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Regulation of GTP-binding Protein (Gαs) Expression in Human Myometrial Cells: A Role for Tumour Necrosis Factor in Modulating Gαs Promoter Acetylation by Transcriptional Complexes

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Running Title: Regulation of the Gαs Promoter

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Background: The GTP-binding protein, Gαs, facilitates myometrial quiescence during pregnancy.
Results: TNF overcomes Trichostatin A (TSA)-induced myometrial relaxation. This is due to changes in levels of CBP and acetylated H4K8 within the Gαs promoter.
Conclusions: Promoter acetylation is important in governing expression of Gαs.
Significance: TSA-induced myometrial relaxation can be overcome by TNF through repression of Gαs promoter activity.

Summary

The onset of parturition is associated with a number of pro-inflammatory mediators which are themselves regulated by the Nuclear Factor kappa B (NF-κB) family of transcription factors. In this context we previously reported that the RelA NF-κB subunit represses transcription and mRNA expression of the pro-quiescent Gαs gene in human myometrial cells following stimulation with the pro-inflammatory cytokine TNF. In this present study we initially defined the functional consequence of this on myometrial contractility. Here we show that, contrary to our initial

expectations, TNF did not induce myometrial contractility but did inhibit the relaxation produced by the HDAC inhibitor, TSA; an effect which was, in turn, abolished by the NF-κB inhibitor, QNZ. This result suggested a role for TNF in regulating Gαs expression via activating NF-κB and modifying histone acetylation associated with the promoter region of the gene. In this context we show that the -837 to -618 region of the endogenous Gαs promoter is occupied by CREB, Egr-1 and Sp1 transcription factors and that CBP transcriptional complexes form within this region where it induces histone acetylation resulting in increased Gαs expression. TNF, acting via NF-κB, did not change the levels of CREB, Sp1 or Egr-1 binding to the Gαs promoter but it induced a significant reduction in the level of CBP. This was associated with increased levels of HDAC-1 and, surprisingly, an increase in H4K8 acetylation. The latter is discussed herein.

Introduction

During pregnancy the uterus adopts a relative state of myometrial smooth muscle inactivity termed quiescence. At the onset of labour, the quiescent state is lost and a series of powerful myometrial contractions occur that act to expel the infant. In the developed world, premature birth complicates 6-10% of pregnancies (1). Significantly, the incidence of birth before 28 weeks gestation (severely preterm) is increasing (2, 3) with those infants having elevated risks of major long-term mental and physical handicap (4).

There is growing evidence indicating that in the human myometrium, the cessation of uterine quiescence (myometrial relaxation) and the onset of both normal and preterm labour are associated with a number of pro-inflammatory factors and cytokines, including TNF, IL1-β, IL-8 and COX-2, which are regulated by a family of transcription factors collectively referred to as Nuclear Factor kappaB (NF-κB) (5-22; reviewed in 23, 24).

While it is accepted that NF-κB is predominantly considered an activator of gene expression, a body of evidence exists that also supports the thesis that NF-κB can act as a repressor of transcription. It can do this directly by binding to DNA and recruiting inhibitory factors such as histone-deacetylase proteins to the promoter (25-27) or through interaction with other transcription factors such as Egr-1, c-Myc and Sp1 (28-31; reviewed in 32). Of significance, however, is that NF-κB can repress transcription of certain genes via in-direct non-DNA binding mechanisms that involve competing for cellular co-activators of transcription such as CBP and p300 (33, 34). Such factors are recognised as enzymes capable of making chromatin more accessible to the transcription machinery through induction of histone acetylation indicative of epigenetic changes. Consequently, removal of these transcription factors from a given promoter by NF-κB may favour promoter histone deacetylation and subsequent transcriptional repression.

The importance of epigenetic mechanisms in the establishment, regulation and maintenance of transcriptional activation or repression is well documented (35, 36). Chromatin is the term given to DNA when it assumes a highly ordered, structured complex within the nucleus. The fundamental unit of chromatin is the nucleosome consisting of 150 bp of DNA wrapped around a core histone octamer (two each of H2A, H2B, H3, and H4; 37). Importantly, DNA-mediated processes, including transcription, must occur within the architectural confines of the nucleosome. In general, chromatin is highly repressive to events such as transcription because the folding of the DNA results in the steric blockade of the template greatly reducing its accessibility by the relevant enzymes and ancillary factors. To circumvent this problem, eukaryotic cells have evolved in a manner that allows the chromatin to be carefully manipulated thereby permitting controlled access to DNA at the appropriate temporal junction. Indeed, eukaryotes have developed multiple mechanisms to finely tune chromatin structure: a principle approach involving post-translational modification of histones including acetylation (38), methylation (39) and phosphorylation (40), all of which are laid down in a dynamic fashion.

The full repertoire of molecular factors that perpetuate myometrial quiescence remains

unknown. It is clear, however, that components of the cAMP/protein kinase A (PKA) pathway are differentially expressed in the human myometrium during pregnancy (41-47): central to this is the GTP-binding protein, Gαs. Indeed, signalling through Gαs is thought to facilitate promotion of myometrial quiescence and Gαs protein levels have been shown to be reduced at the onset of labour (41, 42). There are, however, only a limited number of reports describing the transcriptional control of the Gαs gene. The Gαs promoter itself is located on chromosome 20, is a highly GC-rich segment of DNA and therefore contains many binding sites for transcription factors which bind GC-containing DNA sequences including those for CREB, Egr and Sp families (48-51). Our previous studies have suggested that the promoter region of the Gαs gene is regulated by Sp-like transcription factors that require phosphorylation by PKA (44-46). Moreover, we have demonstrated that TNF-induced activation of NF-κB led to the repression of the Gαs promoter in a manner that utilised competition for the co-activator and histone acetyltransferase, CBP (22). In our view, since levels of CBP are elevated in pregnant human myometrium but decline in labouring samples (52), this suggests that epigenetic control of quiescence may also be important in human parturition.

In this study, we demonstrated that TNF significantly reduced Trichostatin A (TSA)-induced myometrial relaxation. Moreover, the NF-κB inhibitor, N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine, (QNZ), prevented the effects of TNF. Utilising the chromatin immunoprecipitation (ChIP) assay, we show that the native Gαs promoter is occupied by CREB, Egr-1, Sp1 and CBP; moreover, both promoter occupancy by CBP and acetylation status of histone H4K8 were subsequently modified in the presence of TNF and the histone deacetylase inhibitor, TSA. We discuss the implications of these findings.

Materials and Methods:
Selection of Patients and Tissue Collection
Women were recruited from the Department of Obstetrics and Gynaecology at the Jessop Wing, Sheffield Teaching Hospitals NHS Foundation Trust, Sheffield South Yorkshire U.K. This study received approval from the Rotherham Local Research Ethics Committee (**REC Reference No. 05/Q2306/22**). All patients gave informed, written consent. Lower uterine segment myometrial samples were obtained from healthy women undergoing elective Caesarean section at term (n = 45, age 16-43, gestation 37-40 weeks) as described previously (21, 22). Myometrial smooth muscle cell cultures were then subsequently generated as detailed in Phaneuf et al. (43).

Cell Culture, Transient Transfections, Plasmids and Luciferase Assays
Transient transfection of primary human myometrial cells was performed using the LT-1 reagent from Miras (Geneflow, Staffordshire UK) as described by Chapman et al. 2005 (22). The Gαs-luciferase reporter (Gαs-luc) has been described previously (45). The 3x-κB-ConA-luciferase (3x-κB-ConA-Luc) and enh-ConA-luciferase (ΔκB-ConA-Luc) vectors were the generous gift of Prof. Ron Hay (University of Dundee, UK) and the construction of these has been reported in detail (53). The RcRSV-HA-CBP expression plasmid was supplied by Dr. Paul Hurd, (Queen Mary University of London, UK) but was originally generated in the Goodman laboratory (Vollum Institute, Portland, Oregon) and its construction has been described elsewhere (54). The pcDNA-HDAC-1 vector was the gift of Prof. Bradley Bernstein (Department of Pathology, Massachusetts General Hospital, Harvard Medical School) and its construction has been described previously (55). For each transfection, 200 ng of luciferase reporter was used. Twenty-four hours after transfection, cells were then stimulated for a further 24 hours with 10 ng/ml TNF. For experiments investigating the role of CBP, 200 ng RcRSV-HA-CBP or 200 ng RcRSV-NRC-MCS were employed. Transfections using HDAC-1 utilised 0.5 μg, 1.0 μg and 1.5 μg pcDNA-HDAC-1, or 0.5 μg, 1.0 μg and 1.5 μg pcDNA3 to normalise the amount of DNA. Luciferase assays were performed three times in triplicate as previously described (22) and data collected using a Berthold Sirius

luminometer (Geneflow Ltd. Staffordshire, UK).

