligomers, and the generation of peroxide is involved in the downregulation of LMW-PTP activity by PDGF-BB, leading to the activation of PDGFR-BB-dependent signaling pathways [3]. Figure 4A shows that a decrease in the reduced form of LMW-PTP (the active form) was observed on treatment with PDGF-BB for 30 min (lane 2). The decrease in levels of the reduced form caused by PDGF-BB was similar to that observed on treatment with hydrogen peroxide (lane 5). Pretreatment of cells with DHEA for 18 h maintained the reduced form of LMW-PTP (lane 4).

In this experiment, oligomerized LMW-PTP (the inactive form) was not detectable for unknown reason, and LMW-PTP-specific phosphatase activity was not determined. Together with our previous report using synthesized LMW-PTP and GSH/GRX1 redox system [3], the data suggest that DHEA maintained the redox state of LMW-PTP regulated by the GSH/GRX1 system.

***Transfection of siRNAs for GRX1 and LMW-PTP abolishes the effect of DHEA on PDGF-BB-induced phosphorylation of PDGFR- $\beta$***

To down-regulate the expression of GRX1 and LMW-PTP, specific siRNA (100 nM) were introduced into A7r5 cells. siRNAs bearing scrambled sequences were used as the control. At 48 h post-transfection, cells were serum-starved for 6 h, then stimulated with 2 nM PDGF-BB. Compared to the PDGF-BB-induced phosphorylation of PDGFR- $\beta$  in the cells transfected with siRNA bearing scrambled sequences (Figure 4B, lanes 2-4), the transfection of siRNA for GRX1 further stimulated the PDGF-BB-induced phosphorylation of PDGFR- $\beta$  (lanes 5-8). Similarly, the cells transfected with the specific siRNA (100 nM) for LMW-PTP for 48 h showed an increase in PDGF-BB-induced phosphorylation of PDGFR- $\beta$  (lanes 9-12). The results confirm that (i) DHEA increases the GSH/GRX1 redox potential,

(ii) the GSH/GRX1 system is necessary to regulate the phosphorylation of PDGFR- $\beta$ , (iii) the activity of LMW-PTP maintained by DHEA regulates the phosphorylation, of PDGFR- $\beta$  and (iv) the LMW-PTP-dependent dephosphorylation is regulated by the GSH/GRX1 system. To know whether the DHEA-dependent suppression of the PDGF-BB-induced phosphorylation of PDGFR- $\beta$  is mediated through specific receptors for androgen or estradiol, the effect of antagonists against androgen receptor (flutamide) and estrogen receptor  $\alpha/\beta$  (ICI182,780) was examined. It was found that the antagonists had no apparent effect on the DHEA-induced suppression of the phosphorylation (Figure 4D, lane 4 vs. lane 8 and lane 12). The results are consistent with the previous report that the inhibitory effect of DHEA on PDGF-BB-induced proliferation of VSMC is independent of the androgen receptor and estrogen receptor [12].

In summary, this study showed that DHEA inhibits PDGFR- $\beta$  phosphorylation that leads to proliferation and phenotypic changes of VSMC, and that the transcriptional control of the GSH/GRX level and the redox state of LMW-PTP may account, at least in part, for the beneficial effects of DHEA on VSMC.

## **FOOTNOTES**

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## **FIGURE LEGENDS**

### **Figure 1. Effect of DHEA on VSMC proliferation and expression of VSMC marker genes.**

Time-dependent (A) and dose-dependent (B) effects of DHEA on PDGF-BB-induced proliferation were estimated. (C), The expression of SM22 $\alpha$  and SM $\alpha$ -actin was estimated by the quantitative RT-PCR method as described in “Materials and Methods”. After incubation of A7r5 cells with or without 100 nM DHEA for 18 h, the cells were treated with 2 nM PDGF-BB for 24 h. Each value represents the mean for three independent experiments, and the S.D. was always within 10% of the mean. \*,  $p < 0.05$  compared with the control vehicle-treated cells.

### **Figure 2. DHEA down-regulates the PDGF-BB-dependent phosphorylation of PDGFR- $\beta$ (A, B), and up-regulates the mRNA expression of GRX1 and $\gamma$ GCS (C) and GSH levels (D).**

A7r5 cells were serum-starved for 24h. After pretreatment with 100 nM DHEA for 18h, the cells were stimulated with 2 nM PDGF-BB for the periods indicated. (A), the phosphorylation status of PDGFR- $\beta$  was examined by immunoblot analysis. (B), The intensity of the bands was estimated densitometrically, and the

phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR- $\beta$  to PDGFR- $\beta$  protein. (C), mRNA expression of GRX1 and  $\gamma$ -GCS heavy subunit was analyzed by quantitative RT-PCR. The expression was expressed as relative intensity compared to the control. (D), Levels of GSH and GSSG were estimated using a Total Glutathione Quantification Kit and were expressed as relative intensity compared to the control. Each value represents the mean for three independent experiments, and the S.D. was always within 10% of the mean. \*,  $p < 0.05$  compared with the control vehicle-treated cells.

