ORIGINAL RESEARCH COMMUNICATION

Intracellular redox equilibrium is essential for the constitutive expression of AP-1 dependent genes in resting cells: studies on TGF-β1 regulation

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ABSTRACT

Constitutive genes are detected in cells in basal conditions. Stimuli such as reactive oxygen species (ROS) may up-regulate the expression of these genes, but the mechanisms involved in their continuous basal expression are unknown. As ROS concentrations are tightly maintained at intracellular level, we hypothesize that basal levels of ROS could be responsible for the expression of these constitutive genes. In this report, we analyze the regulation of an important constitutive gene, TGF-β1, after decreasing intracellular ROS concentration in human mesangial cells. Decreased intracellular hydrogen peroxide by catalase addition reduced basal TGF-β1 protein, mRNA expression and promoter activity. Furthermore, catalase decreased the basal activity of Activated Protein-1 (AP-1) that regulates TGF-β1 promoter activity. This effect disappeared when AP-1 binding site was removed. Similar results were observed with proteins whose promoter regions contains AP-1 binding sites as eNOS, but not with other constitutive genes as COX1 or PKGα, without AP-1 sites in their promoter. Pharmacological inhibition of ROS synthesis by blocking of NADPH oxidase, respiratory chain or xanthine oxidase induced a significant reduction of basal ROS concentration, similar to the observed with catalase. Similar changes were detected in human fibroblasts mutated in mitochondrial DNA. In any of these conditions, TGF-β1 expression decreased except with xanthine oxidase blockaded.

These findings suggest a novel role for the steady-state basal intracellular ROS concentration, which could be essential for the basal expression of constitutive AP1-dependent genes, as TGF-β1. Moreover, the different systems involved in the production of ROS could play different roles on this regulation.

Introduction

Genes are considered as constitutive when they are transcribed at relatively constant levels in cells under basal conditions. Unlike inducible genes, that are transcribed only when they are needed as a consequence of specific stimuli, constitutive genes are expressed continuously in cells, and it seems that they play significant roles in the maintenance of basic cell processes. Although the transcription level of constitutive genes seems to be rather constant, it has been reported that they can be also regulated by different stimuli, such as reactive oxygen species (ROS) (7,12,18,27,43)**.**

ROS consist of a variety of oxygen-derived small molecules with diverse structures. Some of these species, such as superoxide and hydroxyl radicals, are extremely unstable, whereas others like hydrogen peroxide (H_2O_2) are relatively long-lived and freely diffusible (7). They are generated in cells as a consequence of their basal function, as they are synthesized by a wide variety of enzymatic systems such as NAD(P)H oxidase, xanthine oxidase and arachidonic acid metabolizing enzymes as well as by the mitochondrial respiratory chain (7,26,38,41). Degradation of these ROS is also regulated by antioxidant enzymes, including superoxide dismutase, catalase (CAT) and glutathione peroxidase, among others. The balance between production and elimination is tightly regulated and ensures the proper maintenance of cellular metabolism and homeostasis. In general, moderate levels of ROS are involved in biological processes such as cell proliferation, differentiation and survival (11,37). Exogenous stimuli may also modulate ROS balance and in these cases transient ROS production can act as an intracellular signalling mechanism, regulating a broad variety of genes and cell functions (2,31,39). Among ROS, H_2O_2 is best studied as a second messenger. It mainly acts in the cells by modifying critical residues of proteins thereby regulating their catalytic activities or other functions (7,40).

TGF-β1 is a member of a large family of growth and differentiation factors that are involved in cell division, differentiation, migration, adhesion, death, extracellular matrix production, tissue homeostasis and embryogenesis (20,28,33). It express constitutively in a wide variety of cell types. TGF-β1 has a crucial role in tissue homeostasis and the disruption of the TGF-β1 pathway has been implicated in many human diseases, including cancer, autoimmune, fibrotic and cardiovascular diseases (20,29). Previous reports have showed that ROS can regulate TGF-β1 synthesis

(5,12,17,23,25,32) also through the activation of the transcription factor AP-1 (12). Taking into account this fact, we hypothesized that basal concentrations of ROS could be involved in the constitutive expression of TGF-β1.

Present experiments were devoted to analyze this hypothesis. Additional, the possible mechanisms involved in the observed effects were studied. Finally, the possibility of the different sources of ROS could play different roles in the regulation of the constitutive expression of TGF-β1 was also tested.

Results

CAT-dependent reduction of intracellular H_2O_2 decrease TGF-β1 protein content in human mesangial cells (HMC).

In order to reduce the basal levels of H_2O_2, HMC were incubated for 16 h with various CAT concentrations. As previously reported (24,34), the intracellular CAT activity significantly increased (Fig. 1A) and a dose-dependent reduction of intracellular ROS concentration, measured by flow cytometry with fluorescent dye DCFHDA, was observed (Fig. 1B). As controversy exists regarding the specificity of this dye for H_2O_2, we pursued cellular imaging approaches to analyze intracellular H_2O_2 concentration using the biosensor HyPer (1), transfected into HMCs. Briefly, HyPer is a genetically encoded, highly specific fluorescent probe for detecting H_2O_2 inside living cells. This probe consists of circularly permuted yellow fluorescent protein (cpYFP) inserted into the regulatory domain of the prokaryotic H_2O_2-sensing protein, OxyR. Sensitivity of the probe is sufficient to observe H_2O_2 increase upon physiological conditions (1,13). The incubation of cells with H_2O_2 significantly increased the fluorescent signal, whereas CAT incubation suppressed the basal H_2O_2 generation (Fig. 1C).

In these conditions of CAT-dependent reduction of intracellular H_2O_2, TGF-β1 protein synthesis was evaluated by three means. Figure 2A shows a dose-dependent reduction of TGF-β1 protein evaluated by immunoblot. When TGF-β1 content was analyzed in intact cells by immunofluorescence, CAT also reduced this protein (Fig. 2B). Finally, total immunoreactive TGF-β1, measured by ELISA, decreased in the supernatants of HMC incubated with CAT (Fig. 2C). Phorbol 12-myristate13-acetate (PMA), a potent stimulus for TGF-β1 synthesis (9) was used as positive control (Fig. 2,B-C).

Mechanisms involved in the CAT-dependent reduction of TGF-β1 protein content in human mesangial cells (HMC).