Immunofluorescence

Myometrial cells were cultured in a 24-well plate and upon reaching 80% confluence were stimulated with 10 ng/ml TNF for 24 hours. The cells were washed in PBS and fixed in 1% (v/v) formaldehyde overnight at 4°C. Cells were washed in PBS 3 times for 5 minutes on a rocking platform and permeablised with PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100 with 3, 5 minute washes. After washing again in PBS, RelA antiserum (Santa Cruz Biotechnology Inc. sc-372) was added to each well at a dilution of 1:100 (in PBS containing 1% (w/v) BSA) for 2 hours at 37°C. The antibody was removed with three 5-minute washes in PBS and the goat anti-rabbit IgG secondary antibody was added to the wells at a dilution of 1:200 (in PBS, 1% (w/v) BSA) for 1 hour at 37°C. The unbound antibody was washed with three, five minute PBS washes and the nuclear counter-stain DAPI added to the wells at a 1:20 dilution in PBS.

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was performed using the Magna-ChIP ChIP assay kit provided by Millipore (#17-611) following the manufacturer's guidelines. Essentially, four T-75 flasks of primary myocytes were grown to 100% confluence. Two of these flasks were then stimulated with 10 ng/ml TNF for 24 hours. Cells were then fixed for 10 minutes using 37.5% formaldehyde added directly to the media. This reaction was subsequently quenched by adding 2 ml 1M glycine made up in water. Finally, media was removed and cells washed twice in ice cold PBS (pH 7.4) supplemented with protease inhibitor cocktail (Millipore). Cells were removed from each flask in the PBS:protease inhibitor cocktail using a cell scraper. Appropriate samples were then pooled into control or stimulated groups and the cell suspension clarified by centrifugation at 4°C at 800g for five minutes.

The cell pellet was re-suspended in 500 μl Magna-ChIP lysis buffer supplemented with protease inhibitor cocktail. Essentially a two-step lysis was undertaken to reduce non-specific chromatin:IgG interactions. In the first lysis step, cells were opened and nuclei obtained; cells were then incubated in lysis buffer on ice for 15 minutes and vortexed briefly every five minutes. This suspension was clarified by centrifugation at 800g for five minutes at 4°C. For the second lysis step, which opened the nuclei, the supernatant was discarded and the pellet re-suspended in 500 μl of Magna-ChIP nuclear lysis buffer and the sample subjected to ultrasonic cavitation on high power for three 10 second exposures in order to lyse the nuclei and fragment the chromatin. Chromatin fragments with an average size of ~500bp were obtained.

Immunoprecipitation of Chromatin-bound Complexes

The fragmented chromatin was separated in to 50μl aliquots (approximately 1x10^6 cells) and then diluted in 450 μl of Magna-ChIP dilution buffer. To each sample, 5 μg of immunoprecipitating antibody was added. Antisera used were anti-RelA C-terminal antibody (Santa Cruz Biotechnology Inc. sc-372), anti-Sp1 (Santa Cruz Biotechnology Inc. sc-), anti-CREB (Cell Signalling Technology; #9192), anti-acetylated histone-H3 (H3K9; Millipore; #06-599) and anti-acetylated histone H4 (H4K8; Cell Signalling Technologies; #2594), CBP and Egr-1 (Santa Cruz Biotechnology Inc. sc-369 and sc-588 respectively) and non-specific rabbit IgG (Abcam; #ab46450). To this a 20 μl bed volume of pre-prepared Dyna-beads (Invitrogen) pre-adsorbed with 20 μg/ml sonicated salmon sperm DNA and 1 mg/ml BSA in TE buffer were added. The immunoprecipitation reactions were carried out overnight at 4°C with constant, gentle agitation. After this period, immunoprecipitated complexes were recovered with a magnet and the beads washed to remove non-specific binding using alternative high salt:low salt and LiCl washes in accordance with the manufacturer's instructions. Transcription factor-bound DNA was then eluted and purified as detailed in the Magna-ChIP protocol and stored at -20°C.

PCR of Immunoprecipitated DNA

PCR on the immunoprecipitated DNA was carried out using primers flanking the κB sites

within the IκBα promoter (sense 5'-GACGACCCCAATTCAAATCG-3' and anti-sense 5'-TCAGGCTCGGGGAATTTCC-3') as a positive control for the ChIP assay giving a product size of ~300bp (22). As a positive control for promoter occupancy by CBP, PCR on the immunoprecipitated DNA was also carried out using primers flanking the CXCL-9 promoter (56). The primer sequences used were CXCXL-9 sense 5'-TTCCACATCCAGGTAGCAACTTTG-3' and CXCL-9 antisense 5'-TGTTGGAGTGAAGTCCGAGAATGT-3'. These primers gave the expected product size of 86bp. Reactions conditions for both controls were those originally described in Saccani et al. (57) and are given here: one cycle of denaturation at 94°C 3 minutes, 36 cycles of 94°C 45 seconds, 60°C for 1 minute and 72°C for 1 minute followed by a final elongation at 72°C for 10 minutes. Samples were then resolved using 1.5% Tris-acetate agarose gel electrophoresis and a product size of ~300 bp corresponding to the IκBα promoter was observed.

During the course of the study it became apparent that the GC-rich nature of the Gαs promoter made its amplification by PCR difficult. Consequently, to analyse promoter occupancy in the Gαs promoter (-837 to -618; 48, 49) the following primers were used: Gαs-distal-3 sense 5'-TTG GTT CGT GCT CGC CTC GC-3' and Gαs-distal-3 anti-sense 5'-CAG TGA CGA CCC CTC GCA CG-3'. The following protocol was also utilised to facilitate PCR from the GC-rich -837 to -618 region of the Gαs promoter. Each 25µl reaction consisting of 5X Hercules buffer, 25mM dNTPs, 0.25µl ChIP DNA, 100 pmol Gαs-distal-3 sense, 100 pmol Gαs-distal-3 anti-sense, 0.25 µl Hercules DNA polymerase (NB the manufacturer, Agilent, did not provide a value for the number of enzyme units/µl) and 1µl DMSO. Reactions conditions were: one cycle of denaturation at 98°C for 3 minutes, 38 cycles of 98°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds followed by a final elongation at 72°C for five minutes. Samples were then resolved using 1.5% Tris-acetate agarose gel electrophoresis and a product size of ~219bp (-837 to -618 bp of the Gαs promoter) were obtained.

Band Intensity Analysis of Amplified, Immunoprecipitated DNA
In this study, end-point PCR was used to give semi-quantitative data on enrichment levels of these respective transcription factors on each promoter. These analyses were undertaken using the SynGene GBox Chemi-16 gel documentation system to capture images. Subsequent band quantification was undertaken using GeneTools Version 4 quantification software (SynGene, Cambridge U.K.). In all cases, the value ascribed to non-specific binding of IgG was subtracted from the specific-binding value. That value was then expressed as a percentage of the input fraction; with the input fraction intensity being taken as 100%. Where statistically significant changes were observed, all appropriate ChIP gels are illustrated.

Western Blot Analyses
Western blot experiments were performed as detailed in Chapman et al. (22). Briefly, RelA expression was detected using anti-RelA C-terminal antibody (Santa Cruz Biotechnology Inc. sc-372). The anti-acetyl-RelA anti-serum was obtained from Cell Signalling Technology (#3045) and detects transfected levels of RelA NF-κB only when residue Lys310 is acetylated. Gβ (Santa Cruz Biotechnology Inc. sc-378) was utilised to ensure equal well loading as detailed in Chapman et al. (22). After incubation with an appropriate secondary antiserum (125 ng goat-anti-rabbit-HRP conjugates; #P0448; Dako), membranes were then washed in PBST and developed according to the EZ-ECL detection protocol supplied by Geneflow Ltd. (Staffordshire, U.K.).