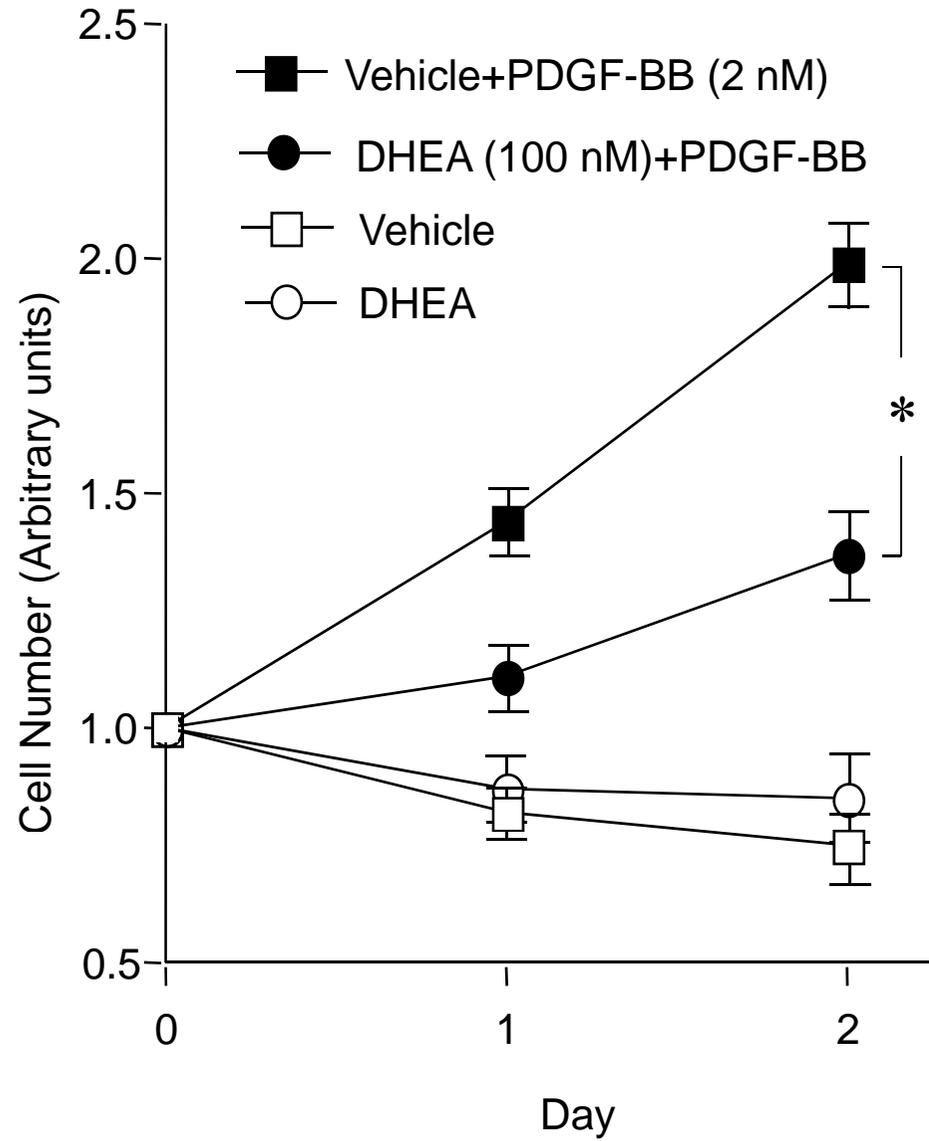
**Figure 3. PPRE-like element is important for the DHEA-dependent induction of the GRX1 and  $\gamma$ -GCS promoter in A7r5 cells.**

The transcriptional regulation of GRX1 and  $\gamma$ -GCS by DHEA was examined. The cells were transiently transfected with the GRX1 promoter-luciferase gene fusion plasmids (A) or the chimeric  $\gamma$ -GCS promoter-luciferase gene (B). After the transfection, luciferase activity was assayed with cellular extracts as described in “Materials and Methods”.

**Figure 4. The role of LMW-PTP, GRX1 and sex hormone receptors in the effects of DHEA on PDGF-BB-induced phosphorylation of PDGFR- $\beta$ .** (A), Effect of DHEA on the redox state of LMW-PTP was estimated. The redox state of proteins was assessed by modifying free thiol with AMS as described in “Materials and Methods”. (B, C), To down-regulate the expression of GRX1, a specific siRNA for GRX1 (100 nM) was introduced into A7r5 cells. siRNA bearing a scrambled sequence was used as the control. Similarly, specific siRNA for LMW-PTP (100 nM) was introduced into the cells. (D), Effects of an androgen receptor antagonist (flutamide) and an estrogen receptor  $\alpha/\beta$  antagonist (ICI182,780) on DHEA-induced suppression of PDGF-BB-dependent phosphorylation of PDGFR- $\beta$  were examined. (E), The intensity of the bands was estimated densitometrically, and the phosphorylation rate is expressed as the relative intensity of phosphorylated PDGFR- $\beta$  to PDGFR- $\beta$  protein.