As we previously demonstrated that H_2O_2 augmented TGF-β1 protein synthesis by increasing the steady-state levels of its mRNA (12), we first analyzed mRNA expression by quantitative-RT-PCR after catalase addition to HMC. We could observe a significant decrease of mRNA expression

(Fig. 3A). This reduction seemed to be due to the CAT-dependent reduction of the TGF-β1 promoter activity, evaluated by measuring of luciferase activity in cells transfected with constructions in which luciferase was under the control of the TGF-β1 promoter (Fig. 3B). Since this promoter contains at least two AP-1 binding sequences (15) and it is also regulated by ROS (21,30,38), we analyzed the activity of TGF-ß1 promoter after deletion of different response sites, in order to test the implication of AP-1 on the decreased TGF-β1 promoter activity under CAT treatment. As shown in Figure 3C, CAT inhibited the activity of the complete promoter, as well as the construction containing AP-1 site (phTG5), but did not modify activity in cells transfected with the plasmid phTG6, that did not contain the consensus sequence for AP-1.

In order to ensure the implication of AP-1 in TGF-β1 down-regulation induced by CAT, we analyzed CAT ability to modulate the basal activity of this transcription factor. For this purpose, EMSA assays were performed in HMC incubated with different CAT concentrations. As shown in Figure 3D, a significant dose-dependent reduction of the interaction between AP-1 specific oligonucleotide and nuclear proteins occurred after CAT addition. This finding was confirmed by studying the activity of the 3TP-Lux plasmid, a construction in which luciferase activity is under the control of three AP-1 sites, in HMC treated with CAT. Again, CAT induced a dose-dependent decrease of the activity of this plasmid (Fig. 3E).

Considering together these results, it appears that basal intracellular concentration of ROS regulates the activity of AP-1 transcription factor, with the subsequent activation of genes under the control by this factor, particularly TGF-β1. To ascertain this hypothesis, the ability of CAT to modulate other proteins with different regulatory elements in their promoters was tested. The results are collected in Figure 4. Catalase-dose-dependent decrease in eNOS protein content was observed in endothelial cells (Fig. 4A), whereas no changes in cyclooxygense-1 (Fig. 4B) and protein kinase Gα1 (Fig. 4C) protein content were detected in HMC.

Importance of the different systems that synthesizes ROS in the basal TGF-β1 expression in HMC

To study the influence of different sources of ROS in the maintenance of the TGF-β1 levels, cells were incubated with pharmacological inhibitors of NADPH oxidase (diphenyleneiodonium, DPI,

10μM), mitochondrial electron-transport chain complex I (rotenone, ROT, 10μM), and xanthine oxidase (allopurinol, ALLO, 100 μM). The results were compared with those from 320 U/mL CAT. After treatments, ROS and H_2O_2 concentration was evaluated by flow cytometry in cells loaded with DCFHDA and by confocal microscopy in cells transfected with HyPer. A relevant and similar suppression of basal ROS and H_2O_2 synthesis was observed in all cases and the inhibition was comparable to that of CAT (Fig. 5A-B). In these conditions of reduced H_2O_2 intracellular levels, only DPI and ROT inhibited TGF-β1 and 3TP-Lux promoter activity (Fig. 5C), as well as TGF-β1 content in cell supernatants (Fig. 5D). However, ALLO did not modify any of these parameters (Fig. 5C-D).

As these experiments were performed with pharmacological inhibitors, we tried to confirm some results by using genetically modified cells. Human fibroblasts mutated in mitochondrial DNA (G7896A) (3) were used to analyse the ability of a defect in the respiratory chain to modulate the basal TGF-β1 cellular expression. In these cells, a decreased concentration of the cytokine in cell extracts (Fig. 6A) and in supernatants (Fig. 6B) was observed, when compared with their control cells. Additionally, when cells were transfected with the HyPer plasmid, they exhibited a lower H_2O_2 concentration than normal fibroblasts.

Discussion

The ability of increased ROS to modulate different aspects of cell function has been widely recognized (11,26,37,38,40). Our group has contributed to the knowledge of this regulation by demonstrating that H_2O_2 induces mesangial cell contraction (6), stimulates cell proliferation (8) and increases extracellular matrix proteins via TGF-β1 (12). However, the experiments devoted to analyze the role of ROS in cell function have been systematically performed by increasing ROS concentration, directly or through mechanisms that stimulate their endogenous synthesis. Resting cells synthesize a significant amount of ROS via mitochondrial electron-transport chain that is maintained under control by antioxidant enzymes (26). We wondered if these ROS could play some role in the basic functions of resting cells, such as the expression of constitutive genes. As we demonstrated that increased ROS stimulates TGF-β1 (12), we chose this gene to analyze our hypothesis, as we supposed that the opposite effect could be also possible.

To decreased intracellular ROS concentration, CAT was added to the extracellular media of the cells. Extracellular CAT decreases H_2O_2 in cell supernatants, with the subsequent transfer of this ROS from the intra to the extracellular media, but also penetrates into the cells (24,34). Our experiments confirm that intracellular CAT activity increased about three-fold after addition of 320 U/ml CAT to the cell supernatants for 16 h. Moreover, they confirmed that intracellular ROS concentration decreased about 40% with this same CAT concentration, measured by fluorescence of DCFHDA. Although this dye has been proposed as specific for H_2O_2, it seems that it may be also activated by other ROS (4). Thus, to be sure about changes in H_2O_2 elicited by extracellular CAT, we transfected the cells with the HyPer plasmid (1), that specifically responds to changes in H_2O_2 concentrations and we confirmed the significant decrease of intracellular H_2O_2 by CAT addition.

Cells with low intracellular H_2O_2 concentration did not show any significant toxicity, at least in the intervals that these studies were performed. However, the intracellular concentration of TGF-β1, the release of this cytokine to cell supernatants, the steady-state levels of its mRNA and its promoter activity significantly decreased in the cells. The quantitative changes observed in these parameters were comparable to the reduction in H_2O_2 concentration. Thus, the presence of 320U/ml

CAT in the extracellular media decreased about 50% of TGF-β1 protein content evaluated by immunoblot and TGF-β1 mRNA quantified by RT-PCR.

Present experiments suggest that the decreased TGF-β1 promoter activity, consequence of the decreased H_2O_2 intracellular concentration, is the basic mechanism that leads to the TGF-β1 down-regulation. Since this promoter is under the control of different transcription factors (16), we tried to perform a general approach of these factors by analyzing the transcriptional activity of continuous deletions of the promoter. The complete deletion of the region between -1362 and -453 did not modify the promoter response to CAT addition, but when the deleted region included the fragment between -453 and -323 the CAT inhibition disappeared. In this region is located the specific DNA sequence that binds AP-1 thus supporting the relevance of this transcription factor in the genesis of the observed effects. In fact, the binding of AP-1 to cellular extracts progressively decreases in cells incubated with increasing concentrations of CAT, as well as the activity of 3TP-Lux construction, which specifically activates in response to AP-1. This was a rather expected result, as this factor seems to be one of the most relevant transcription factors involved in TGF-β1 regulation (16,30), and it is up-regulated by ROS over-production (14,21,30,38).