Myometrial Contractility
The effects of TNF were tested in a model of myometrial contractility at 10 ng/ml and 30 ng/ml. Experiments were carried out as described in Pearson et al. (58). Briefly, strips were mounted in vitro at 37°C in a physiological salt solution aerated with 95% O_2/5% CO_2 and allowed to contract spontaneously. Following equilibration, separate concentration-response curves for both TSA and the NF-κB inhibitor, N4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine, (QNZ; 59) were generated. The ability of

QNZ to modulate the contractile response was carried out by pre-incubating tissue ± 10 ng/ml or 30 ng/ml TNF for one hour prior to cumulative additions of TSA. Data were analysed by calculating activity integrals for contractions over a 30 min period and concentration-effect curves fitted to a four parameter logistic equation (58).

Statistical Analysis
All experiments were performed a minimum of three times and results are expressed as the mean ± SEM (error bars). All data analyses were conducted on GraphPad Prism Version 5.02 (GraphPad Software, San Diego, California). Data from transfection experiments and pharmacological studies were compared using one-way ANOVA and analysed further using Tukey's multiple comparisons post-test. Comparison of data from two matched samples were compared using a paired, two-tailed t-test; $p<0.05$ was considered statistically significant.

Results
TNF Inhibits TSA-induced Relaxation in Isolated Strips of Human Myometrium: A Process Inhibited by Treatment with QNZ
A number of pro-inflammatory cytokines, including TNF, are associated with the onset of both normal and preterm birth (23, 24). Together with data highlighting the reduction of Gαs protein levels in term and pre-term labouring myometrium, we speculate that the localised inflammation within the myometrium is an important step in this transition from quiescence to contractility. Much of that work, however, has been conducted in primary cell monolayers. As such we wanted to determine how TSA, QNZ and TNF could influence contraction of isolated strips of human myometrium. Firstly, cumulative addition of TSA was seen to relax spontaneously contracting, isolated myometrial strips (Fig. 1A; red line; n = 6). Significantly, while TNF had little effect on TSA-induced myometrial relaxation at 10 ng/ml (Fig. 1A; blue line), it did, however, greatly reduce the TSA-induced relaxation at a concentration of 30 ng/ml (Fig. 1A; green line).

To support the notion that this contraction was mediated by the NF-κB family of transcription factors, the NF-κB inhibitor, QNZ, which is known to inhibit endogenous TNF synthesis (59), was also seen to inhibit myometrial contractions with an IC_{50} of $3.9\times10^{-8}M$ (n = 6) suggesting a role for NF-κB in this contractile response (Fig. 1B).

Finally, pre-incubation of myometrial strips in 50nM QNZ (a concentration close to the IC_{50}) followed by addition of TSA also produced relaxation (Fig. 1C; green line; n = 6). In the presence of 30 ng/ml TNF and 50 nM QNZ a leftward shift in the concentration-response curve to TSA was seen (Fig. 1C; red line; n = 6) supporting our view that myometrial relaxation induced by TSA can be modulated by TNF in a mechanism involving the NF-κB family of transcription factors.

TNF Represses Expression of the Gαs Promoter While Inducing NF-κB Activity A decline in both the level of mRNA and Gαs protein within human myometrium has been shown to accompany the transition from quiescence to labour (22, 42). While the full details of this mechanism remain unclear, we have previously described the TNF-induced repression of a Gαs-Luc reporter vector transfected into primary myometrial myocytes being mediated by the NF-κB RelA subunit competing for limiting quantities of the key transcription co-factor CBP (22). Consequently, control experiments were undertaken to determine that the myometrial cell cultures employed in this study behaved in a similar manner: briefly, TNF was seen to repress the Gαs-luc reporter (Fig. 2A) whilst inducing transcription from the NF-κB-sensitive 3x-κB-luc vector (Fig. 2B). No activity was seen cells harbouring the ConA-luc vector (which is not NF-κB sensitive) were exposed to TNF (Fig. 2C). Since we wished to investigate the regulation of the Gαs promoter in the presence of activated NF-κB RelA, we confirmed, as expected, that TNF was inducing nuclear localisation of the RelA in primary myometrial myocytes (compare diffuse staining in un-stimulated or non-specific IgG-stained cells (Fig. 2D; Left and Right panels) to TNF-stimulated cells (Fig. 2D; Middle panel).

The -837 to -618 Region of the Endogenous Gαs Promoter is Occupied by CREB, Egr-1 and Sp1 and Also Recruits CBP The Gαs promoter originally utilised in the Gαs-luc vector represents the region from -790 to the Transcription Start Site (TSS; 45). Previous studies have concentrated on the more proximal aspects of that promoter sequence (45). Therefore, in this study, we wished to define how the more distal aspect of the Gαs promoter, region -837 to -618, regulated expression (illustrated in Fig. 3A).

The native Gαs promoter is a GC-rich, TATA-less regulatory region (48). Using the TransFac algorithm (49), we identified many putative transcription factor binding sites. Those of particular relevance to this study included Sp, Egr and CREB families. Sp1 has been shown to play a pivotal role in regulating the expression of TATA-less GC-rich promoters through its ability to position RNA polymerase II at the appropriate initiator site (50, 51). Moreover, Sp1 is also thought to serve as an active boundary between non-transcribed heterochromatin and transcriptional competent euchromatin (50). As such, it was decided to examine Sp1 in this context. Egr1 was chosen because it is a potent GC-rich binding zinc finger transcription factor. Moreover, it has been shown to compete with NF-κB RelA for DNA binding and also binds to CBP (28, 60). We decided to investigate CREB since it requires an interaction with CBP for full transactivation (61, 62) and, in terms of myometrial biology, both CREB and CBP have been demonstrated to have a pivotal role in Gαs regulation (22, 45, 52). Consequently, to determine if these factors were present on the endogenous Gαs promoter we isolated primary myometrial smooth muscle cells as detailed above and cultured them until confluent. The ChIP assay was then utilised to determine if these factors were present on the distal Gαs promoter fragment. Fig. 4A-4C demonstrates that CREB, Sp1 and Egr-1 occupied the -837 to-618 aspect of the endogenous Gαs promoter respectively. Positive controls for this experiment included occupation of the IκBα promoter by RelA NF-κB (Fig. 4D). Other endogenous Sp and Egr family members were

not detected in western analyses prior to the ChIP study (data not shown).

Our previous work demonstrated that when CBP and the Gαs-luc reporter vectors were co-transfected into primary myometrial cells, expression of exogenous CBP was shown to enhance activity of the Gαs promoter (Fig. 4A; Ref. 22). Factors such as CBP, however, do not have an intrinsic specific DNA binding domain but can still be recruited to a promoter through direct interactions with promoter-bound heterologous factors (62). In the context of the Gαs promoter, those factors identified in Fig. 3A-3C, namely CREB, Sp1 and Egr-1 have all been documented to interact with CBP in various cells of various origins including the myometrium (60-62). Consequently, we wished to determine if CBP was also present within the -837 to -618 section of the native Gαs promoter. To address this notion endogenous chromatin from primary myometrial myocytes was again subjected to the ChIP assay. Significantly, CBP was associated with this segment of the endogenous Gαs promoter in native chromatin derived from myometrial myocytes (Fig. 5B; Upper Panel). To support this observation, we also examined the CXCL9 promoter which has previously also been shown to be a target of CBP (56). Again, in our studies we were also able to see CXCL9 promoter occupancy by CBP (Fig. 5B Lower panel).