Figure 1

A



B

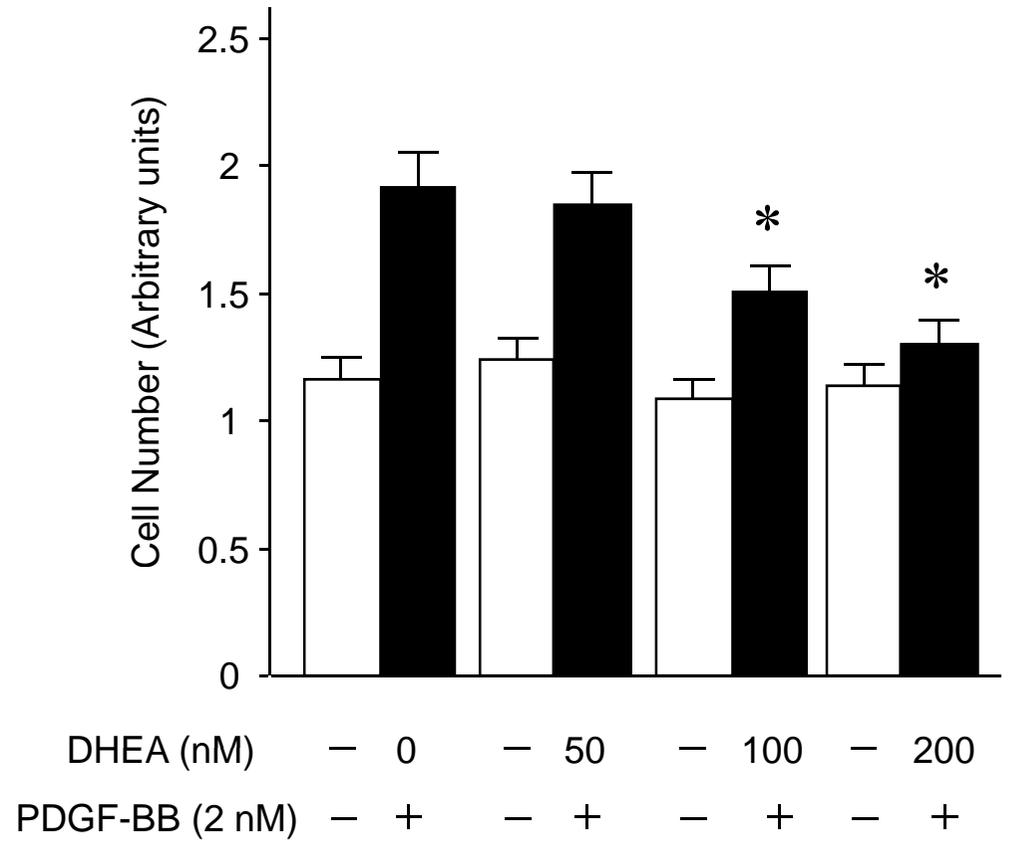


Figure 1

C