These experiments support the role of the basal AP-1 activity on the basal expression of TGF-β1, under the control of intracellular steady-state levels of H_2O_2. If AP-1 were so critical in the regulation of this process, genes under its control would have behaviours similar to TGF-β1, and the lack of response elements to AP-1 in the promoter region would determine lack of response to CAT treatment. For this purpose we analysed other proteins. The cellular content of eNOS decreased when endothelial cells were incubated with CAT, whereas no changes were observed in COX1 and PKG-α1 content in HMC after treatment with CAT. From these three proteins, only eNOS contains AP1-site in their promoter region (22,35,42).

ROS are derived from several compartments inside the cells: mitochondria, peroxisomes, plasma membrane, endoplasmic reticulum, Golgi complex, lysosomes and the nucleus, where there are enzyme systems that synthesize ROS (7,26,37). Although this synthesis is usually considered as a whole, probably the different enzymes are responsible for ROS production for specific cellular functions. Regarding the basal activity of AP-1 we tested the possibility that it could depend on the

mitochondrial synthesis of ATP or in two well studied enzymatic systems, NADPH oxidase or xanthine oxidase. Interestingly, only the pharmacological blockade of mitochondrial function and NADPH oxidase reproduce the TFG-β1 down-regulation elicited by CAT, although a similar reduction in ROS concentration was observed with the different pharmacological inhibitors tested.

These findings allow us to speculate. Although different metabolic and enzymatic systems are active in resting cells, only some of them, as ATP synthesis by mitochondria or NADPH oxidase are responsible for the ROS production that regulates basal AP-1 activity. In this context, we have performed some experiment in cells with genetically-determined deficient mitochondrial activity. In these cells, which exhibit decreased H_2O_2 intracellular concentrations, the synthesis of TGF-ß1 is significantly reduced.

Present results support the hypothesis that endogenous ROS help to maintain the basal expression of some constitutive genes, such as TGF-β1 or eNOS, through the regulation of the activity of the transcription factor AP-1. H_2O_2 could be the most relevant ROS involved in this regulation. Although more studies will be necessary, our results point to the possibility that different sources of ROS production can have different roles in gene regulation, supporting the idea of the functional compartmentalization of ROS. Understanding these mechanisms is relevant because of importance of ROS in normal cell function.

Innovation

This is the first time that a role for intracellular ROS steady-state levels in the basal expression of constitutive genes is proposed. Our findings suggest that basal levels of intracellular ROS regulate the activity of the transcription factor AP-1, and subsequently of the genes under its control, particularly TGF-ß1 in HMC and eNOS in endothelial cells. Moreover, our data suggest that the source of ROS may be relevant in this regulation. Interestingly, ATP synthesis by mitochondria seems to be one of the sources involved, suggesting a link between constitutive gene expression and cell respiration.

Materials and Methods

Materials

Culture media were from Lonza (Basel. Switzerland). Foetal bovine serum (FBS), antibiotics, goat anti-Rabbit, gold antifade reagent and lipofectamine were from Invitrogen (San Diego, CA). Fluorescent probe dichlorodihydrofluorescein-diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR). Human TGF-β1 and cyclooxigenase-1 antibodies were from Santa Cruz (La Jolla, CA). AntiPKG-1α antibody was from Cell Signalling (Danvers, MA); anti-eNOS antibody from Transduction Laboratories (Palo Alto, CA) and anti-rabbit horseradish peroxidase antibody from Chemicon (Billerica, MA).Catalase assay kit was from Cayman Chemical (Ann Arbor, MI). Dual-Luciferase Reporter Assay System and ELISA kit were from Promega (Madison, WI). Radioactivity (γ-32P)-ATP was from Amersham (Buckinghamshire, UK). Other antibodies and reagents were from Sigma Chemical Company (St. Louis, MO).

Cell culture and treatment

Human mesangial cells (HMC) were prepared as described in (12,10). The experiments were performed in cells on passages 3-7. Human endothelial cells, EAHY926 were from American Type Culture Collection (Rockville, MD). Human fibroblasts from skin were provided by Dr Garesse. They have a mutation G7896A in the subunit II of cytochrome c oxidase (3). Fibroblasts from healthy individuals were used as control.

Cells were maintained in culture medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS. In the case of human fibroblasts, 50µg/ml uridine was also added. Cells were serum-deprived for 24 h and then they were incubated for 16 h with catalase (160 U/ml-640 U/ml) prepared as recommended by 36. In some experiments HMC were incubated for 16 h with diphenyleneiodonium (DPI, 10µM), rotenone (ROT, 10µM), and allopurinol (ALLO, 100 µM). Cell toxicity was evaluated by analyzing the exclusion of the trypan blue dye and in every case was over 90 %.

Measurement of intracellular CAT activity and ROS concentration

For determination of catalase enzymatic activity, cells were scraped off in PBS, pelleted by centrifugation for 10 min at 1500 rpm at 4°C, resuspended in 50 mmol/l potassium phosphate, pH 7 and sonicated for two 15s bursts. Lysates were clarified by centrifugation at 13,000g for 10 min at 4°C and supernatants were used to determine catalase activity using catalase assay kit.

ROS production was determined by two methods. First, flow cytometry was used in cells loaded with dichlorodihidrofluorescin, DCFHDA (5 mmol/l, 60 min). Cells were detached by trypsinization after treatments and cellular fluorescence intensity was measured by using FACScan flow cytometer (Becton Dickinson, NJ). For each analysis, 10,000 events were recorded and cell viability was controlled with propidium iodide. ROS production was calculated as percentage of DCFHDA fluorescence intensity with respect to untreated cells. Second, cells were transfected with 1μg of HyPer plasmid (1) with lipofectamin. Twenty-four hours after transfection, cells were serum deprived for 24 h and then treated. The analysis of the plasmid expression was performed by using a confocal laser scan microscopy LEICA TCS-SL (Heidelberg, Germany). Hydrogen peroxide (100μM) was added to the cells as control of plasmid induction.

Protein extraction and immunoblot analysis

Immediately after treatments, cells were washed with cold PBS and lysed in lysis buffer (50 mM Trizma, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10 mM EDTA, 0.25% sodium deoxycholate and protease inhibitors). Cell lysates were sonicated and proteins were resolved onto 8–12 % SDS-polyacrylamide gels. The proteins were transferred to PVDF membrane, blocked with 5% non-fat dry milk in TBS-T (50 mM Trizma, pH 7.6, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4°C with the corresponding primary antibodies and 1h at room temperature with secondary antibodies. Immunolabeling was detected by enhanced chemiluminescence.