TNF Does Not Reduce Gαs Promoter Occupancy by CREB, Sp1 or Egr-1 But Does Reduce Binding by CBP TNF has been shown to repress a Gαs promoter construct transfected into myometrial cells (22). The factors involved in regulating the natural Gαs promoter within its native context, however, have not been defined. In this present study, we employed the ChIP assay on chromatin extracted from TNF-stimulated primary myometrial myocytes, to determine whether there were any changes in the level of Gαs promoter occupancy by the factors identified above, namely CREB, Egr-1 and Sp1. In the presence of TNF, we did not observe any noticeable change in the levels of CREB (Fig. 6A), Egr-1 (Fig. 6B) or Sp1 (Fig. 6C) on the -837 to -618 portion of the native Gαs

promoter when the myometrial cells were exposed to TNF.

As stated above, CBP is not thought to possess intrinsic DNA-binding ability instead relying on recruitment to the promoter through interactions with promoter-bond ancillary factors, including those identified herein. Expression of exogenous CBP alone was seen to enhance expression from the Gαs-luc reporter vector (Fig. 5A; discussed above). Moreover, we previously demonstrated that expression of exogenous CBP could relieve TNF-induced repression of the Gαs-luc promoter vector (22). That observation suggested that Gαs repression was occurring because CBP was being removed or lost from the transcription complex based on the Gαs promoter. To test this notion further, we undertook a series of ChIP analyses on TNF-stimulated primary myometrial myocytes to determine if the level of CBP occupying the Gαs promoter was reduced when cells were exposed to TNF. Fig. 6D clearly illustrates that the level of CBP present within the -837 to -618 region of the endogenous Gαs promoter is reduced approximately two-fold when myometrial myocytes were exposed to TNF. Again, to confirm that this system was working efficiently, TNF-induced occupancy of the IκBα promoter by RelA served as the positive control (Fig. 6E; Upper panel). Moreover, we have previously demonstrated that RelA does not bind to the Gαs promoter, consequently, that observation was utilised in this study to confirm that the effects observed herein were specific since, once again, there was no binding of RelA to the endogenous Gαs promoter (Fig. 6E; Lower panel).

Gαs-luc Promoter Activity is Repressed by Histone Deacetylase-1 (HDAC-1) But Induced by the HDACi, TSA As discussed above, histone acetylation is generally associated with transcription of a given promoter while reciprocal histone deacetylation generally alters chromatin structure in a manner where it becomes refractory to transcription thereby silencing a given gene. In terms of myometrial function, there is increasing data that suggest deacetylation of myometrial genes may be of importance during quiescence. Indeed, both

Phillips et al. (2005; 45) and Karolczak-Bayatti et al. (2009; 47) have reported the binding of HDAC-1 to two other GC-rich control regions, namely the hCG/LH promoter and the PKA regulatory subunit RIIα promoter. Consequently, we wished to test the notion that HDAC-1 could repress the function of the endogenous Gαs-luc promoter construct. Importantly, when monolayers of primary myometrial smooth muscle cells co-transfected with the Gαs-luc and increasing amounts of HDAC-1, HDAC-1 caused a dose-dependent repression of the Gαs-luc promoter construct (Fig. 7A). Interestingly, the effect was observed with 500 ng of HDAC-1 plasmid DNA but reaching a maximal effect at the lower dose of 200 ng of HDAC-1 plasmid DNA. The reason for this is not clear but may reflect a phenomenon of promoter squelching when elevated levels of exogenous factors are introduced into the cell.

To ensure that those observations where exogenous HDAC-1 repressed expression from the Gαs-luc vector (Fig. 6A) were not merely due to promoter squelching, we also employed the ChIP assay to immunoprecipitate the endogenous Gαs promoter using anti-HDAC-1 anti-serum. Cultures of primary myometrial cells were treated with TNF or not stimulated at all. As seen in Fig. 7B, TNF treatment causes an association of HDAC-1 with the endogenous Gαs promoter supporting the notion that the HDAC-1 deacetylase is recruited to this region in the presence of inflammatory mediators.

Since we observed that exogenous HDAC-1 could repress activity of the Gαs promoter, the obvious corollary was that the HDAC inhibitor, TSA, should induce activity of the Gαs-luc construct. To test this notion, primary myometrial smooth muscle cells transfected with the Gαs-luc vector were exposed to TSA (330nM). Fig. 7C clearly demonstrates that TSA can induce expression from the Gαs-luc promoter construct. Moreover, the TNF-induced repression of Gαs could also be relieved when TSA was added to the cultures after TNF (Fig. 7C). Together, these data suggest the need for acetylation of this promoter and/or factors involved in regulating this region.

It has been reported that the NF-κB RelA subunit undergoes acetylation at key lysine residues prior to transactivation (63, 64). Moreover, HDAC inhibitors have been observed to facilitate this acetylation by reducing such deacetylase activity. As such, in terms of myometrial biology, where there is a thesis that HDAC inhibitors could serve a tocolytic (labour-stopping) function, we wished to determine whether TSA could facilitate acetylation, and hence activation of, RelA. To do this, primary myometrial myocytes were transfected with the 3x-κB-luc reporter and stimulated with TNF, TSA or DMSO vehicle. As can be seen in Fig. 7D, only TNF activated the NF-κB-sensitive reporter construct; neither TSA nor DMSO had any effect. Significantly, using western analysis with an antiserum specifically recognising acetylated RelA (acetyl-Lys 310), we did not see any changes in RelA acetylation status in the presence of TNF, TSA or DMSO (data not shown). We did, however, observe elevated nuclear localisation of RelA when cells were exposed to TSA (Fig. 7E; left panel) but this was still markedly below those levels seen for TNF (Fig. 7E; right panel; same samples but reduced exposure time to illustrate TNF effect). Equal loading of samples was confirmed by probing for Gβ protein (Fig. 7E; 22).

TNF Increases Histone H4K8 Acetylation on the Gαs Promoter But Not That of Histone H3K9 It has been well documented that CBP is a potent histone acetyltransferase targeting a variety of intra-cellular factors including histone H3 and H4 (38). Acetylation of both H3 and H4 can be associated with both transcriptional activation or repression of a number of genes as they modify the inherent promoter architecture thereby enhancing the accessibility of the promoter to RNA polymerase II (65, 66 and references therein). At present, however, there are no data describing whether the Gαs promoter is acetylated. Since we have identified CBP occupancy of the -837 to -618 region of the native Gαs promoter (Fig. 5B) and that the level of CBP occupying that region of the Gαs promoter is reduced in the presence of TNF (Fig. 4D) we wanted to determine (i) if the Gαs promoter was acetylated and (ii) if there

were changes in the acetylation status of either histone H3 or H4 within the Gαs distal promoter fragment in primary myometrial myocytes upon exposure to TNF. Once again, myocytes were stimulated with TNFα for 24 hours and further ChIP analyses performed for pan-acetylated lysine residues, acetylated Histone H3 or acetylated histone H4 within the endogenous distal Gαs fragment.

In our system, we did not observe any change in the levels of acetylated lysine within the -837 to -618 Gαs promoter region when cells were exposed to TNF (Fig. 8A). Moreover, we did not observe any change in the level of histone-H3K9 acetylation as judged by those ChIP analyses between controls and TNF-stimulated cells (Fig. 8B). Significantly, however, exposure of primary myometrial smooth muscle cells to TNF was seen to induce an increase of approximately 2.5-fold in the Gαs band intensity when ChIP was utilised for acetylated Histone H4K8 (Fig. 8C) suggesting this modification of the endogenous Gαs promoter is elevated in the presence of TNF.

Discussion
TNF Overcomes TSA-induced Myometrial Relaxation The cumulative addition of TSA was seen to induce relaxation in isolated myometrial strips: an effect abolished by the addition 30 ng/ml TNF although TNF was not seen to induce myometrial contraction on its own. Whether contraction would be seen at 24 hours (a similar time course utilised for the transfection and ChIP studies herein) remains unclear although certainly at 24 hours, tissue viability would be brought into question. Significantly, QNZ, the NF-κB inhibitor, prevented the TNF-induced modulation of TSA-mediated relaxation. This observation suggests two things; 1) the uterotonic actions of TNF have an NF-κB-regulated component to them; 2) the uterotonic effects of TNF can only occur once other factors, not identified in this study, are acetylated (TSA being an inhibitor of deacetylation). It remains unclear which NF-κB dimers are responsible for the TNF-induced effect. While QNZ is marketed as a specific NF-κB inhibitor, its molecular mechanism remains unclear. This is a salient point: essentially, for example, if QNZ

functions through inhibition of the IκBα, then it would only serve to inhibit complexes regulated by that protein. There are certain NF-κB complexes, including p52 homodimers, p52:RelB heterodimers, that are not sensitive to IκBα inhibition (23, 32) and would therefore not be targeted by QNZ.