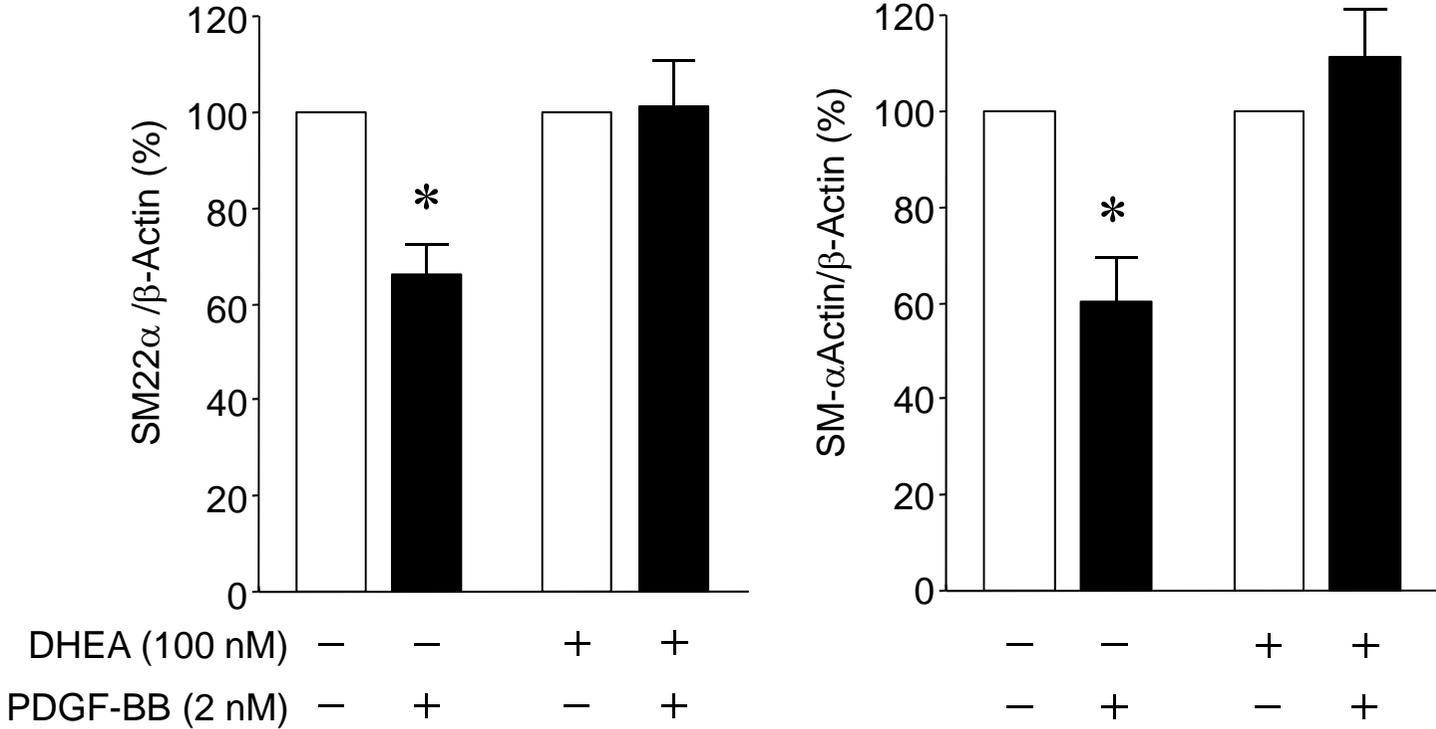
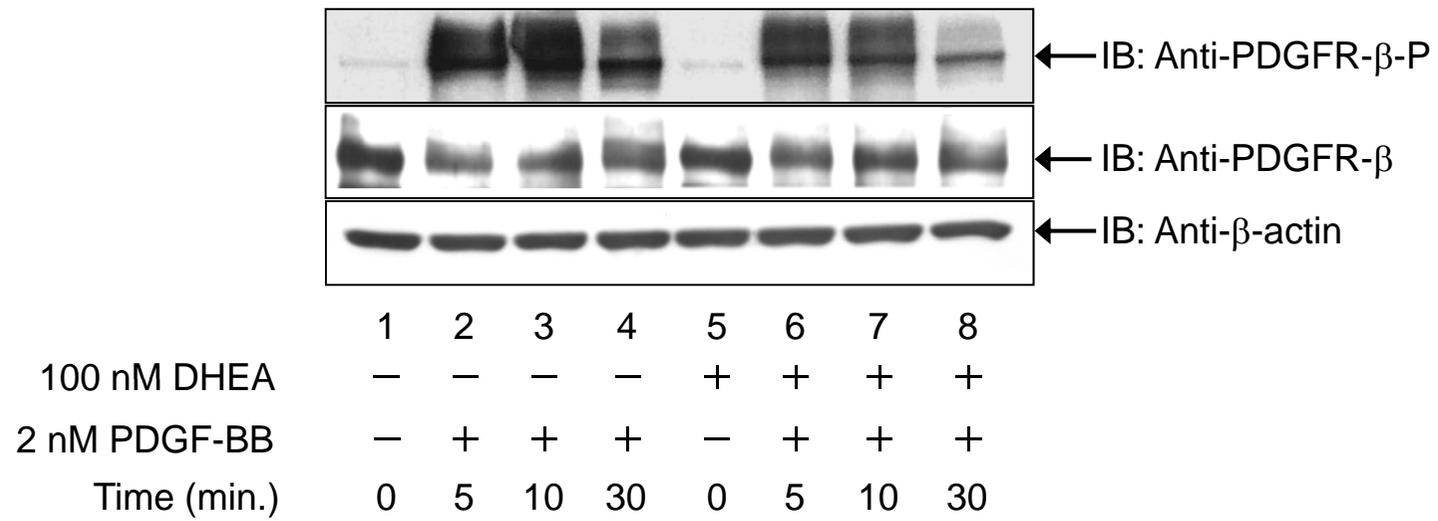


