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Oxidative stress regulates IGF1R expression in vascular smooth muscle cells via p53 and HDAC recruitment

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Synopsis

Apoptosis of vascular smooth muscle cells (VSMC) leads to features of atherosclerotic plaque instability. We previously demonstrated that plaque-derived VSMC have reduced insulin-like growth factor-1 (IGF1) signalling through reduced expression of the IGF1 receptor (IGF1R) compared to normal aortic VSMC [Patel, Zhang, Siddle, Soos, Goddard, Weissberg, and Bennett (2001) *Circ Res* **88**, 895-902]. In this study we show that apoptosis induced by oxidative stress is inhibited by ectopic expression of IGF1R. Oxidative stress repressed IGF1R expression at multiple levels, which was blocked by mutant p53. Oxidative stress induced p53 phosphorylation and apoptosis in VSMC. p53 negatively regulated IGF1R promoter activity and expression, and consistent with this, p53^{-/-} VSMC demonstrated increased IGF1R expression both *in vitro* and in advanced atherosclerotic plaques *in vivo*. Oxidative stress-induced interaction of endogenous p53 with the TATA-binding protein (TBP) was dependent on p53 phosphorylation. Oxidative stress also increased the association of p53 with HDAC1. Trichostatin A, a specific HDAC inhibitor, or p300 overexpression relieved repression of the IGF1R following oxidative stress. Furthermore, acetylated-histone-4 association with the IGF1R promoter was reduced in cells subject to oxidant stress. These results suggest that oxidative stress-induced repression of IGF1R is mediated by association of phosphorylated p53 with the IGF1R promoter via TBP, and subsequent recruitment of chromatin modifying proteins such as HDAC1 to the IGF1R promoter-TBP-p53 complex.

Keywords: apoptosis, IGF1R, p53, VSMC, HDAC, gene expression

Introduction

Atherosclerosis is a complex, chronic arterial disease and a major cause of morbidity and mortality. Vascular smooth muscle cells (VSMC) are the principle source of collagen and extracellular matrix required to maintain the tensile strength of atherosclerotic plaques. Apoptosis of VSMC decreases the synthesis of collagen and extracellular matrix, and induces features of plaque instability [1]. Rupture of atherosclerotic plaques can result in thrombotic complications leading to stroke, myocardial infarction and sudden death.

Oxidative stress is increasingly implicated in the development of atherosclerosis [2]. Increased oxidative damage and elevated levels of DNA strand breaks occur in human atherosclerotic plaques [3]. Furthermore, apoptosis in plaques co-localises with macrophages, a major source of reactive oxygen species (ROS) and oxidative stress can induce VSMC apoptosis in culture [4-6]. Understanding how ROS regulate apoptosis of VSMC may therefore identify new treatments for advanced atherosclerosis.

We recently demonstrated that human plaque-derived VSMC exhibit reduced IGF1-dependent survival compared with VSMC from normal vessels [7]. IGF1 receptor (IGF1R) activation initiates signalling pathways involved in cell proliferation, survival, differentiation and transformation [8]. IGF1R-dependent signalling is crucial for survival of many cell types including VSMC [9, 10]. IGF1R activity in VSMC is mediated by a number of intra and extra-cellular factors [11-14] and its expression is also regulated at the level of transcription [15]. However, the role of oxidative stress on IGF1R gene expression in VSMC is not clear. We show for the first time that oxidative stress represses IGF1R expression via increased association of p53 with the IGF1R promoter and recruitment of HDAC1. Thus, oxidative stress may contribute to plaque instability by repression of IGF1R.

Experimental

Cell culture

Normal and plaque VSMC were isolated from aortas of cardiac transplant patients or following carotid endarterectomy in patients with recent transient ischaemic episodes respectively. Informed consent and approval of the Local Ethics Committee was obtained in all cases. Pup rat

(WKY12-22) aortic VSMC, rat aortic VSMC and VSMC from aortas of wild type or p53^{-/-} knockout mice (C57Bl6/J background) were also used. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) or Waymouth's medium (Sigma) supplemented with 10 U/ml penicillin, 10 µg/ml streptomycin, 5 µg/ml L-glutamine, and 10% foetal calf serum. Transient transfection was performed using FuGENE6 (Roche Applied Science). Cells were treated with tert-butyl hydroperoxide (T-(BuOOH), Sigma Aldrich) at 70-90% confluency. For Luciferase assays, Renilla activity generated by pRL-TK (Promega) was used as an internal control to normalise transfection efficiency. For studies involving Trichostatin A (TSA, Cayman Chemical Company), the cells were treated with indicated concentrations of TSA and T-(BuOOH), and harvested 24 h later. For experiments involving the IGF1R antagonist H-1356 (an IGF1 peptide analogue, Bachem), rat VSMC were treated with 10 µg/ml H-1356 for 6 hours in the presence of 10 % FBS DMEM. Cells were subsequently harvested for Western blotting.

Plasmid constructs

The rat IGF1R promoter construct (-476/+640 Luc) [16] and wild type and YF mutant form of IGF1R [17] have been reported previously. pCMV-p53 and pCMV-p53G135A were purchased from Clontech. p300 CMVβ was purchased from Upstate Biotechnology and CMVβ was generated by restriction enzyme digest and removal of p300 cDNA.

Generation of stable cell lines

Wild-type or kinase dead (YF) IGF1R cDNA [17] were cloned into the retroviral vector pBMN IRES-puro [18]. The resulting plasmids were transfected into the ecotropic packaging cell line, Bosc23 and infectious, replication-deficient virus used to infect rat VSMC in the presence of 8 µg/ml polybrene. After selection in 5 µg/ml puromycin IGF1R expression was demonstrated by immunoblotting with an antibody specific for IGF1Rβ.

Cell viability and apoptosis

Cells were washed in phosphate-buffered saline, harvested with trypsin/EDTA and viable cells determined by trypan blue exclusion. For apoptosis assays, VSMC were treated with T-(BuOOH) for 24 h prior to harvest. Apoptotic cells were quantified using the Annexin V-FITC Kit (Bender MedSystems) following the manufacturer's instructions. Annexin V

positive/propidium iodide negative cells were analysed by flow cytometry using a Facscalibur (Becton Dickinson).

Quantitative polymerase chain reaction

Total RNA was isolated using STAT-60 (Tel-Test, Inc., Friendswood, TX) following the manufacturer's instructions. cDNA generated from 5 µg of RNA was analysed by quantitative PCR (absolute quantification) using the Rotor-Gene 3000 (Corbett Research) and Taqman gene expression assay systems for IGF1R, p53 and GAPDH as specified by the manufacturer (Applied Biosystems).

Western Blot

Equal amounts of protein lysates prepared in Laemmli sample buffer were resolved on SDS-polyacrylamide gels, transferred onto Immobilon-P PVDF membranes (Millipore) and incubated with antibodies to p53 (BD Biosciences Pharmingen), phospho-p53 (Serine-392) (Cell Signalling), IGF1R β (Santa Cruz Biotechnology), HDAC1 (Santa Cruz Biotechnology), β -Actin (Sigma-Aldrich), Akt (Cell Signalling), and phospho-Akt (Cell Signalling). Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare).