The Gαs Promoter is Bound by a Number of Transcription factors Our current hypothesis suggests that acetylation of the Gαs promoter by CBP is a pre-requisite for Gαs expression in the human myometrium: a process which would facilitate uterine quiescence seen during pregnancy (22, 52). The corollary to this, therefore, is that inflammatory mediators, including TNF which we have previously shown to serve as a potent repressor of the Gαs promoter (25), will exert their action through repression of the Gαs promoter.

In this study, to determine how TNF may repress expression from the Gαs promoter, we have been able to demonstrate for the first time that the -837 to -618 region of the endogenous Gαs promoter is occupied by the transcription factors CREB, Egr-1 and Sp1. We were unable to determine the exact loci of these sites in this study; as such the following may provide explanations for our observations. Firstly, the size of the chromatin fragments we obtained after sonication was ~500bp (data not shown), consequently, we cannot rule out that the chromatin enrichment we observed in our ChIP assays may also include sequences located both more proximally or distally to the -837 to -618 Gαs fragment studied herein. For example, the ChIP is still specific for CREB, Egr-1 and Sp1 etc. and the primers remain specific for the appropriate sequences flanking the -837 to -618 region, but if the specific enriched fragment contains both a more proximal or distal transcription factor binding site and the site of primer recognition, then such a result suggests direct occupancy of the region under study.

Secondly, to put this into context of nucleosomes, we have investigated a fragment of 219 bp; essentially this represents one complete nucleosomal particle with an extra 69 bp of DNA. We cannot, therefore, be certain that the effects we have observed occur throughout the entire regulatory locus of the Gαs region or whether they are restricted to this particular nucleosomal region. Such explanations, however, does not detract from our findings because it still supports the thesis that these factors are binding and regulating the Gαs promoter.

We also demonstrated that exogenous histone deacetylase activity, namely HDAC-1, could repress the Gαs-luc promoter while TNF stimulation induced occupancy of the Gαs -837 to -618 fragment by endogenous HDAC-1. Furthermore, the HDAC inhibitor, TSA, had the opposite effect and induced Gαs promoter activity. Together, these observations support the view that deacetylation of the Gαs promoter is an important regulatory mechanism. At present, however, we were unable to fully define whether the deacetylation was specific to nucleosomal histones, or whether the deacetylation was more global; inducing its effects through promoter-bound ancillary factors other than histones.

H4K8 Acetylation and Gαs Promoter Repression The obvious question posed by our data is that if deacetylation is required to reduce or silence Gαs expression, why should an increased recovery of acetylated histone H4 be observed in the distal Gαs fragment in the presence of TNF. Firstly, in terms of an explanation for this observation of increased H4K8 acetylation with TNFα, is that acetylation at H4K8 is required to stabilise the interaction between DNA and the repressor element 1-silencing (REST) protein (reviewed in Ooi and Wood, 2007; 65). This mechanism requires that the interaction between REST and DNA is stabilised by the binding of the ATP-dependent chromatin-remodelling enzyme, BRG1 (which forms part of the REST complex) to H4K8 (65). In doing so the REST complex can subsequently serve as a platform for other transcriptional repressors, including the HDAC-1 and -2-containing complex of mSin3 (65). As such, increased H4K8 acetylation as seen in our data, induced by CBP prior to removal from the Gαs promoter region, would facilitate the binding and stabilisation of the REST:mSin3 complex to

the distal Gαs fragment and induce repression of that promoter. Further work is needed to test this hypothesis because there is evidence that mSin3-associated HDAC-1 can deacetylate histone H3K9; our data described herein did not see such H3K9 deacetylation in the presence of TNF.

In terms of interaction with those factors in the distal Gαs promoter, REST has been shown to bind Sp1 and Egr-1. In both studies, bidirectional regulation of the specific target genes were noted; i.e. Sp1 could activate expression of sodium channels; while Egr-1 stimulated expression of the Cav3.2 T-type Ca^{2+} channel. In the presence of REST both these effects were repressed (66, 67). As such, we speculate a similar mechanism may be occurring to govern expression of the Gαs GTP binding protein.

Influence of Non-genomic Protein Acetylation Previous work has also demonstrated a key role for lysine acetylation of Hsp20 (68). This post-translational modification was seen to govern the interaction between Hsp20 and cofilin, Indeed, inhibitors of the non-nuclear lysine deacetylase (KDAC) KDAC8 modified Hsp20 acetylation profile in such a manner that myometrial contractility was subsequently inhibited.

At present, the exact mechanism by which TNF influences acetylation and Gαs expression is unclear. TNF is a potent inducer of NF-κB activity; indeed, RelA NF-κB is documented to undergo CBP-mediated acetylation at Lys218, 221 and 310 (Chen et al. 2001). Moreover, Dai et al. (2005) subsequently reported that newer HDAC inhibitors including SAHA and MS-275 induced acetylation and nuclear localisation of RelA. That group suggested that this was necessary for NF-κB-induced anti-apoptotic effects in certain leukaemia cells (Dai et al., 2005; 64). We did not observe TSA-induced acetylation of RelA in this study although the antiserum used was specified by the manufacturer to only be effective against transfected RelA not the endogenous protein: this may account for our lack of data in that experiment. We did, however, also note that

TSA stimulation of myometrial cells caused an increase in RelA nuclear localisation without activating the NF-κB-sensitive 3x-κB-luc reporter. The significance of that observation remains unclear at present but may be due to a delay in IκBα re-synthesis similar to that seen for pervanadate-induced NF-κB activation (69). Moreover, it would also suggest that, in the context of myometrial function, a cautious approach to employing HDAC inhibitors as tocolytic (labour-stopping) drugs should be employed. Briefly, there is growing body of literature interested in understanding whether HDAC inhibitors may be of use clinically as tocolytic drugs (17, 68, 70). While such studies do present convincing effects that HDACi inhibitors such as TSA exert robust pro-relaxant effects on isolated strips of human myometrium (17, 68, 70; Fig. 1 this study), we must, however, also consider potential unwanted effects including acetylation and subsequent activation of non-chromatin based factors that could also promote labour – i.e. NF-κB RelA such as that described by Chen et al. (63). Indeed, our data demonstrate that TNF can overcome TSA-mediated myometrial relaxation. If HDAC inhibitors are having a positive effect on the activity of NF-κB, utilising them in the clinical setting may only serve to exacerbate those premature myometrial contractions that the drug was given to prevent.

Conclusions In conclusion, our data demonstrate that the HDAC inhibitor, TSA induces relaxation in isolated strips of human myometrium; an effect that can be overcome by stimulation of such tissues with TNF. While TNF itself did not induce contraction, the NF-κB inhibitor, QNZ did repress the TNF effect while also sensitising the tissue to TSA implying that the NF-κB family serve an important function in this modulatory role. Subsequent studies into the regulation of the pro-quiescent factor, the Gαs GTP binding protein, demonstrated that the -837 to -618 region of the endogenous Gαs promoter is occupied by CREB, Egr-1 and Sp1 transcription factors. Moreover, CBP is also found associated with this region: we believe CBP is required to acetylate the Gαs promoter leading to Gαs expression. In the presence of TNF, while there is no discernible change in

the level of CREB, Sp1 or Egr-1 bound to the Gαs promoter, there was significant reduction in the amount of CBP: an observation that was supported by increased levels of HDAC-1 and elevated H4K8 acetylation. We were also able to show that exogenous HDAC enzymes, namely HDAC-1 could repress the Gαs-luc construct while reciprocal activation was seen with exogenous CBP or the HDACi, TSA. Taken together, our data suggest that expression from the Gαs promoter is, in part, governed by protein acetylation.