Immunofluorescence analysis

After treatment, cells were fixed with 4% paraformaldehyde in PBS, 10 min at room temperature, rinsed and permeabilized with 0.5% Triton X-100 in PBS (10 min). Cells were then

incubated for 1h with 3% BSA in PBS to block nonspecific binding. Afterwards, cells were incubated overnight at 4°C with anti-TGF-β1 antibody (1:50) and then rinsed with PBS. Finally, cells were incubated with goat anti-Rabbit IgG (1:500) for 1h in the darkness. Slides were washed and mounted with Gold antifade Reagent. Colour detection was performed by confocal microscopy.

Measurement of immunoreactive TGF-β1 by ELISA.

Culture medium (250 µl) from treated cells was removed from each well and incubated with 5 µl 1N HCl for 15 min to activate latent TGF-β1. After, supernatants were neutralized with 1N NaOH. Samples were analyzed with a commercial TGF-β1 ELISA kit (19). Protein concentrations were determined spectrophotometrically. Each sample was measured in duplicate and the results were expressed as pg of TGF-β1 per µg of protein.

Analysis of total TGF-β1 mRNA by quantitative RT-PCR

Total RNA from each sample was extracted and quantified. Two micrograms of total RNA was reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). 250 ng of cDNA was analyzed for TGF-β1 (Hs00171257_m1) and GAPDH (4326317E) as endogenous control by using TaqMan gene expression Assays (Applied Biosystem). The normalized gene expression method ($2^{-\Delta\Delta CT}$) for relative quantification of gene expression was used.

Transient transfections and assay of luciferase activity

Complete promoter region of the human TGF-β1 gene linked to the luciferase reporter gene and deletion mutants were kindly provided by Dr. Kim (Bethesda, MD). Specifically, luciferase-linked deletion mutants used were: phTG5, which lacks negative regulatory regions located between −1362 and −1132 and from −731 to −453 positions and an enhancer element located from −1132 to −731 region, but does contain the AP-1 consensus sequence; phTG6, which lacks AP-1 site (16). An AP-1–sensitive promoter (3TP-Lux) and cytomegalovirus-renilla were also used.

Subconfluent (60%) cells were incubated 6 h with 0.5 µg TGF-β1 or 3TP-Lux plasmids, 0.05 µg cytomegalovirus-Renilla and 5µl lipofectamin reagent. After serum deprivation for 24 h, cells were

treated and then lysed. Luciferase activity was measured with a commercial Dual-Luciferase Reporter Assay System according to the manufacturer's recommendations. Results were expressed as percentage of luciferase activity with 100% being the luciferase activity in control cells.

Electrophoretic mobility shift assays (EMSA)

Treated cells were washed with cold PBS and disrupted with lysis buffer (0.5 % Nonidet P-40, 10mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, pH7.9, 1mM DDT, 200mM PMSF, 200mM sodium orthovanadate and protease inhibitors). Lysates were centrifuged 11,000*g* for 10 min at 4°C and supernatants were maintained at −80°C until analysis. Pellets containing nuclei were lysed with hypertonic buffer (0.5 % Nonidet P-40, 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.9, 1 mM DDT, 200 mM PMSF, 200 mM sodium orthovanadate, and protease inhibitors), centrifuged 11,000*g* at 4°C for 6 min, and supernatants were conserved at −80°C until assayed. For EMSA the oligonucleotide 5′-CTTTCCCAGCCTGACTCTCCTTCCGTTC-3′, which contains the consensus AP-1 sequence into the TGF-β1 promoter, was annealed and radiolabeled using the Ready To Go T4 polynucleotide kinase and 10 μCi/μl (γ-32P)-ATP. Nuclear protein extracts (10 μg) were incubated with 1 μg poly dI-dC and 0.1 ng of radiolabeled probe (10^4 to 5×10^5 cpm) in a total volume of 20 μl binding buffer (Tris–HCl pH 7.5; 10 mM $MgCl_2$, 200 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% glycerol, 5 mM NaF and protease inhibitors) for 30 min at room temperature. Samples were run on 8% non-denaturing polyacrylamide gels for 2 h at 200 V. The gels were then dried and exposed to X-OMAT films.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed by either nonparametric Wilcoxon or Friedman tests. A value of *$p<0.05$ was considered statistically significant.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

LIST OF ABBREVIATIONS

ALLO	Allopurinol
CAT	Catalase

DCFH-DA	5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate
DPI	Diphenyleneiodonium
eNOS	Endothelial nitric oxide synthase
HMC	Human Mesangial Cells
HUVEC	Human umbilical vascular endothelial cells
PMA	Phorbol 12-myristate 13-acetate
ROT	Rotenone

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FIGURE LEGENDS

Figure 1. Intracellular catalase activity increases and intracellular reactive oxygen species (ROS) concentration decreases in human mesangial cells (HMC) treated with catalase. HMC were incubated with catalase (160 U/ml to 640 U/ml) for 16h in absence of serum. (A) Intracellular catalase activity was measured by colorimetric techniques. Results are mean ± SEM; n = 6 experiments, *$p<0.05$ vs control. (B) Intracellular ROS concentration was determined by flow cytometry using the dichloro-dihidro-fluorescin diacetate probe. Results are mean ± SEM; n = 6

experiments, *p<0.05 vs control. (C) Intracellular H_2O_2 concentration was measured with confocal microscopy, in HMC transfected with HyPer. Single cell images were obtained 48 h after transfection, and after incubation with catalase (320 U/mL, 16 h) or H_2O_2 (100 nM, 30 min). A representative experiment is shown, from 6 independent experiments. (To see this illustration in colour the reader is referred to the web version of this article at www.liebertonline.com/ars)

Figure 2. Transforming growth factor-β1 (TGF-β1) protein expression decrease in human messangial cells (HCM) treated with catalase. HMC were treated with several concentrations of catalase (160 U/ml to 640 U/ml) or with phorbol 12-myristate 13-acetate (3 nM, PMA) for 16h in absence of serum. Protein expression of TGF-β1 was analyzed. (A) Western blotting was performed with total cell lysates by using specific antibody against TGFβ-1. Actin was used as loading control. Representative Western blot is shown. Bar graphs represent densitometric analysis of bands corrected with actin. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (B) Confocal microscopic images showing the staining of TGFβ-1 (green) in cells. Treated cells (320 U/ml CAT of 3 nM PMA) were fixed and incubated with anti-TGF-β1 antibody. A representative experiment is shown from 6 independent experiments. (C) Total immunoreactive TGF-β1 was measured in the supernatants by ELISA. Bar graph represents TGF-β1 content in cell supernatants after treatment. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (To see this illustration in colour the reader is referred to the web version of this article at www.liebertonline.com/ars)