Immunoprecipitation studies

VSMC were harvested in immunoprecipitation buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerol phosphate, 1 mM sodium orthovanadate) and 600 µg of pre-cleared protein lysate incubated with 2.5 µg of TBP antibody (TFIID, Santa Cruz Biotechnology) or p53 (Santa Cruz Biotechnology) for 16 h at 4°C. In some instances, the pre-cleared lysate was incubated with 20 units of calf intestinal phosphatase (CIP) for 30 min at 37°C prior to addition of the TBP antibody. Washed protein-G sepharose beads were added to the lysate/antibody mixture for a further 2 h. Samples were washed extensively with immunoprecipitation buffer and subsequently run on PAGE. Western blot was performed for the indicated antibodies

Chromatin immunoprecipitation (ChIP)

Cell lysates were prepared as previously described [19] and immunoprecipitated with 2.5 µg of TBP, p53 or acetyl Histone-4 antibody (Upstate Biotechnology). Co-precipitated IGF1R promoter was amplified by PCR (Forward IGF1R prom; 5'-CCGGGGCATTGTTTTT-3' and Reverse IGF1R prom; 5'-CTCAGCGAGTTAATGCTGGT-3') as follows: 91°C 1min followed by 35 or 37 cycles of 91°C 30 sec, 55°C 30 sec, 72°C 1 min, final extension 72°C 4 min. The product was verified by sequencing.

Immunohistochemistry

Paraffin-embedded sections of brachiocephalic arteries from p53^{+/+}/ApoE^{-/-} and p53^{-/-}/ApoE^{-/-} mice maintained on a high fat diet [20] were sectioned at 5 µm intervals. Specimens were de-waxed, microwaved in citrate buffer and endogenous peroxidase activity blocked with hydrogen peroxidase. Sections were incubated in bovine serum albumin for 1 h at room temperature and then with antibodies to smooth muscle actin (SMA; Dako) or IGF1Rβ (Santa Cruz Biotechnology) overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies. Peroxidase activity was detected with diaminobenzidine (DAB).

Statistical analysis

Parametric tests were employed. Comparison between three or more unpaired groups were made using ANOVA. * indicates P < 0.05.

Results

IGF1R inhibits death of VSMC in response to oxidative stress

To investigate the role of IGF1R signalling in VSMC survival we generated rat VSMC stably overexpressing either wild type IGF1R or an inactive dominant-negative mutant (IGF1R-YF) [17] (Figure 1A), and tested their sensitivity to oxidative stress. Tert-butyl hydroperoxide (T-BuOOH), a membrane-permeable analogue of hydrogen peroxide induced profound apoptosis in VSMC expressing IGF1R-YF (Figure 1B), suggesting that the kinase inactive IGF1R-YF expression interferes with the endogenous IGF1R in VSMC. Consistent with a pro-survival role, ectopic expression of wild type IGF1R protected cells from apoptosis induced by oxidative stress (Figure 1B). To demonstrate the existence of a continuously active IGF1-signalling circuit in VSMC, we used the IGF1R antagonist, H-1356. Rat VSMC displayed reduced IGF1R and p-

Akt protein expression following treatment with H-1356 (Figure 1C). In contrast, there was no change in total Akt levels (Figure 1C). These findings importantly show that suppression of IGF1R-dependent signalling occurs in the absence of exogenous IGF1.

Oxidative stress represses IGF1R gene expression

IGF1R protein is expressed at markedly lower levels in human plaque VSMC versus VSMC from normal aorta [7] consistent with reduced IGF1R mRNA levels (Figure 2A). Since atherosclerotic plaques show evidence of oxidative stress [3, 21], we investigated whether this might be responsible for the observed decrease in IGF1R expression. Normal human VSMC demonstrated a dose-dependent reduction in IGF1R promoter activity (Figure 2B), mRNA (Figure 2C) and protein (Figure 2D) after 24 h treatment with T-(BuOOH), demonstrating that oxidative stress can repress IGF1R gene expression in VSMC.

p53 is associated with reduced IGF1R expression and mediates oxidative-induced apoptosis.

Oxidative stress induces the expression and activity of p53 [22]. Post-translational modifications of p53 can regulate its transcriptional activity, essential for the response to many stimuli such as oxidative stress and DNA damage [22, 23]. Since atherosclerotic plaques are associated with evidence of oxidative stress, elevated p53 levels (including increased p53 phosphorylation) and apoptosis [3, 24], we investigated whether p53 is involved in oxidant-induced repression of IGF1R. Although we see no change in total p53 protein levels 24 h after oxidant stress (Figure 3A), p53 mRNA expression is induced by T-(BuOOH) at earlier time points (2-4 h) (data not shown). In addition, we observed increased phosphorylation of p53 at serine-392 in human VSMC treated with T-(BuOOH) (Figure 3B), suggesting that oxidative stress activates p53 in VSMC. In support of this, T-(BuOOH) also induced p21 (data not shown), a direct transcriptional target of p53 that mediates growth arrest [25].

To examine whether p53 regulates IGF1R expression in VSMC, we derived VSMC from aortae of p53^{-/-} or p53^{+/+} mice. p53^{-/-} VSMC exhibited elevated levels of IGF1R protein compared with p53^{+/+} cells (Figure 3B). IGF1R protein was reduced by T-(BuOOH) in p53^{+/+} cells, but not in VSMC lacking p53 (Figure 3B), suggesting that IGF1R expression is, at least partly, regulated

by p53. Immunohistochemical analysis indicated that IGF1R expression was reduced in both medial and fibrous cap VSMC of atherosclerotic plaques in brachiocephalic arteries of p53^{+/+}/ApoE^{-/-} mice compared to p53^{-/-}/ApoE^{-/-} mice (Figure 3C). Moreover, p53^{+/+} VSMC demonstrated increased apoptosis when treated with T-(BuOOH) *in vitro*, a finding not seen in p53^{-/-} VSMC (Figure 3D). These results suggest that p53 negatively regulates IGF1R expression in VSMC of atherosclerotic plaques, most likely in response to oxidative stress.

Repression of IGF1R by T-(BuOOH) is mediated by p53

To determine whether p53 directly regulates IGF1R in VSMC, we transfected wild type p53 into rat VSMC. Exogenous expression of p53 significantly reduced IGF1R mRNA (Figure 4A) and protein (Figure 4B) compared to the control backbone pCMV. In addition, a firefly luciferase reporter containing approx 1 kb of the IGF1R promoter (-476/+640 Luc) was inhibited by wild type p53 but not by an inactive mutant, p53G135A (Figure 4C). The wild type and p53G135A mutant proteins were expressed at equivalent levels (Figure 4E). These findings suggest that IGF1R gene expression in VSMC is regulated in part by p53. We also investigated whether IGF1R repression in response to T-(BuOOH) is mediated by p53. Whereas the inhibition of IGF1R mRNA (Figure 4D) and promoter activity (data not shown) in response to 10 μ M T-(BuOOH) was enhanced by expression of wild type p53, no repression was observed in the presence of p53G135A. These findings taken together, suggest that oxidative stress-induced repression of IGF1R is mediated by p53.