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Footnotes

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Figure Legends
Fig. 1: TNF, TSA and QNZ all Effect Myometrial Contraction. Isolated myometrial strips were prepared as described in Materials and methods. (**A**) Myometrial tissue strips (n = 6 for each observation) were incubated in either 10 ng/ml or 30 ng/ml TNF for 1 hour followed by the cumulative addition of 10^{-9}-10^{-5}M TSA to the bath. Data were analysed using an unpaired, two-tailed t-test and results are expressed as the mean ± S.E.M. (error bars); p<0.05 was considered statistically significant. Control strips were incubated with vehicle and showed no significant change in contractility with time. TSA alone caused a concentration-dependent relaxation of spontaneous myometrial contractions which was inhibited in the presence of 30 ng/ml TNF but unaffected by 10 ng/ml. (**B**) QNZ inhibited myometrial contractility in a concentration-dependent manner with IC_{50} 3.9x 10^{-8}M. (**C**) Cumulative addition of TSA to myometrial tissues pre-incubated with QNZ (50 nM) and TNF (30 ng/ml) produced a leftward shift in the response compared with QNZ alone.

Fig 2: TNF Represses Gαs Expression Whilst Inducing NF-κB Activity in Primary Myometrial Myocytes. Primary myometrial cultures were transfected with 200 ng of either (**A**) Gαs-luc, (**B**) 3x-κB-ConA-luc (NF-κB-responsive) or (**C**) ΔκB-ConA-luc (NF-κB unresponsive). After 24 hours cells were stimulated with TNF (10 ng/ml) for 24 hours. Promoter activity was quantified using a Berthold Sirius tube luminometer. All experiments were performed three times in triplicate. Data were analysed using an unpaired, two-tailed t-test and results are expressed as the mean ± S.E.M. (error bars); p<0.05 was considered statistically significant. As expected, TNF repressed Gαs expression (**A**; p = 0.002) and activated NF-κB (**B**; p = 0.036). No NF-κB activity was observed in a control reporter lacking κB sites (**C**). Immunostaining was used to demonstrate TNF-mediated induction of RelA nuclear localisation in primary myometrial myocytes (**D**; white arrows. Compare Middle Panel; TNF-stimulated with Left Panel; un-stimulated and Right Panel; negative control; scale bar = 100μm).

Figure 3: Schematic Illustration of the Gαs Promoter Highlighting the -837 to -618 Region. The Gαs promoter originally utilised in the Gαs-luc vector represents the -790 to the Transcription Start Site (TSS; **A**). In terms of experimental analyses, this study focussed on the more distal aspect of this promoter (-837 to -618; **B**) and this was the target for use in the ChIP assays herein unless stated otherwise.

Fig. 4: The -837 to -618 Gαs Promoter Fragment is Occupied by CREB, Sp1 and Egr1. Primary cultures of myometrial cells were subjected to the ChIP assay. It was clear that CREB (**A**), Sp1 (**B**) and Egr-1 (**C**) occupied the endogenous distal Gαs promoter. TNF-induced RelA occupancy of the IκBα promoter illustrated the ChIP assay was functioning correctly (**D**).

Fig. 5: Exogenous CBP Induces Gαs-luc Activity While Endogenous CBP is Recruited to the -837 to -618 Region of the Native Gαs Promoter. Primary myometrial cultures were transfected with 200 ng of Gαs-luc and 200 ng, 400 ng or 500 ng of CBP. Cells were harvested after 24 hours and luciferase activity was quantitated. All experiments were performed three times in triplicate. Data were compared using one-way ANOVA and analysed further using Tukey's multiple comparisons test and results are expressed as the mean ± S.E.M. (error bars); p<0.05 was considered statistically significant. CBP strongly induced Gαs expression at all doses (**A**; p = 0.001). Employing the ChIP assay on cultures of primary myometrial myocytes, it was shown that endogenous CBP was also observed to occupy the endogenous Gαs promoter (**B**; Upper Panel). CBP occupancy of the CXCL promoter served as the positive control (**B**; Lower Panel).

Fig. 6: TNF Does Not Reduce Gαs Promoter Occupancy by CREB, Sp1 or Egr-1 But Does Reduce Binding by CBP. Primary cultures of myometrial cells were stimulated with TNF (10 ng/ml) for 24 hours and subjected to the ChIP assay with antisera depicted above. All experiments were performed three times and data were compared using a paired, two-tailed t-test and results are

expressed as the mean ± S.E.M. (error bars); again p<0.05 was considered statistically significant. TNF was not seen to significantly alter the amount of bound CREB (**A**), Sp1 (**B**) and Egr-1 (**C**). TNF did, however, significantly reduce the amount of CBP present within the Gαs promoter (**D**; p = 0.0128). As expected, TNF induced RelA occupancy of the IκBα promoter (**E**; upper panel) although, as expected, there was no binding of RelA to the endogenous Gαs promoter (**E**; Lower Panel).

Fig. 7: Gαs-luc Activity is Repressed by HDAC-1 But Induced by TSA. Primary myometrial cultures were transfected with 200 ng of Gαs-luc and 200 ng, 400 ng or 500 ng of HDAC-1. Cells were harvested after 24 hours and luciferase activity was quantitated. HDAC-1 strongly repressed Gαs expression at all doses, and maximally at 200 ng (**A**; p = 0.0047). Primary cultures of myometrial cells were stimulated with TNF (10 ng/ml) for 24 hours and subjected to the ChIP assay with anti-HDAC-1 antiserum. Data were compared using a paired, two-tailed t-test and results are expressed as the mean ± S.E.M. (error bars); again p<0.05 was considered statistically significant. TNF was seen to induce an increase in HDAC-1 occupancy of the endogenous Gαs promoter (**B**; p = 0.047). Primary myometrial cultures were transfected with 200 ng of Gαs-luc and then 24 hours later stimulated with TNF (10 ng/ml) for 24 hours. TSA (330nM) was then added to the culture medium for 24 hours. Cells were harvested and luciferase activity was quantitated. Data were compared using one-way ANOVA and analysed further using Tukey's multiple comparisons test and results are expressed as the mean ± S.E.M. (error bars); p<0.05 was considered statistically significant. TSA was seen to activate Gαs-luc activity and overcome TNF-induced Gαs-luc repression (**C**; p<0.001). Primary myometrial cultures were transfected with 200 ng of Gαs-luc and then 24 hours later stimulated with either TNF (10 ng/ml) for 24 hours, TSA (330nM) for 24 hours, or TNF and then TSA. Only TNF activated the NF-κB-sensitive 3x-κB-luc reporter (**D**; p = 0.0001). Cultures of primary myometrial cells were stimulated with either TNF (10 ng/ml) for 24 hours; TSA (330nM) for 24 hours or TNF and then TSA. Nuclear extracts were subsequently prepared and probed for the expression of acetyl-RelA (K310) and total RelA. No acetyl-RelA was detected in response to any stimulant (not shown). TSA, however, induced significant RelA nuclear localisation but this remained less than that seen with TNF (**E**; upper panels). Equal loading was confirmed by blotting for Gβ (**E**; lower panel). All experiments were performed three times and results are expressed as the mean ± SEM.