Figure 2

A



B

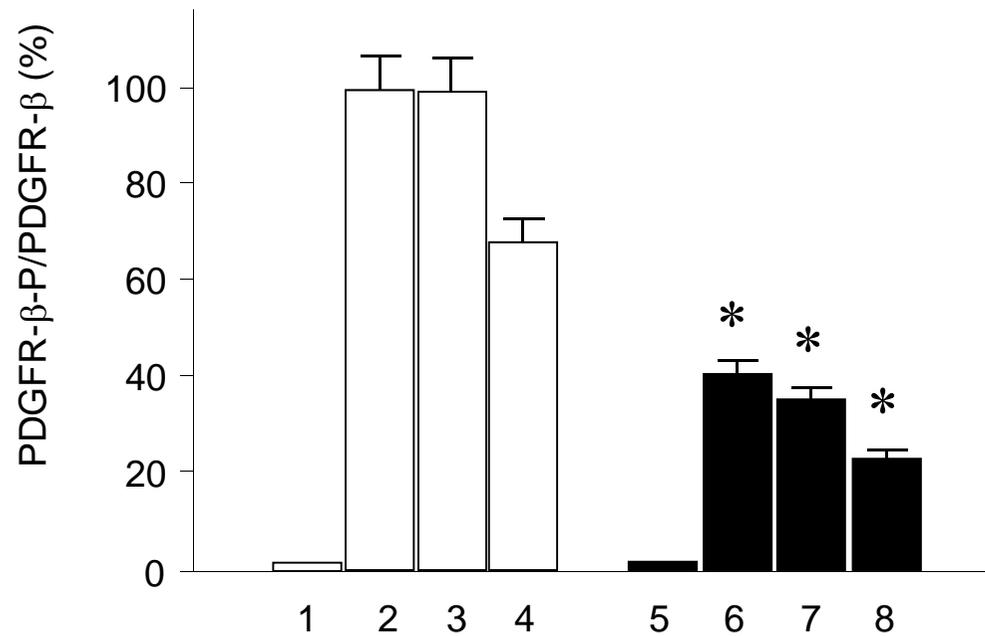
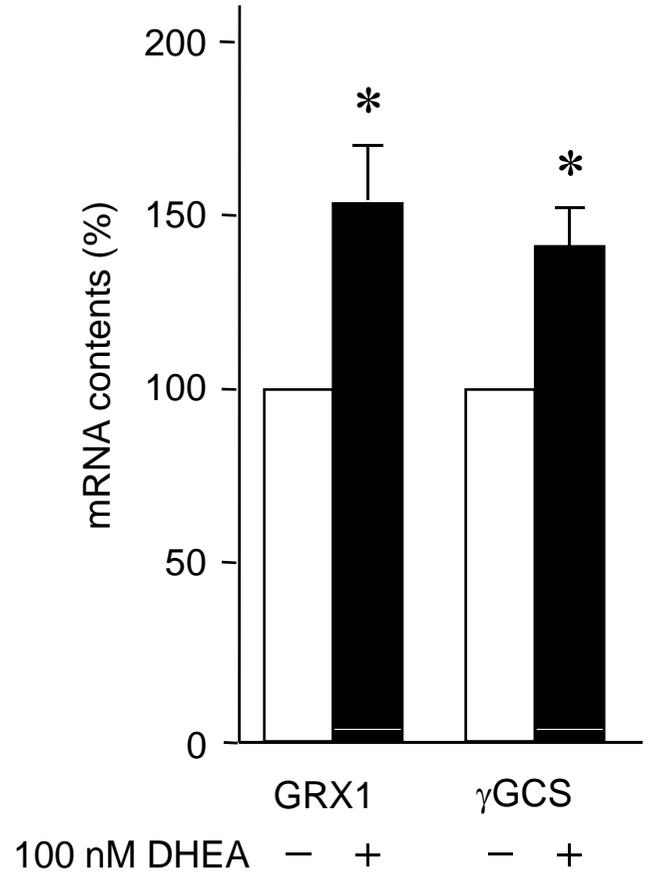