T-(BuOOH) stimulates the interaction of p53 with TBP

p53-dependent repression can be mediated by binding to *cis*-acting elements and also by binding to the transcriptional machinery [26]. The IGF1R promoter does not contain p53 consensus binding sites and p53 does not bind the IGF1R promoter directly [16]. In VSMC, endogenous p53 bound TBP, the TFIID component of the basal transcriptional machinery (Figure 5A) and this interaction was increased following T-(BuOOH) treatment (Figure 5A). The p53-TBP interaction was phosphorylation-dependent since treatment with phosphatase reduced the amount of p53 co-precipitated with TBP to basal levels (Figure 5A). No change in TBP protein expression was observed with T-(BuOOH) (Figure 5B). To determine whether p53 is recruited to the IGF1R promoter following oxidative stress *in vivo*, we treated VSMC with T-(BuOOH)

for 24 h and prepared them for chromatin immunoprecipitation studies. We were able to amplify the IGF1R promoter using antibodies directed to TBP, suggesting that TBP binds to the endogenous IGF1R promoter in VSMC (Figure 5C). In contrast, we were only able to detect an IGF1R amplicon following immunoprecipitation with an antibody directed to p53 in cells treated with T-(BuOOH) (Figure 5C). Concomitant with this, detection of an IGF1R amplicon following T-(BuOOH) and immunoprecipitation with TBP was reduced, but not abolished (Figure 5C). Our results suggest that, in response to oxidative stress, increased binding of p53 to TBP mediates down-regulation of the IGF1R promoter.

Repression of IGF1R by T-(BuOOH) involves histone deacetylation and p53-HDAC1 association

In eukaryotic cells, transcriptional regulation also involves chromatin remodelling by histone acetyltransferases and histone deacetylases (HDAC) [27]. Repressed chromatin is associated with deacetylated histones. In contrast, acetylation of histones can enhance transcriptional activity. Since HDACs associate with p53 either directly or by co-localisation [28, 29], we next investigated whether T-(BuOOH)-activated p53 could recruit HDAC1 in VSMC. Figure 6A demonstrates increased p53 association with HDAC1 following T-(BuOOH) treatment compared to untreated cells. In order to determine whether oxidative stress-dependent repression of the IGF1R promoter requires HDAC activity, we used Trichostatin A (TSA) a specific HDAC inhibitor. T-(BuOOH)-dependent reduction of IGF1R protein was inhibited by 800 ng/ml TSA compared to the control (Figure 6B). Moreover, forced expression of p300, a histone acetyltransferase, relieved IGF1R repression induced by T-(BuOOH). (Figure 6C), confirming a role for chromatin remodelling in oxidant stress-induced IGF1R repression. Consistent with this, ChIP demonstrated reduced amplification of the IGF1R promoter in T-(BuOOH) treated VSMC immunoprecipitated with an acetyl-Histone-4 antibody (Figure 6C). These findings suggest that Histone-4 is deacetylated upon treatment with T-(BuOOH) in VSMC and that chromatin remodelling and recruitment of gene silencing complexes to the IGF1R promoter regulate oxidative stress-induced repression of IGF1R expression.

Discussion

Atherosclerotic plaques are associated with up-regulation of p53 and phospho-p53 [3, 21] and

exhibit DNA damage, oxidative stress and increased apoptosis [3, 21, 24]. IGF1R mRNA and protein is reduced in human carotid plaque-derived VSMC compared to VSMC from normal aorta [7]. Given that VSMC from the thoracic aortic and coronary arteries behave comparably *in vitro* both differing from that of plaque VSMC from coronary atherectomies [30], and that VSMC are cultured for several weeks prior to experiments, we consider that differences in IGF1R expression are unlikely to arise from differences in the vascular bed or prior patient medication. Consistent with our studies showing reduced IGF1R expression following oxidative stress *in vitro*, a reduction in IGF1R protein expression in sections from advanced human atherosclerotic tissue has been reported [31]. Since down-regulation of IGF1R in VSMC correlates with increased sensitivity to apoptosis [7], loss of IGF1 signalling may be an important determinant of cell fate within the atherosclerotic plaque. Establishing the mechanism of IGF1R repression in response to oxidative stress in VSMC is, therefore, vital to understanding advanced atherosclerosis.

Following exposure to oxidative stress and DNA damaging agents, p53 expression and activity is induced [22]. Although we see no change in total p53 protein levels 24 h after oxidant stress, p53 mRNA expression is induced by T-(BuOOH) at earlier time points, consistent with previous reports demonstrating increased protein expression of p53 within 6 h by hydrogen peroxide [32]. p53 phosphorylation occurs at several sites by multiple kinases including casein kinase I, II, Cdc2 and PKC (reviewed in [22, 23]). These post-translational modifications activate p53 allowing it to accumulate in the nucleus and alter transcription of target genes. In particular, phosphorylation of p53 at serine-392 stabilises p53 tetramer formation and activates p53 as a transcription factor in response to DNA damage [33]. Oxidant stress can exert multiple effects in atherosclerosis, including inactivation of nitric oxide, oxidation of DNA and proteins, lipid oxidation (and therefore retention) and apoptosis of vascular cells [21]. Our studies therefore provide further insight into the complexity of gene regulation by p53 in response to oxidant stress.

The role of oxidative stress in regulating IGF1R expression in VSMC is not clear. For example, exposure of VSMC to ROS either by hydrogen peroxide or ox-LDL has been reported to either increase [12] or decrease [13] IGF1R expression. The molecular and transcriptional mechanisms

of IGF1R expression in response to oxidative stress have not been examined. We demonstrate for the first time, that exposure of VSMC to oxidative stress results in repression of the IGF1R via epigenetic modifications involving phosphorylation of p53, and its increased association with TBP and HDAC1. p53^{+/+} VSMC display reduced IGF1R expression compared with p53^{-/-} cells *in vitro* suggesting that basal regulation of IGF1R in this cell type is mediated by p53, a finding supported by previous studies [15, 16, 34]. Consistent with this, overexpression of wild type but not mutant p53 negatively regulates IGF1R expression.

p53 does not bind the IGF1R promoter directly but interacts with TBP [16]. Although under basal conditions some p53 associates with TBP, this is increased by T-(BuOOH) and is dependent on p53 phosphorylation. Increased p53 binding (to TBP) may interfere with the ability of the TBP antibody to recognise its epitope, which may account for the reduced TBP-IGF1R promoter association following oxidative stress (Figure 5C). For example, p53-dependent alterations in the conformation of the transcriptional complex, or recruitment of additional factors such as co-repressors to the complex, may mask the TBP epitope. Indeed we find increased HDAC1 associated with p53 following T-(BuOOH) treatment. Moreover, T-(BuOOH) treatment results in reduced acetylated histone-4 associating with the IGF1R promoter. In contrast, both TSA and forced expression of p300, significantly block oxidative stress-induced repression of IGF1R protein expression. These findings suggest that chromatin remodelling and epigenetic modifications take place on the IGF1R promoter following oxidative stress. The fact that TSA markedly exacerbates atherosclerosis in *Ldlr*^{-/-} mice [35] indicates that histone acetylation (and deacetylation) is an important regulator of atherosclerosis. Our findings support a model whereby oxidative stress-dependent p53 recruitment of HDAC1 and subsequent chromatin modification contributes to transcriptional repression of the IGF1R promoter. A similar mechanism has been described for repression of the α -fetoprotein gene [36].