Fig. 8: TNF Does Not Alter Global Levels of Histone Acetylation or Histone-H3K9 Acetylation But Does Increase Histone-H4K8 Acetylation in the -837 to -618 Gαs Promoter Fragment. Primary cultures of myometrial cells were stimulated with TNF (10 ng/ml) for 24 hours and subjected to the ChIP assay with antisera depicted above. Data were compared using a paired, two-tailed t-test; again p<0.05 was considered statistically significant. All experiments were performed three times and results are expressed as the mean ± S.E.M. (error bars). TNF did not alter the amount of global levels of acetylated lysine-containing proteins (**A**) or acetylated histone-H3K9 (**B**) within the endogenous Gαs promoter but it induced an approximately threefold increase in acetylated histone-H4K8 levels in the endogenous -837 to -618 Gαs promoter region (**C**).
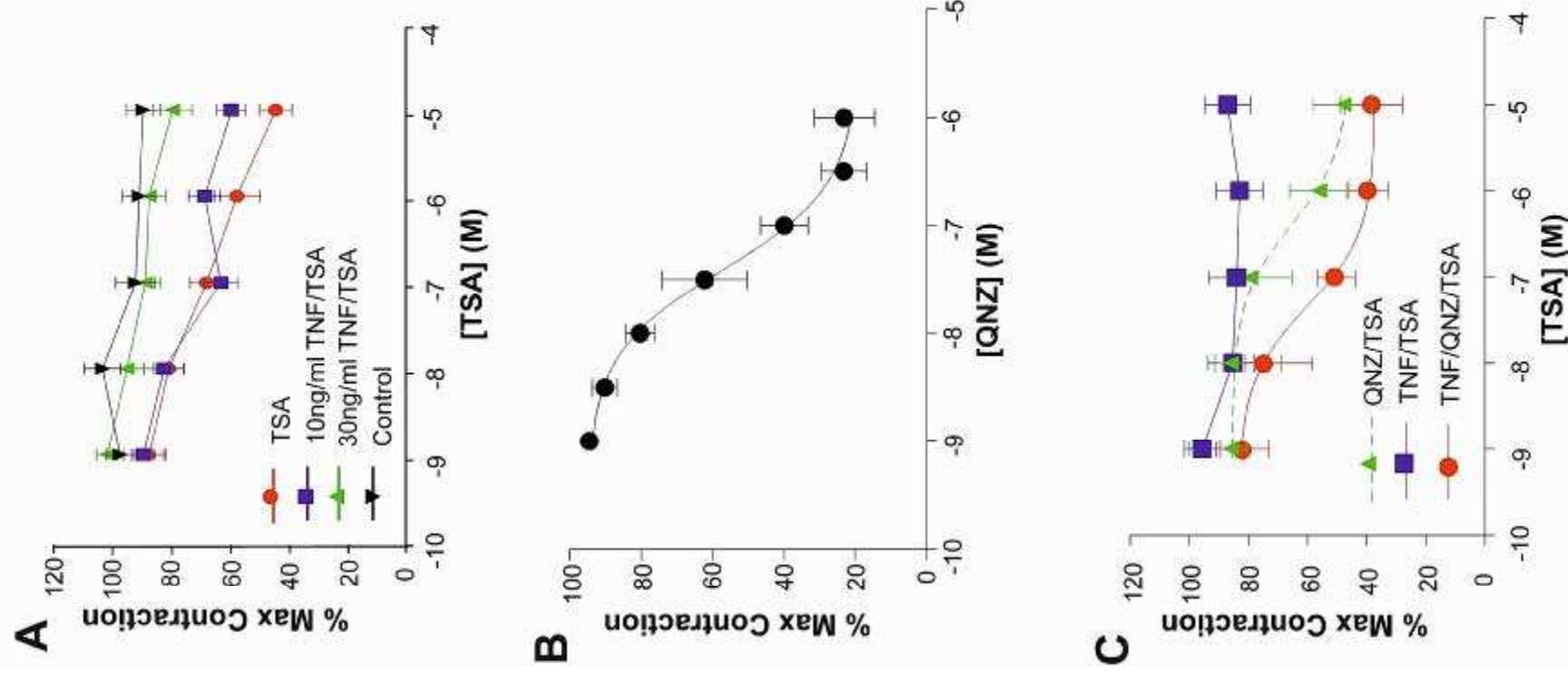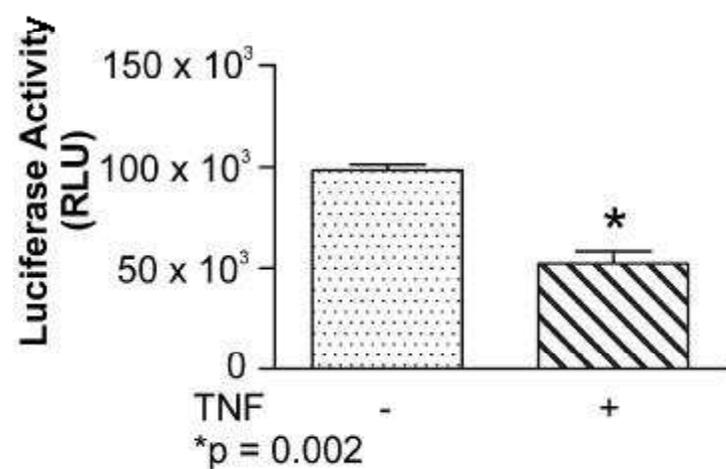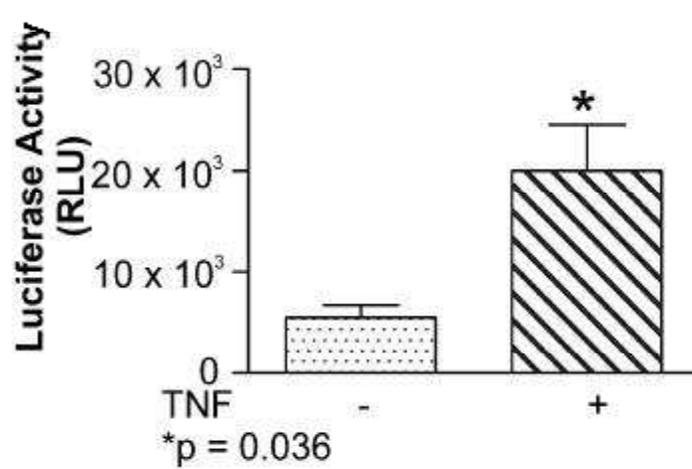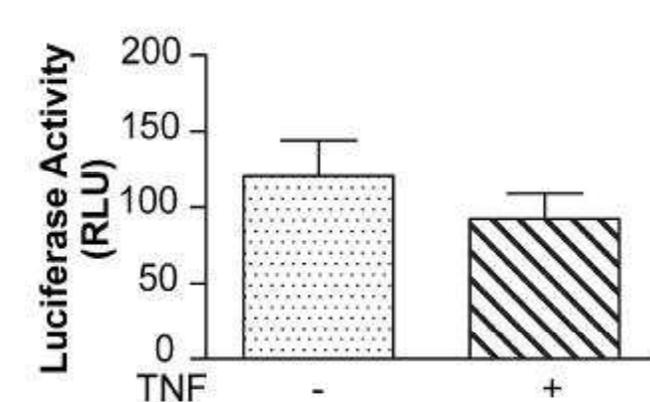
Figure 1