Figure 2

C



D

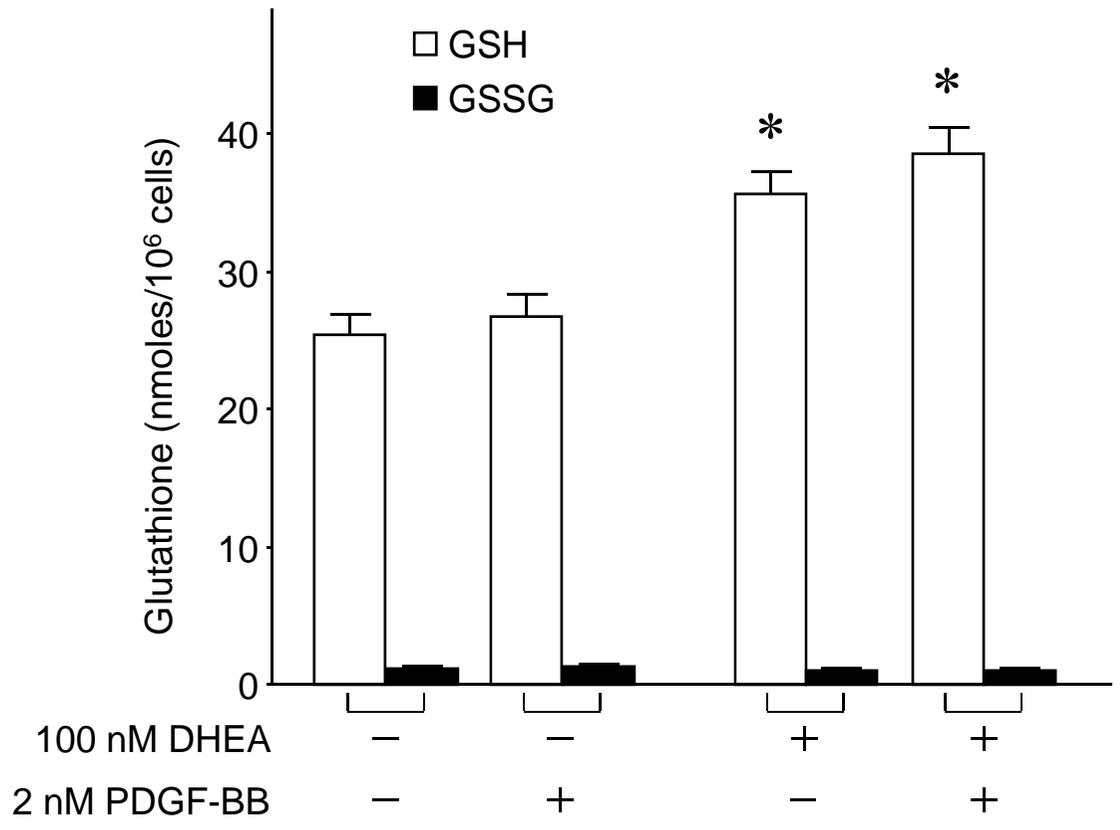
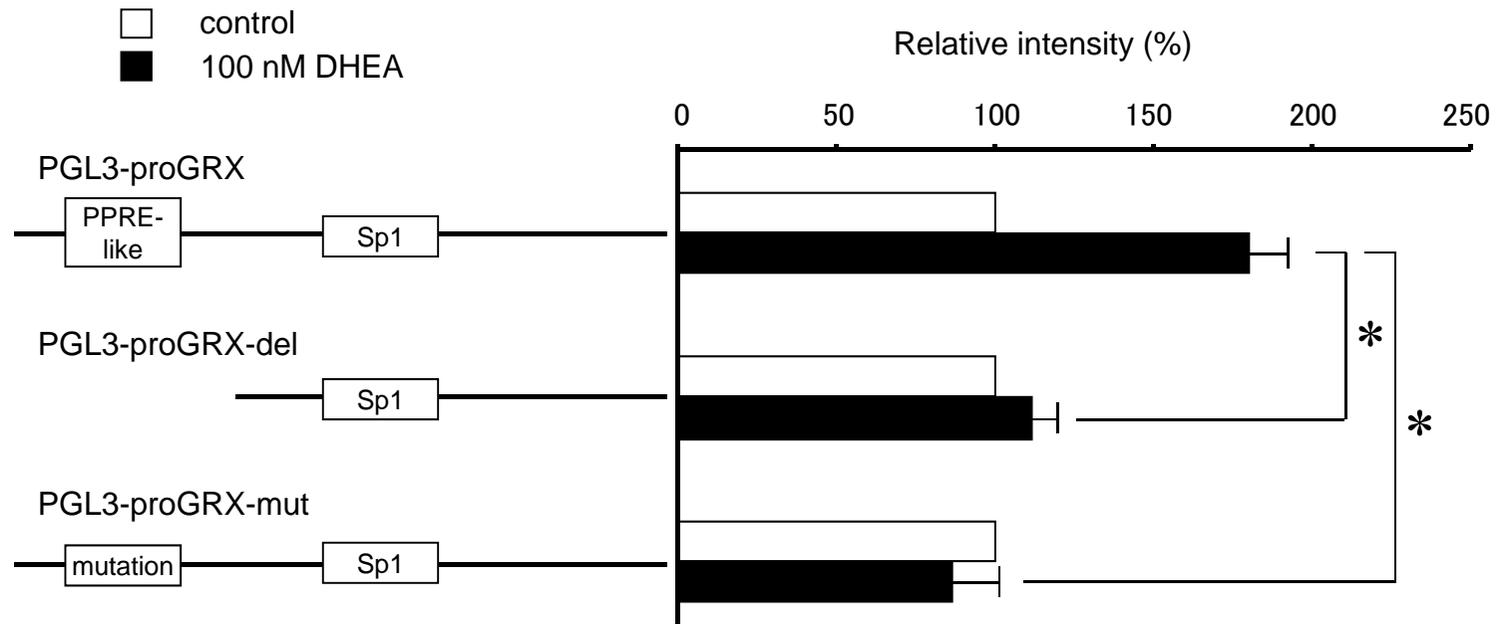