The role of p53-dependent apoptosis in atherosclerotic plaques remains controversial. Under certain circumstances p53 can afford protection, for example, by activation of TIGAR [37]. Different experimental conditions and protocols may account for the reported variation in apoptotic rates between p53^{+/+} and p53^{-/-} VSMC [20, 38, 39]. Here we show that p53 is required for oxidative stress-induced apoptosis of VSMC, since T-(BuOOH) is unable to induce apoptosis

in p53^{-/-} cells *in vitro*. It is also likely that p53 contributes to apoptosis of VSMC by increasing the transcription of IGF1R [40-42] and we have previously found increased expression of IGF1R, 2, 3 and 4 in plaque VSMC cultures compared to normal aortic VSMC (10). Although we demonstrate a reduction of IGF1R expression in VSMC from p53^{+/+}/ApoE^{-/-} mice compared to p53^{-/-}/ApoE^{-/-} mice, due to the small number of apoptotic VSMC observed in atherosclerotic plaques [20, 38, 39] we have not been able to correlate reduced IGF1R expression to apoptosis *in vivo*. Nonetheless, the fact that ectopic expression of IGF1R inhibits oxidative stress-induced (and p53-dependent) apoptosis *in vitro*, suggests that p53-mediated repression of IGF1R may contribute to apoptosis of VSMC in the plaque.

In conclusion, we have demonstrated for the first time that p53 represses IGF1R gene expression in VSMC following oxidative stress, via epigenetic modifications and increased association with HDAC1 and TBP. We propose a model in which oxidative stress-dependent activation of p53 recruits HDACs to the IGF1R promoter resulting in repression of gene expression. These studies may help delineate the complex pathways regulating atherosclerosis and atherosclerotic plaque instability.

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Figure Legends

Figure 1. IGF1R inhibits oxidative stress induced apoptosis in VSMC. **A**, Western blot demonstrating ectopic expression of wild type or mutant (YF) IGF1R compared to control rat VSMC (RASMC). Lower panel shows IGF1R expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SE, n= 2. **B**, Flow cytometry of Annexin V positive/propidium iodide negative control, IGF1R or IGF1R-YF expressing RASMC treated with T-(BuOOH) for 24 h. Untreated \square ; 10 μ M T-(BuOOH) \blacksquare ; 20 μ M T-(BuOOH) \blacksquare . Results are mean + SE, n= 3. * p<0.05. **C**, Western blot demonstrating expression of IGF1R, total Akt and p-Akt following treatment with H-1356 for 6 hours in DMEM containing 10% FBS. β -actin staining demonstrates unbiased loading. Lower panel shows p-Akt normalised to total Akt (arbitrary densitometry units). Results are mean + SE, n= 2.

Figure 2. Oxidative stress represses IGF1R gene expression. **A**, IGF1R mRNA expression in two normal human aortic and three plaque-derived VSMC isolates determined by quantitative PCR. Results are means \pm SD, n=3. * p<0.05. **B**, IGF1R promoter activity is reduced by T-(BuOOH). WKY12-22 rat VSMC were transfected with -476/+640 IGF1R promoter (1 μ g/well of a 6-well titre plate) for 6 h followed by T-(BuOOH) treatment for 18 h. Luciferase activity was normalized to Renilla activity (pRL-TK). Results are means \pm SE, n=3. **C**, Expression of IGF1R mRNA in normal human VSMC following T-(BuOOH) treatment for 24 h determined by quantitative PCR. Results are means \pm SE, n=3. **D**, Expression of IGF1R protein in normal human VSMC following T-(BuOOH) treatment for 24 h determined by Western blot. Lower panel shows IGF1R expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SE, n=2. * p<0.05 for B, C and D vs untreated.

Figure 3. p53 mediates T-(BuOOH)-induced apoptosis of VSMC *in vitro* and is associated with reduced IGF1R in atherosclerotic plaques. **A**, Western blot analysis for total p53 and phospho-p53 (S-392) in normal human VSMC following T-(BuOOH) treatment for 24 h. β -actin staining demonstrates unbiased loading. Lower panel illustrates phospho-p53 expression normalised to total p53 (arbitrary densitometric units). Results are mean \pm SD, n=2. * p<0.05 vs untreated. **B**, Western blot for IGF1R protein in wild type and p53^{-/-} mouse VSMC \pm 20 μ M T-