Figure 3. **Catalase treatment decreases transforming growth factor-β1 (TGF-β1) mRNA and promoter activity, inhibits the binding of AP-1 to TGF-β1 promoter and reduces the 3TP-Lux promoter activity in human mesangial cells (HMC).** HMC were treated with several concentrations of catalase (160 U/ml to 640 U/ml) for 16h in absence of serum. (A) mRNA was determined by Q-RT-PCR normalized by the GAPDH content and expressed as fold induction over the levels found in control cells. Catalase was used at a concentration of 320 U/ml. Results are mean ± SEM; n = 6

experiments, *p<0.05 vs control. (B) HMC was cotransfected with plasmids containing the complete sequence of TGF-β1 promoter linked to the luciferase reporter gene and renilla, and treated. Luciferase activity was corrected by renilla luciferase activity. Results are expressed as the percentage with respect to the control. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (C) The same experiments were performed, as in B, but with serial deletions of the human TGF-β1 promoter. The two deletion mutants were truncated at the locations shown. The negative (N) or positive (P) regulatory regions are represented as black and hatched boxes, respectively. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (D) Nuclear proteins were extracted and EMSA was performed with specific oligonucleotides for the AP-1 site. To detect the bands an excess of cold oligonucleotide was added to the reaction mixture in the catalase sample. A representative EMSA from 6 experiments is shown. (E) Luciferase activity of cells transfected with 3TP-Lux promoter activity was measured by luciferase assay after treatment with catalase. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control.

Figure 4. Inhibition of intracellular ROS by catalase decreases endothelial nitric oxide synthase (eNOS) protein content but not change cycloxygenase 1 (COX1) and protein kinase G (PKG-α1). Cells were treated with catalase at different concentrations (80 U/ml to 640 U/ml) for 16h. Protein content was assayed by Western blot with specific antibodies (A) eNOS expression in EAH endothelial cells. (B) COX-1 expression and (C) PKG-α1 expression in human mesangial cells. A representative blot is shown and bar graphs represent densitometric analysis of bands corrected with actin. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control.

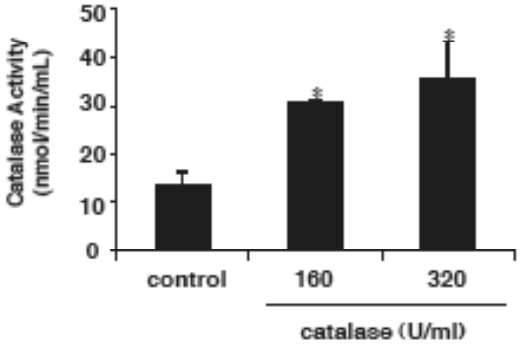
Figure 5. Inhibition of different sources of intracellular ROS produces different response on transforming growth factor-β1 (TGF-β1) protein content and promoter activity in human mesangial cells (HMC). HMC were incubated with catalase (320 U/mL CAT), diphenyleneiodonium (10 μM DPI, NADPH oxidase inhibitor), rotenone (10 μM, ROT, mitochondrial complex I inhibitor), allopurinol (100 μM, ALLO, xanthine dehydrogenase inhibitor) or phorbol 12-myristate 13-acetate (3 nM, PMA). (A) Intracellular ROS concentration was determined by flow cytometry using the

dichloro-dihidro-fluorescin diacetate probe. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (B) Intracellular H_2O_2 concentration was measured with confocal microscopy, in HMC transfected with HyPer. Single cell images were obtained 48 h after transfection, and after incubation with the different reactives for 16 h. A representative experiment is shown, from 6 independent experiments. (C) HMC were cotransfected with plasmids containing the complete sequence of TGF-β1 promoter linked to the luciferase reporter gene and renilla, and treated. Luciferase activity was corrected by renilla luciferase activity. Results are expressed as the percentage with respect to the control. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (D) Total immunoreactive TGF-β1 was measured in the supernatants by ELISA. Bar graph represents TGF-β1 content in cell supernatants after treatment. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. (To see this illustration in colour the reader is referred to the web version of this article at www.liebertonline.com/ars)

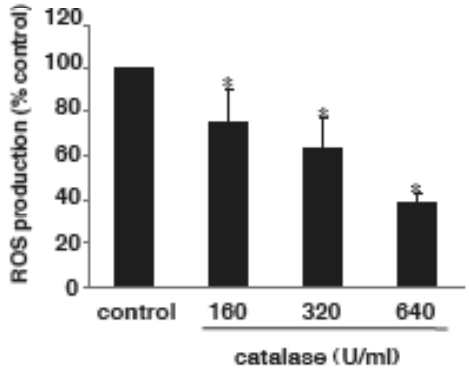
Figure 6. Transforming growth factor-β1 (TGF-β1) protein expression decreases in mutant human fibroblast G7896A with respect to wild-type fibroblast. Human fibroblasts were deprived of serum for 16h. Protein expression of TGF-β1 was then analyzed. Protein content was measured by Western blot in cell extracts (A) and ELISA in supernatants (B). In the western blot experiments, a representative experiment is shown and bar graph represents the densitometric analysis of bands corrected with actin. Results are mean ± SEM; n = 6 experiments, *p<0.05 vs control. In the panel C, intracellular H_2O_2 concentration was measured with confocal microscopy, in fibroblasts transfected with HyPer. Single cell images were obtained 48 h after transfection. A representative experiment is shown, from 6 independent experiments. (To see this illustration in colour the reader is referred to the web version of this article at www.liebertonline.com/ars)

FIGURE 1

A


Catalase Activity (nmol/min/mL)
50
40
30
20
10
0
control
160
320
catalase (U/ml)

B


ROS production (% control)
120
100
80
60
40
20
0
control
160
320
640
catalase (U/ml)