Figure 3

A



B

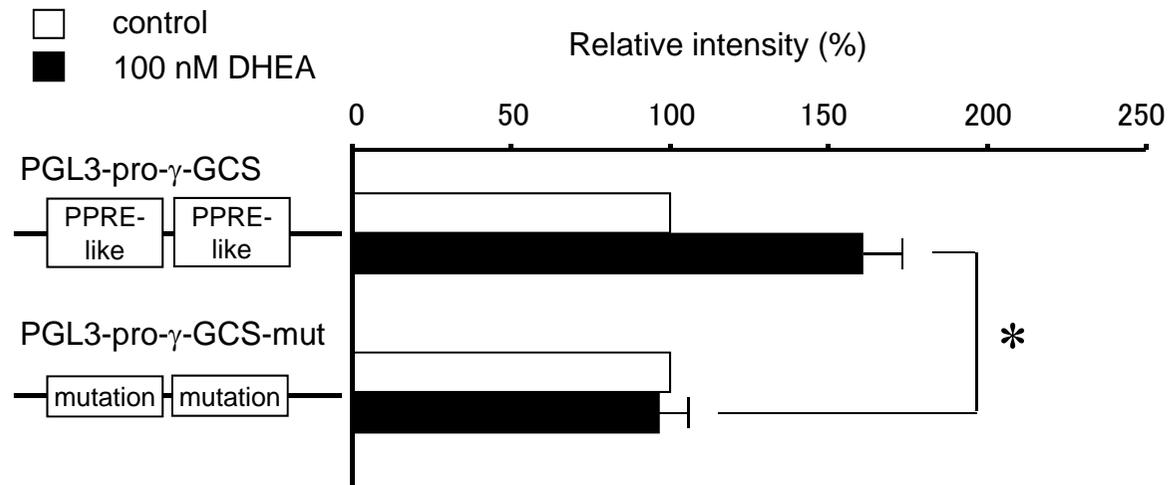
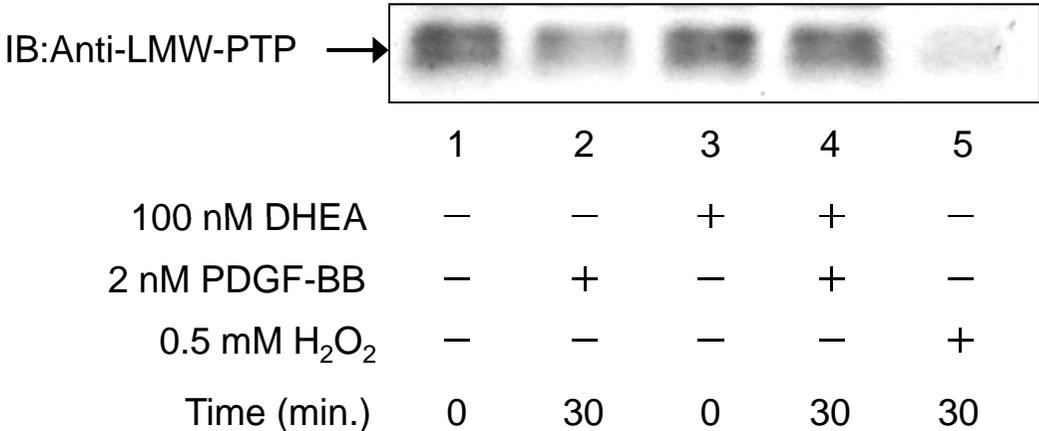