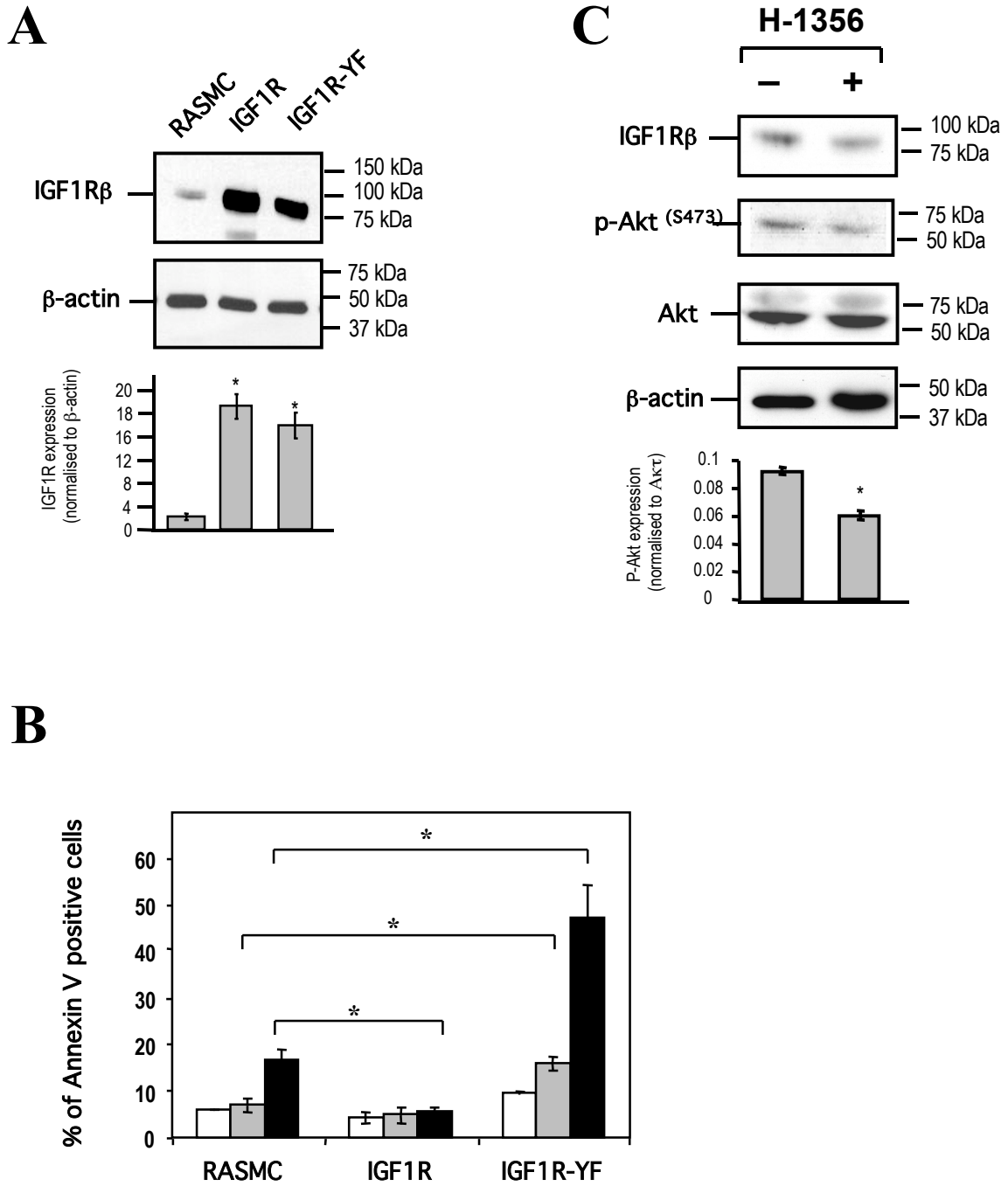
(BuOOH). Lower panel shows IGF1R expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SD, n=2. * p<0.05. **C**, Expression of IGF1R in atherosclerotic plaques of p53^{+/+}/ApoE^{-/-} and p53^{-/-}/ApoE^{-/-} mice determined by immunohistochemistry. Presence of α -smooth muscle actin (SMA) was used to identify VSMC. Middle and lower panels demonstrate high power views of areas outlined in the upper panel. **D**, Flow Cytometric analysis of Annexin V positive/propidium iodide negative wild type and p53^{-/-} mouse VSMC following 24 h treatment with 20 μ M T-(BuOOH). Results are means \pm SD, n=3. * p<0.05.

Figure 4. Repression of IGF1R by T-(BuOOH) is mediated by p53. **A**, IGF1R mRNA expression in control and p53-transfected WKY12-22 rat VSMC determined by quantitative PCR. Results are mean \pm SE, n=3. * p<0.05. **B**, Western blot for IGF1R, p53 and Ets-1 (loading control). **C**, IGF1R promoter activity in WKY12-22 rat VSMC co-transfected with -476/+640 IGF1R promoter-luciferase (1 μ g/well of a 6-well titre plate) and 0.5 μ g/well pCMV, pCMV-p53, or pCMV-p53G135A determined with the dual luciferase assay system. Results are mean \pm SE, n=3. * p<0.05. **D**, IGF1R mRNA expression in WKY12-22 rat VSMC transfected with CMV, CMV-p53 or CMV-p53G135A in the presence or absence of T-(BuOOH) for 24 hours determined by quantitative PCR. Results are mean \pm SE, n=3. * p<0.05. **E**, p53 protein expression in WKY12-22 rat VSMC transfected with 5 μ g pCMV, pCMV-p53 and pCMV-p53G135A. Lower panel shows p53 expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SE, n=2. * p<0.05.

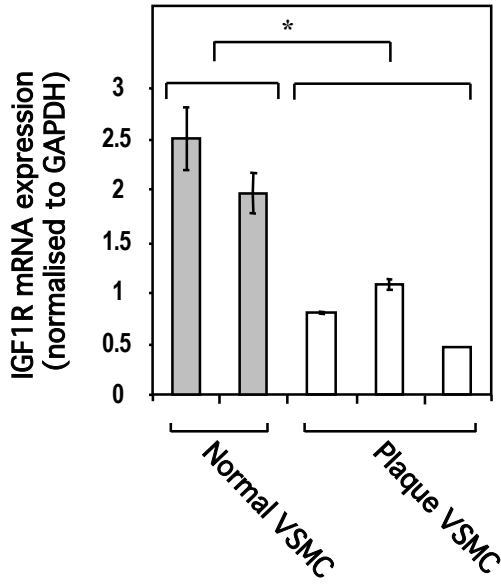
Figure 5. T-(BuOOH)-dependent repression of IGF1R is associated with increased p53-TBP interaction. **A**, Co-immunoprecipitation of phospho-p53 (serine 392) with or without calf-intestinal phosphatase (CIP) pretreatment with cell lysate. The blot was stripped and re-probed for TBP. The intensity of phospho-p53 (serine 392) relative to TBP (lower panel) was determined by densitometry. Results are mean \pm SE, n=2. * p<0.05. **B**, T-(BuOOH) has no effect on TBP protein expression. The blot was stripped and re-probed for β -actin. The intensity of TBP relative to β -actin was determined by densitometry (lower panel). Results are mean \pm SE, n=2. * p<0.05. **C**, Association of TBP and p53 on the IGF1R promoter was determined by chromatin immunoprecipitation. WKY12-22 rat VSMC were treated with T-(BuOOH) or left

untreated for 24 h and cross-linked protein-DNA complexes immunoprecipitated with TBP or p53 antibodies prior to PCR amplification of the IGF1R promoter.