C

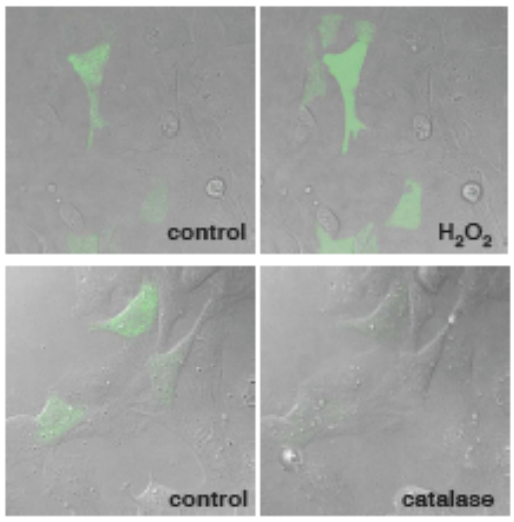

control
H_2O_2
control
catalase

FIGURE 2

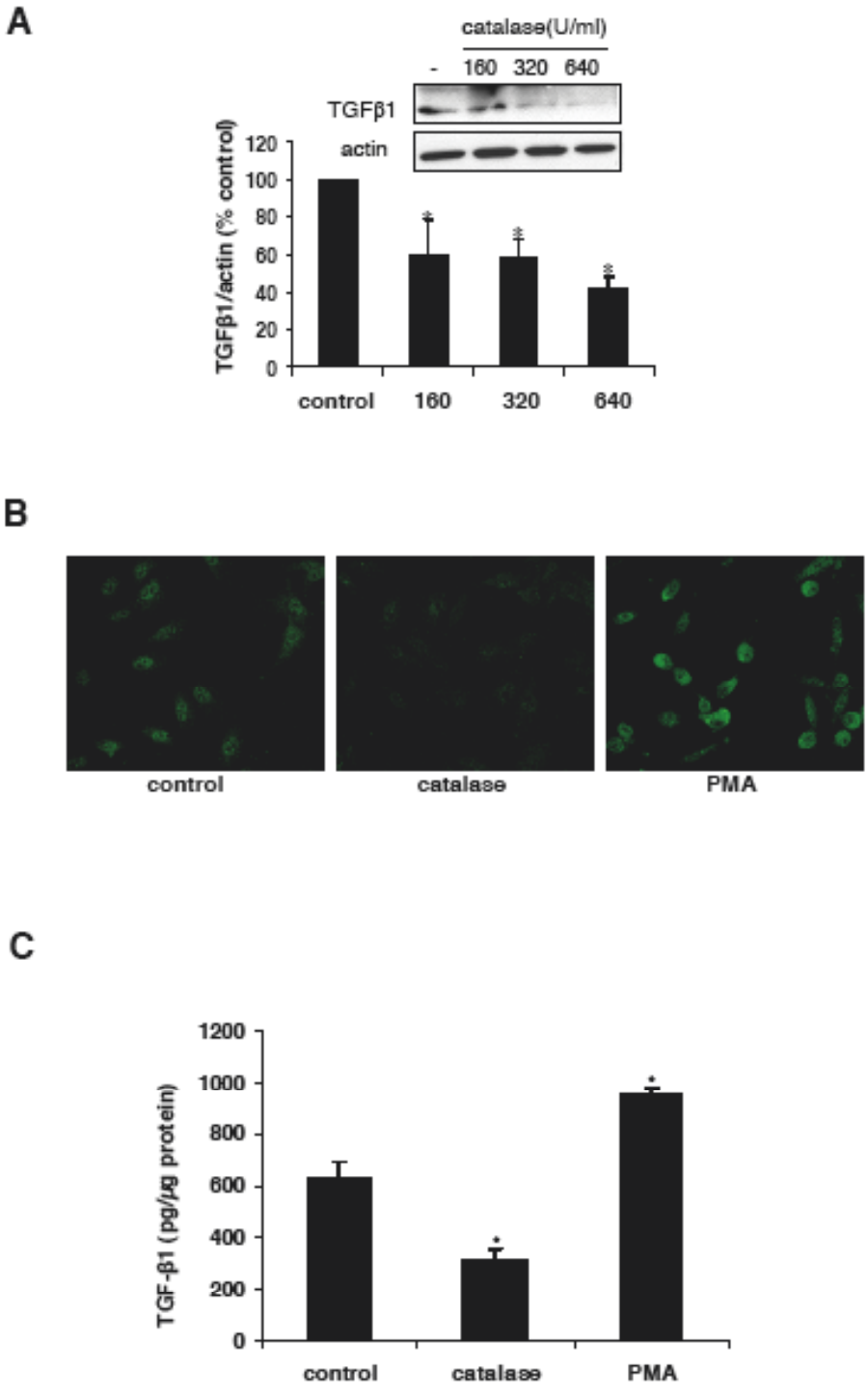

A
catalase(U/ml)
- 160 320 640
TGFβ1
actin
TGFβ1/actin (% control)
120
100
80
60
40
20
0
control
160
320
640
B
control
catalase
PMA
C
TGF-β1 (pg/μg protein)
1200
1000
800
600
400
200
0
control
catalase
PMA