Figure 4

A



**Figure 4**

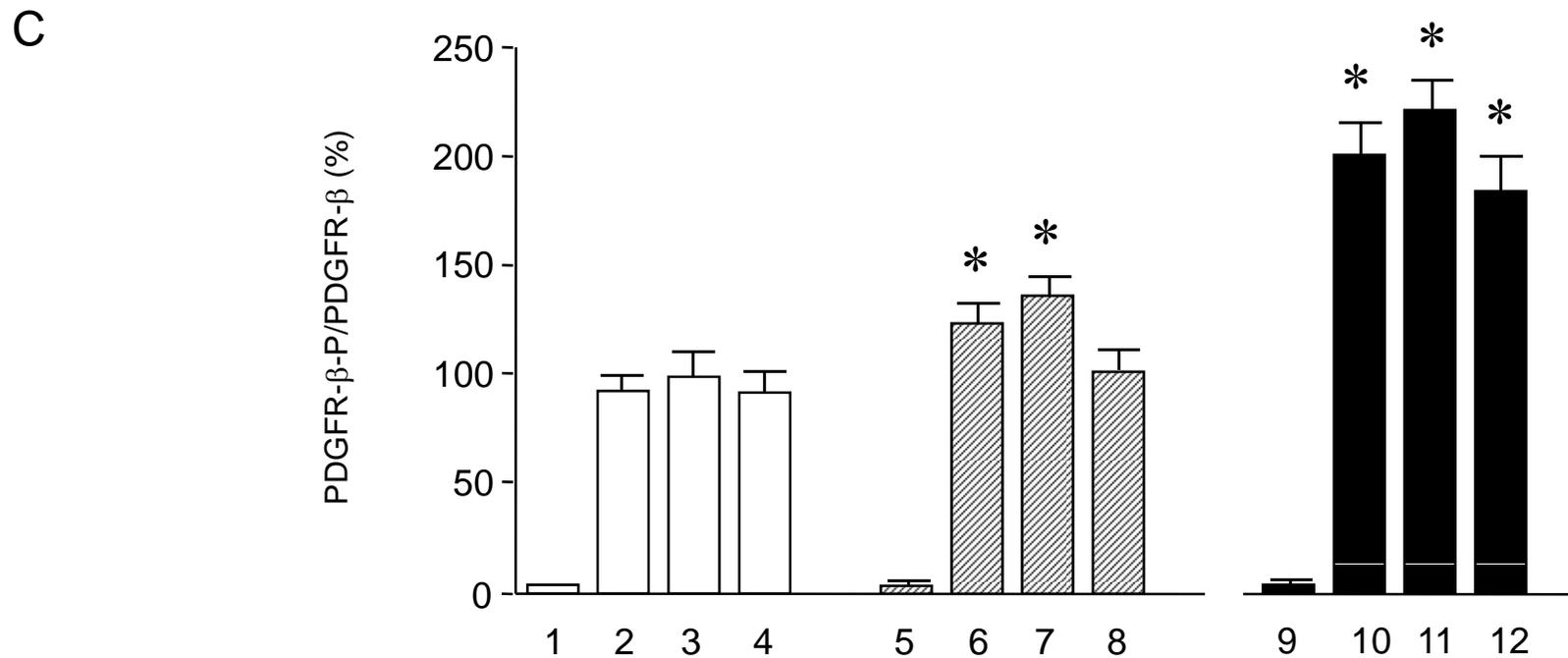
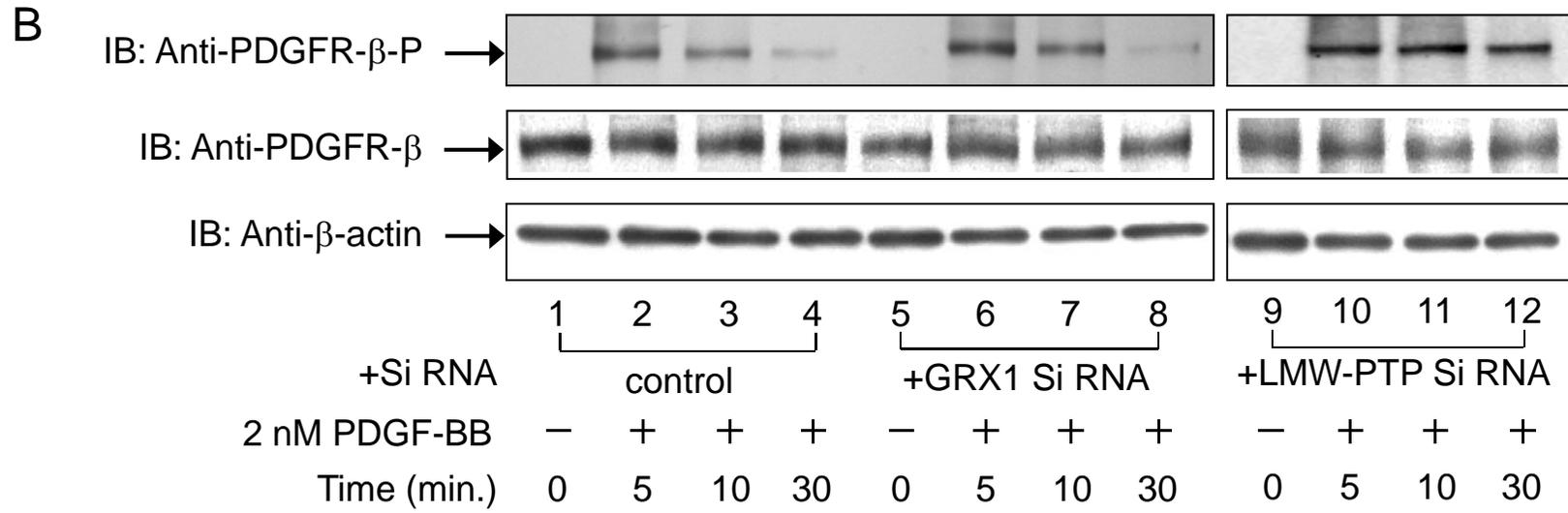
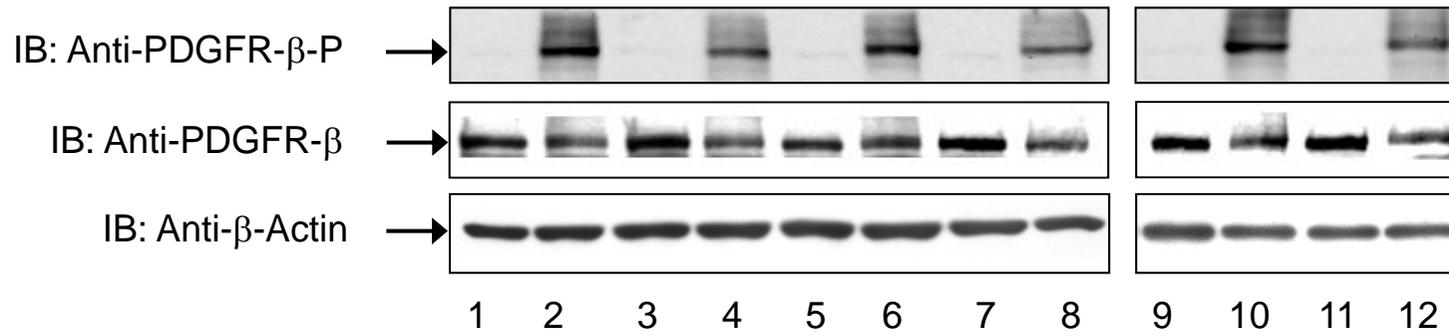


Figure 4

D



E