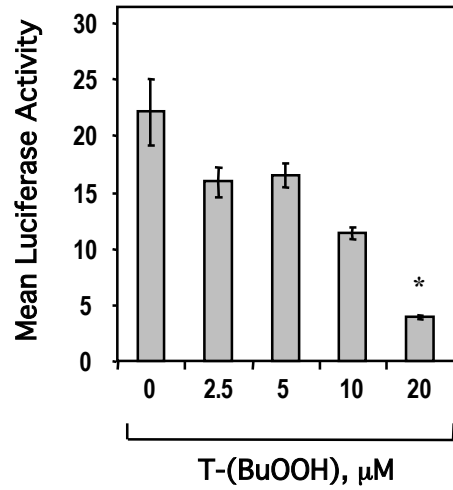
Figure 6. Repression of IGF1R by T-(BuOOH) involves chromatin remodelling. **A**, T-(BuOOH) promotes p53-HDAC1 association. p53 was immunoprecipitated, and associated HDAC1 detected by Western blot. WKY12-22 rat VSMC were treated with T-(BuOOH) for 24 h. Lower panel shows HDAC1 expression normalised to p53 (arbitrary densitometric units). Results are mean \pm SE, n=2. * p<0.05. **B**, Western blot for IGF1R and β -actin showing that TSA treatment dose-dependently inhibits T-(BuOOH)-induced (20 μ M) repression of IGF1R protein expression in human VSMC. Lower panel shows IGF1R expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SE, n=2. * p<0.05. **C**, Western blot for IGF1R and β -actin showing that p300 overexpression relieves T-(BuOOH)-induced repression of IGF1R. WKY12-22 rat VSMC were transfected with 3 μ g p300 CMV β or the backbone control CMV β and treated with 20 μ M T-(BuOOH) at the same time for 24 h. Lower panel shows IGF1R β expression normalised to β -actin (arbitrary densitometric units). Results are mean \pm SE, n=3. * p<0.05. **D**, Oxidative stress reduces the association of acetyl-histone-4 (ac-H4) with the IGF1R promoter. WKY12-22 rat VSMC treated with 20 μ M T-(BuOOH) for 24 h and cross-linked protein-DNA complexes immunoprecipitated with acetyl-histone-4 (ac-H4) prior to PCR amplification of the IGF1R promoter. Chromatin immunoprecipitation data is representative of two independent determinations



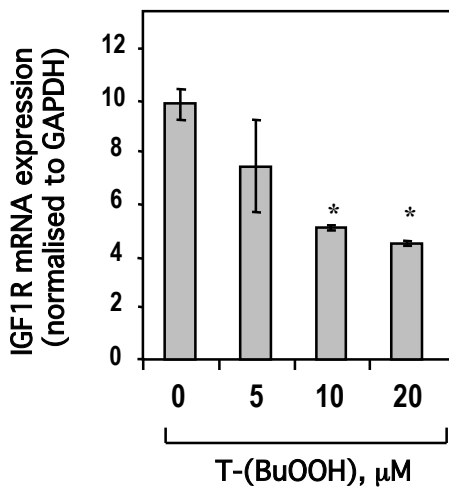
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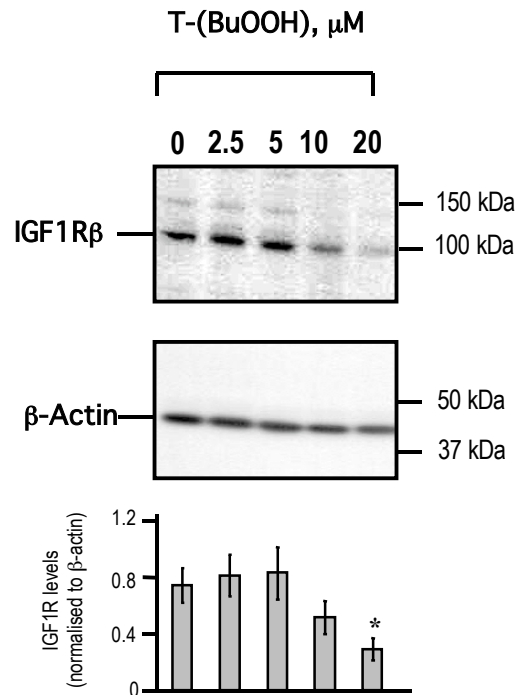
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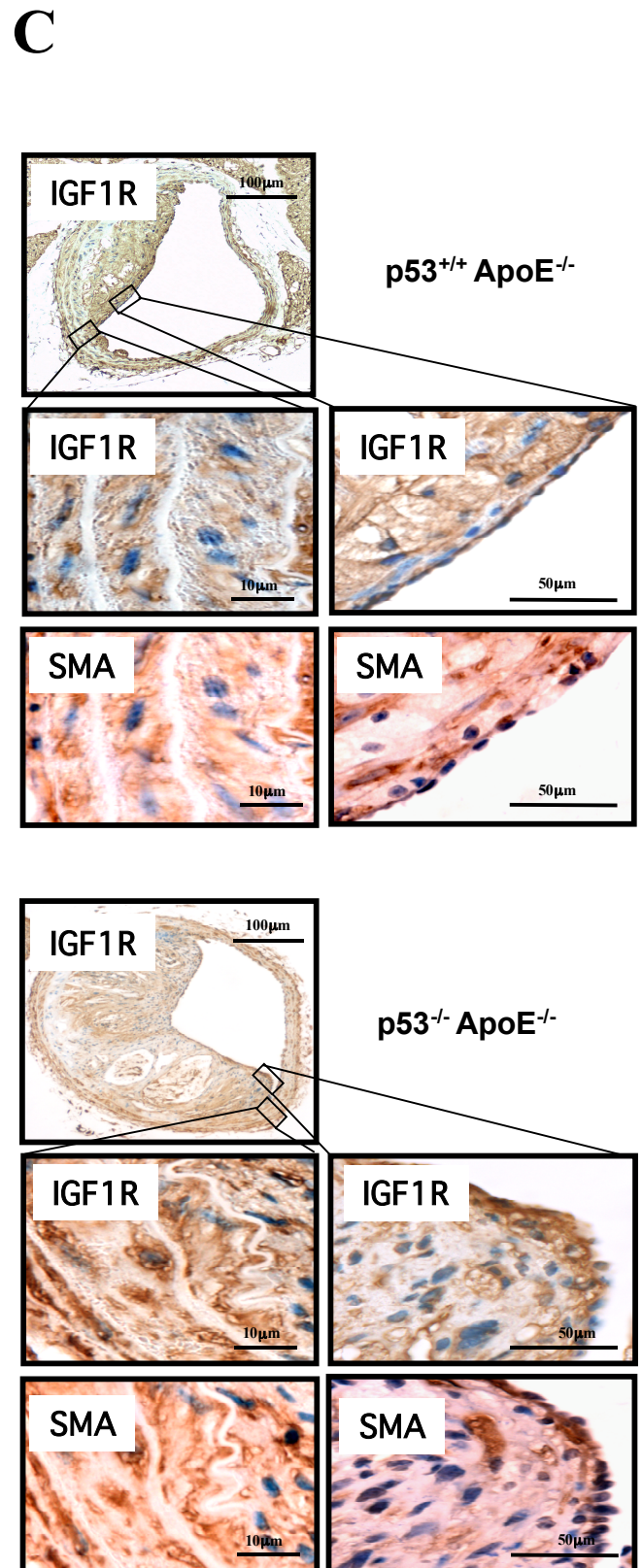
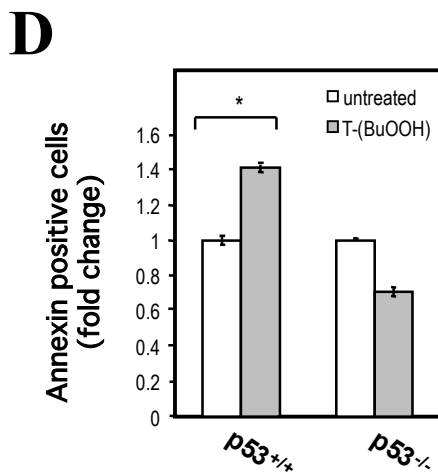
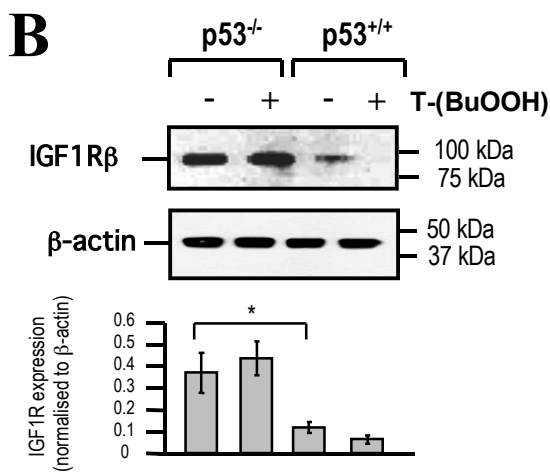
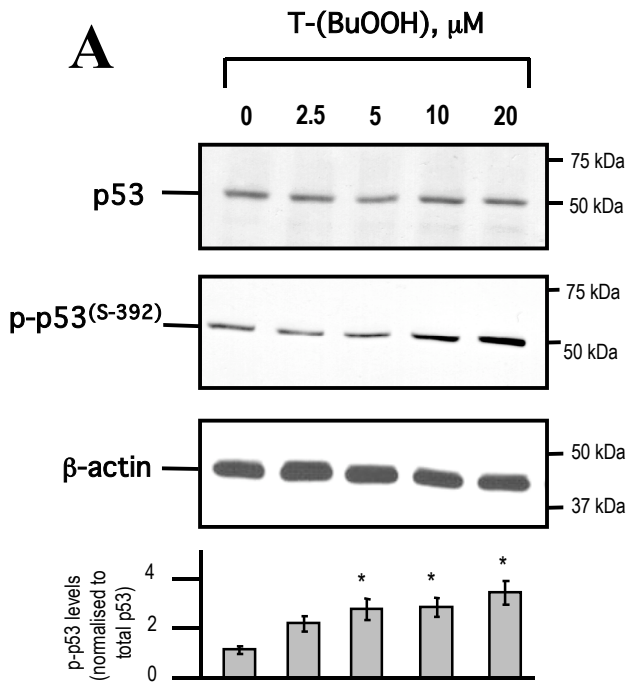