FIGURE 3

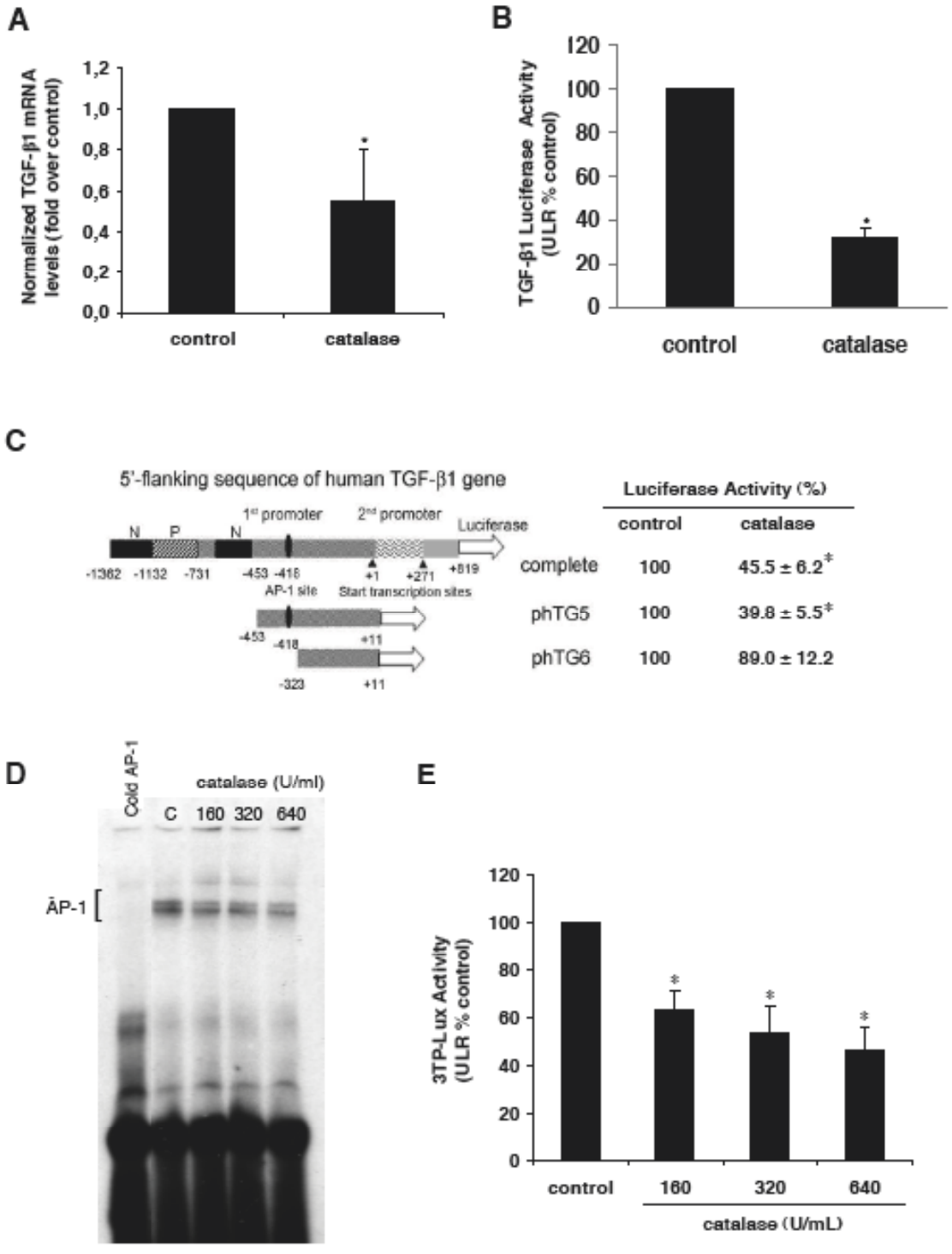

A
Normalized TGF-β1 mRNA levels (fold over control)
1,2
1,0
0,8
0,6
0,4
0,2
0,0
control
catalase
B
TGF-β1 Luciferase Activity (ULR % control)
120
100
80
60
40
20
0
control
catalase
C
5'-flanking sequence of human TGF-β1 gene
1st promoter
2nd promoter
Luciferase
N
P
N
-1362
-1132
-731
-453
-418
+1
+271
+819
AP-1 site
Start transcription sites
-453
-418
+11
-323
+11
Luciferase Activity (%)
control
catalase
complete 100 45.5 ± 6.2*
phTG5 100 39.8 ± 5.5*
phTG6 100 89.0 ± 12.2
D
Cold AP-1
catalase (U/ml)
C
160
320
640
AP-1
E
3TP-Lux Activity (ULR % control)
120
100
80
60
40
20
0
control
160
320
640
catalase (U/mL)

FIGURE 4

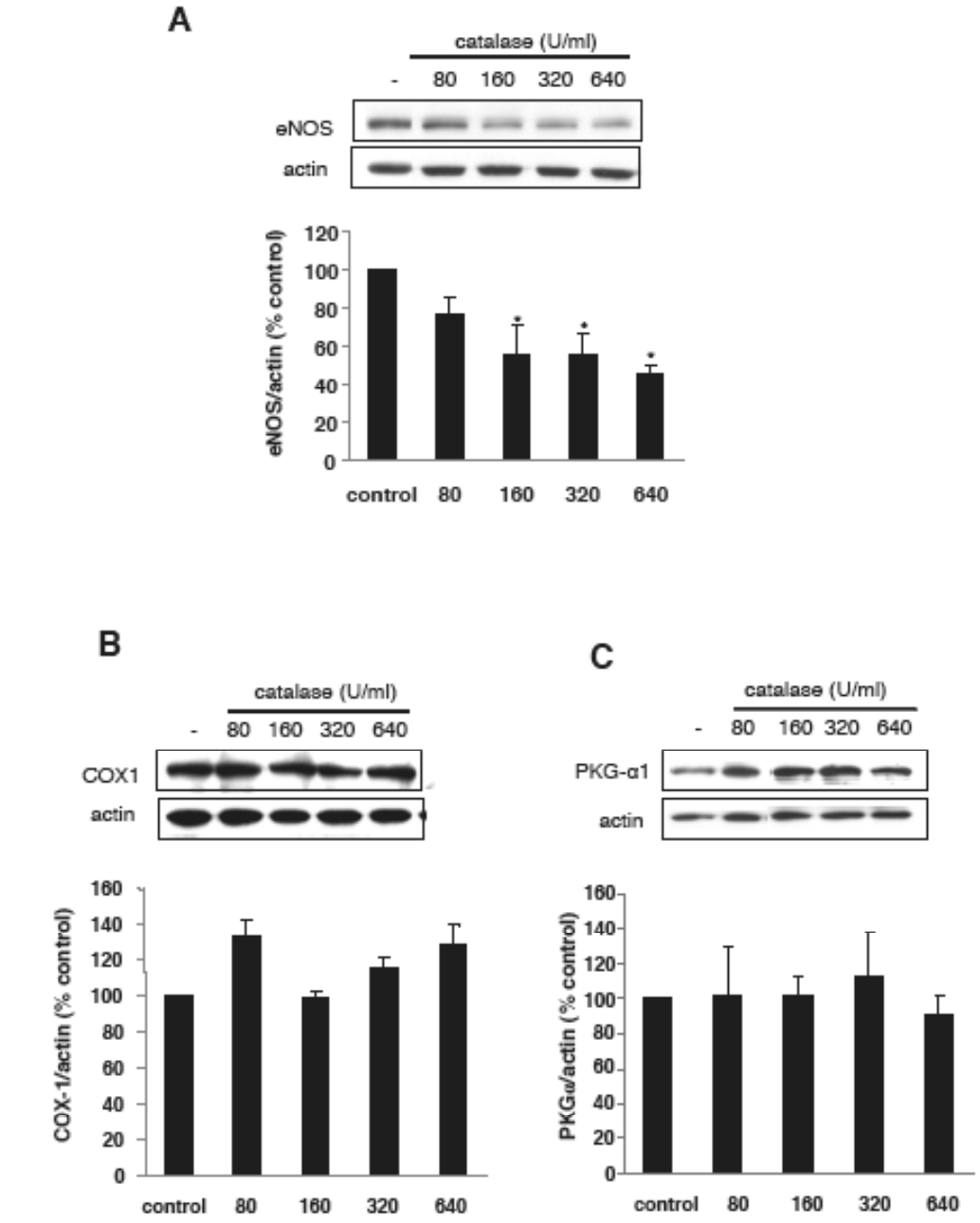
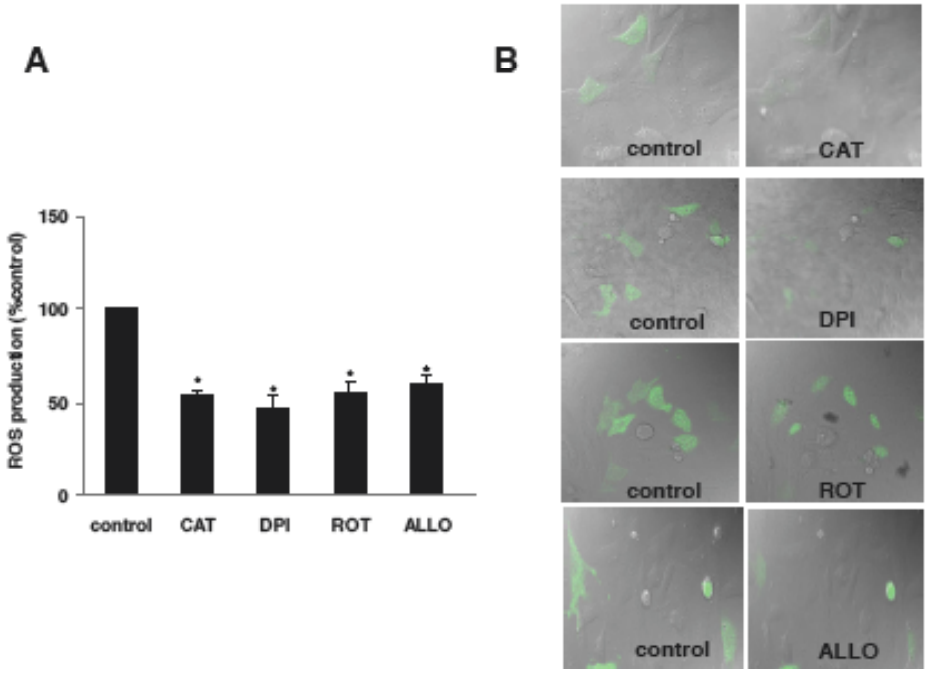