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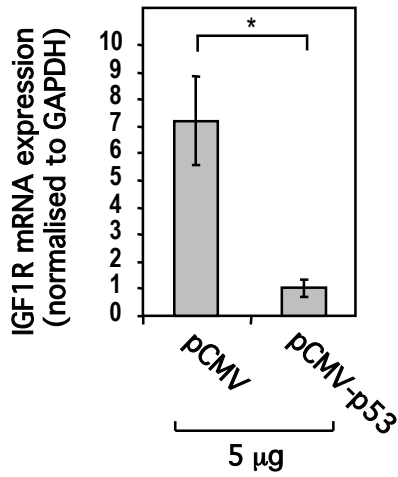


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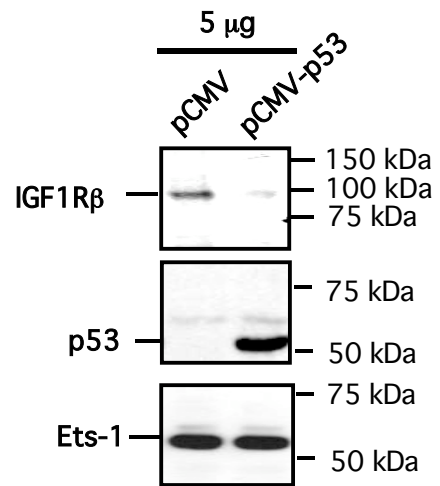




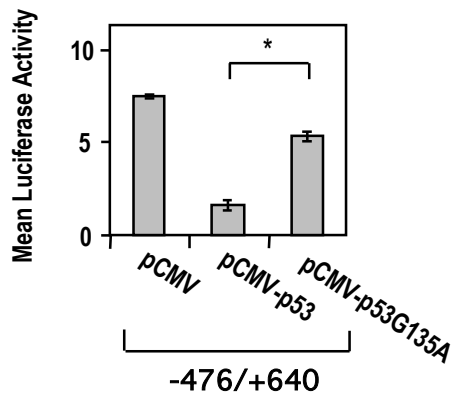
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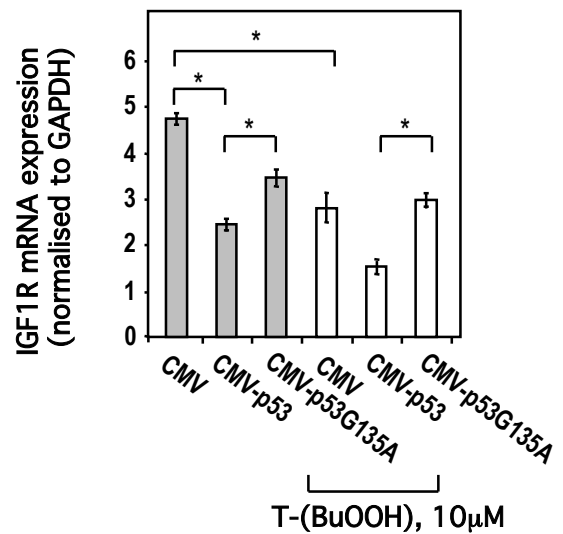
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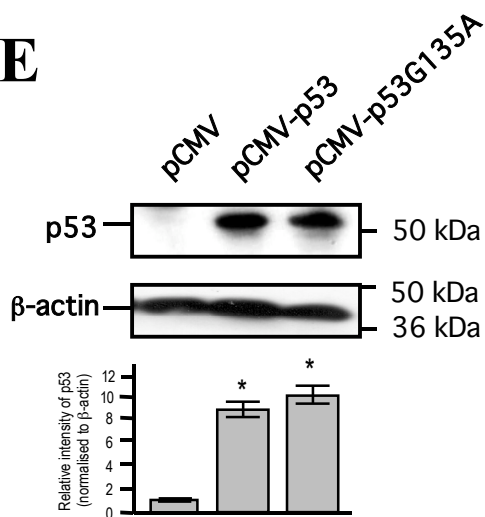
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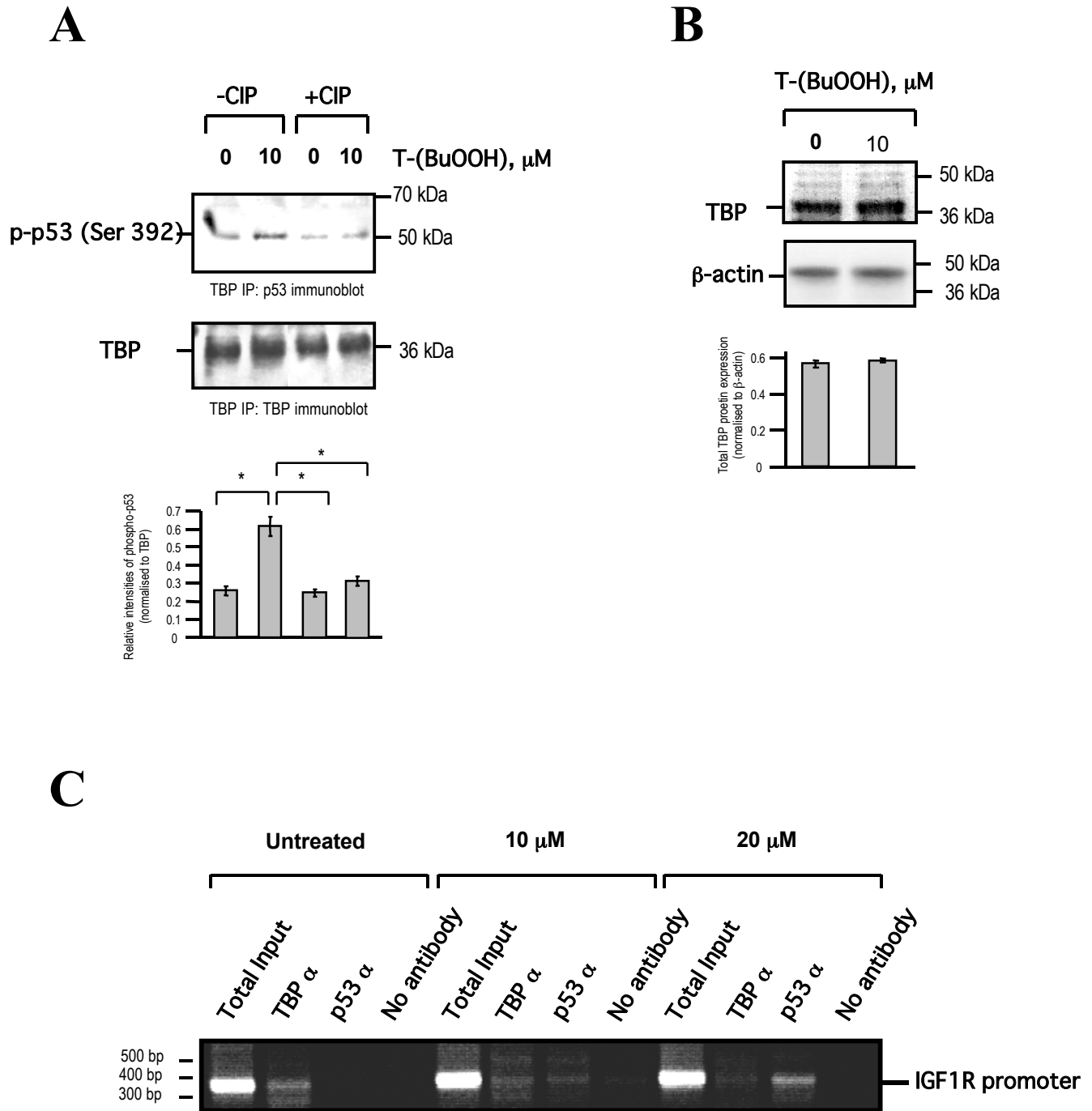


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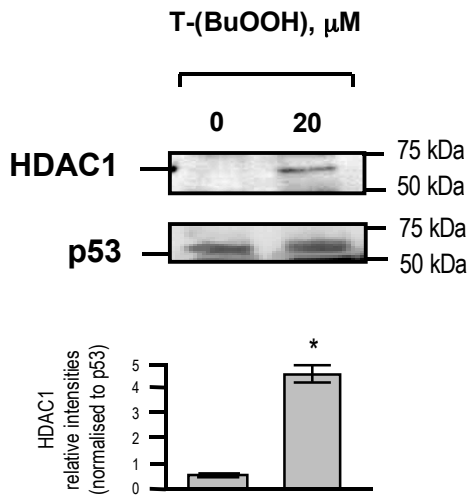


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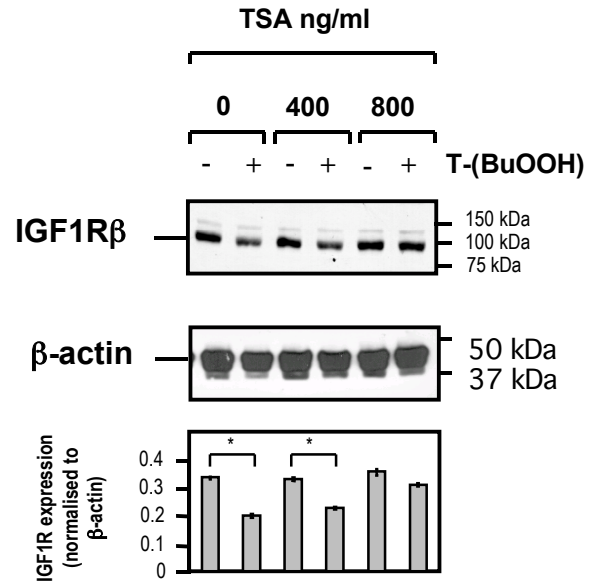




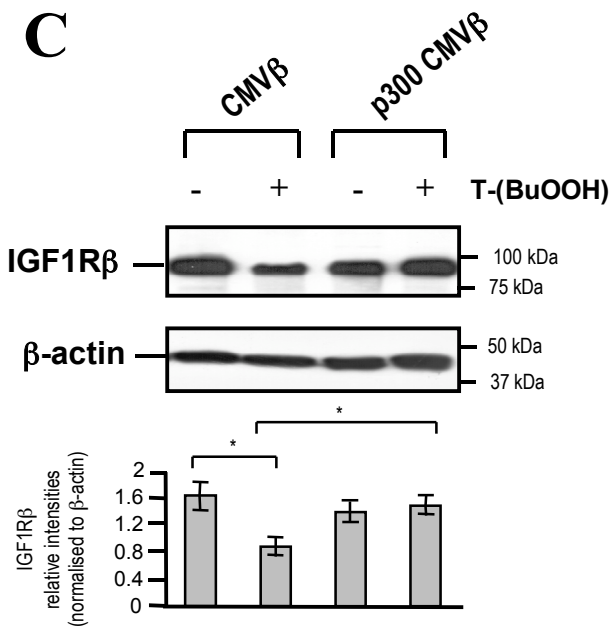
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