A
catalase (U/ml)
- 80 160 320 640
eNOS
actin
eNOS/actin (% control)
120
100
80
60
40
20
0
control 80 160 320 640
B
catalase (U/ml)
- 80 160 320 640
COX1
actin
COX-1/actin (% control)
160
140
120
100
80
60
40
20
0
control 80 160 320 640
C
catalase (U/ml)
- 80 160 320 640
PKG-α1
actin
PKGα/actin (% control)
160
140
120
100
80
60
40
20
0
control 80 160 320 640

FIGURE 5



C

Luciferase Activity (%)	control	CAT	DPI	ROT	ALLO	PMA
TGF-β1	100	32.0±5.5*	56.1±5.9*	60.8±5.4*	99.9±23.2	234.4±13.8*
3TP-Lux	100	54.0±9,8*	68.3±9,5*	54.7±7.3*	104.5±5.7	256,1± 5.8*

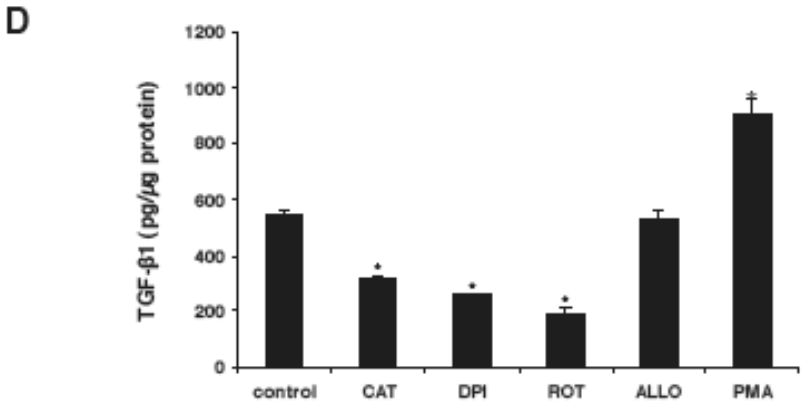
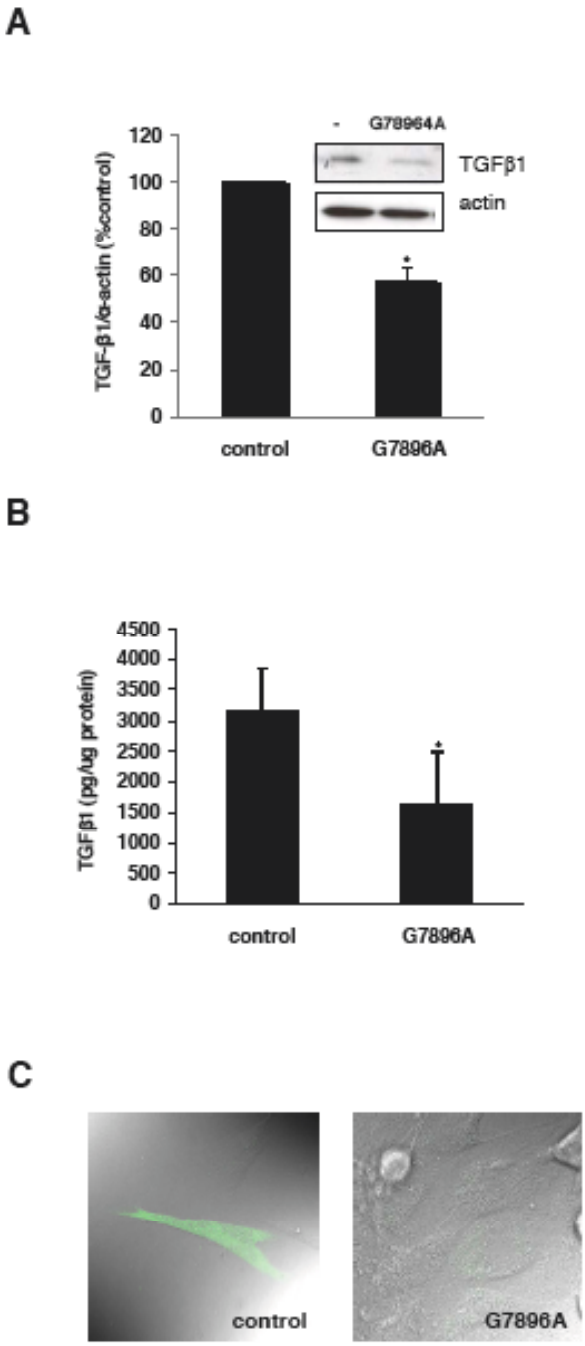


FIGURE 6


A
-
G78964A
TGFβ1
actin
TGF-β1/α-actin (%control)
120
100
80
60
40
20
0
control
G7896A
B
TGFβ1 (pg/ug protein)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
control
G7896A
C
control
G7896A