Figure 2

A Gαs-luc

*p = 0.002

B 3x-κB-luc

*p = 0.036

C ΛκB-ConA-luc

D Anti-RelA staining

Un-stimulated

+TNF

Negative control

Left panel

Middle panel

Right panel
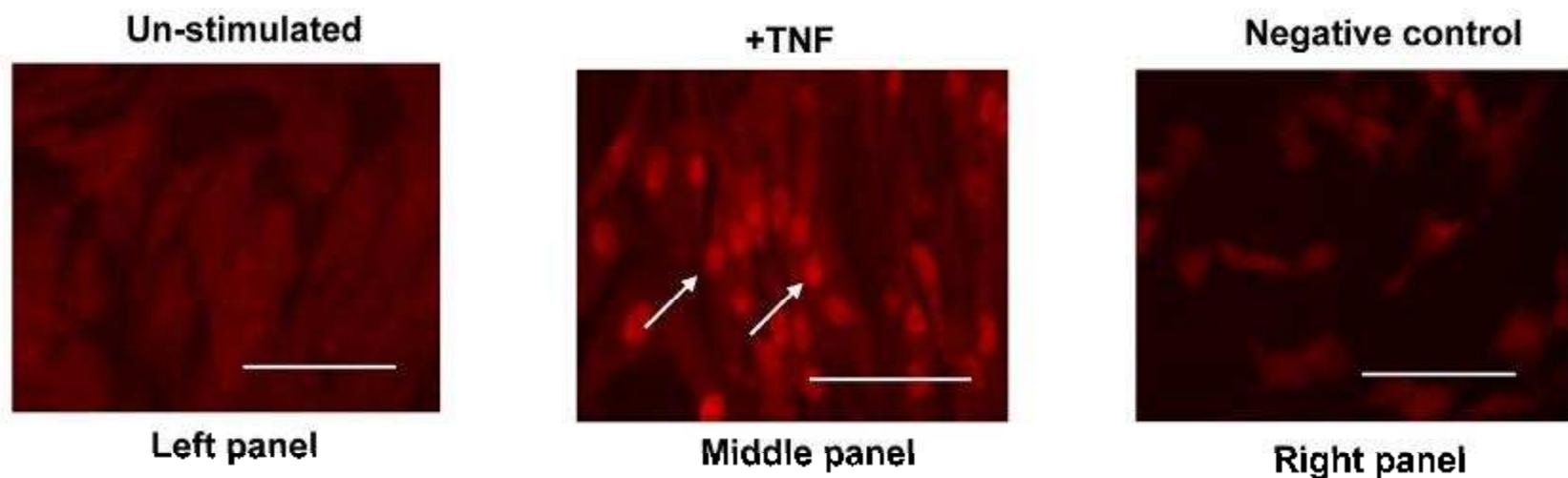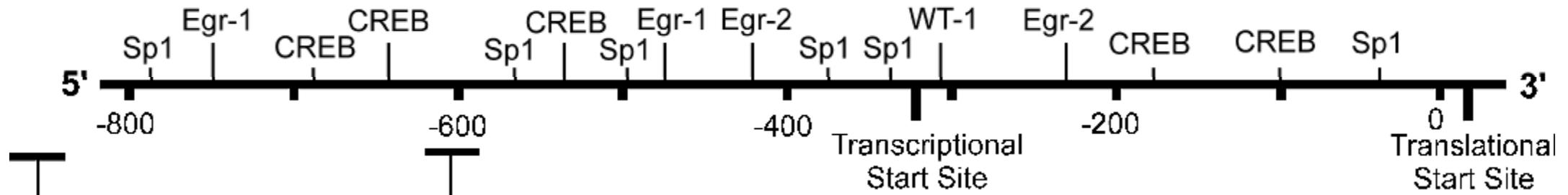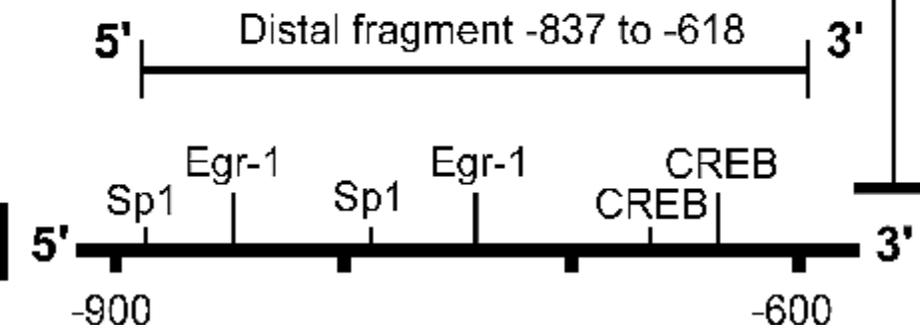
Figure 3

Figure 4

A CREB ChIP analysis

Gαs Promoter Fragment (219 bp)

Primers

I = Input; IP = αCREB

B Sp1 ChIP analysis

Gαs Promoter Fragment (219 bp)

Primers

I = Input; IP = αSp-1

C Egr-1 ChIP analysis

Gαs Promoter Fragment (219 bp)

Primers

I = Input; IP = αEgr-1

D RelA ChIP analysis

IκBα Promoter Fragment (300 bp)

Primers

I = Input; IP = αRelA

Figure 5

A Gαs-luc

CBP 200ng	-	+	-	-	-
CBP 400ng	-	-	+	-	-
CBP 500ng	-	-	-	+	-
Empty Vector 500ng	-	-	-	-	+

*Control vs. CBP: *p = 0.001

B CBP ChIP analysis

Gαs Promoter Fragment (219 bp)

Primers

I = Input; IP = αCBP
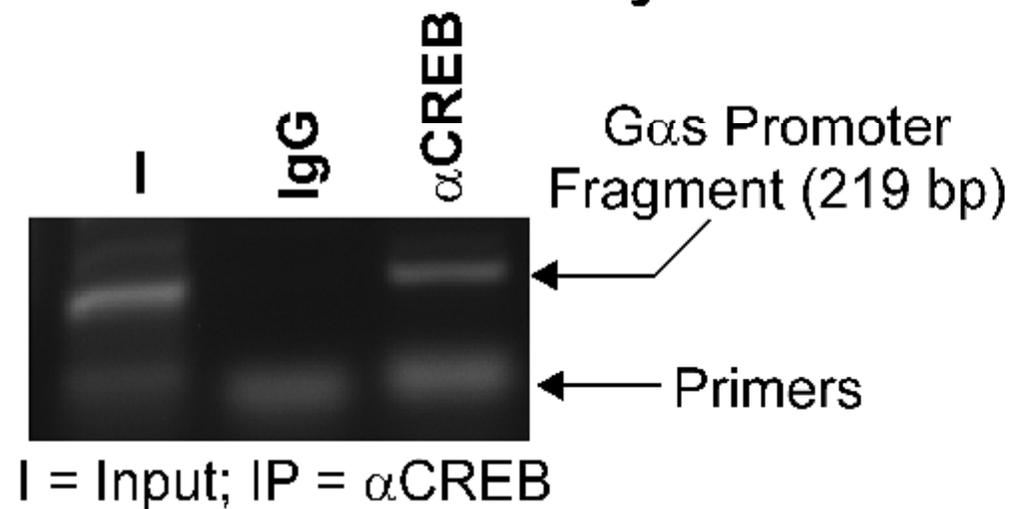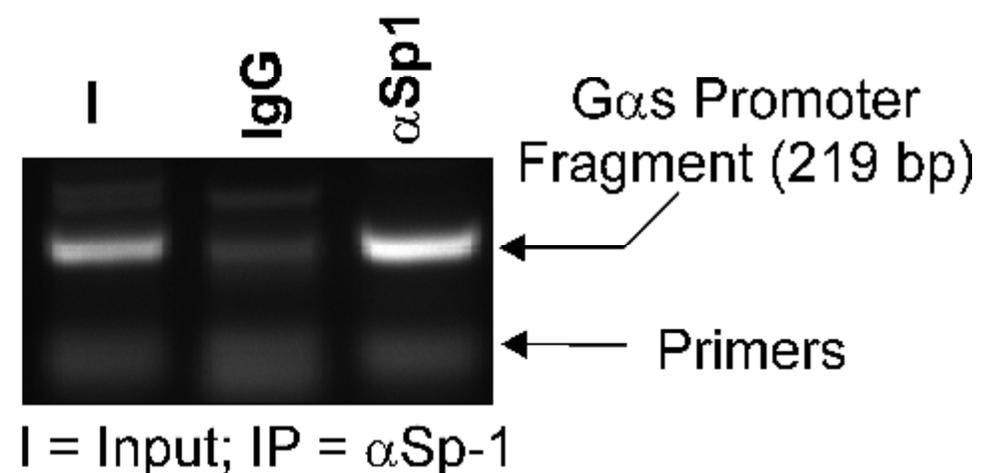
CXCL9 Promoter Fragment (86 bp)

Primers

I = Input; IP = αCBP

Figure 6

A CREB ChIP Analysis

B Sp1 ChIP Analysis

C Egr-1 ChIP Analysis

D CPB ChIP and Band Intensity Analyses

E RelA ChIP on IκBα and Gαs promoters

Figure 7

A Gαs-luc

Control vs. HDAC-1; 500ng: *p = 0.001
Control vs. HDAC-1; 400ng and 200ng: **p = 0.0047

B HDAC-1 ChIP and Band Intensity Analyses

I = Input; IP = αHDAC-1

C Gαs-luc

Control vs. TSA: *p = 0.001
Control vs. TNF: **p = 0.0205
Control vs. TNF + TSA: ***p = 0.001

D 3x-κB-luc

Control vs. TNF: *p < 0.0001

Control HDAC-1 IP vs. TNF IP: *p = 0.047

E Nuclear Extracts

Blots: αRelA (sc-372); Gβ (sc-378)

Figure 8

A Acetyl-Lysine ChIP Analysis

B Acetyl-H3K9 ChIP Analysis

C Acetyl-H4K8 ChIP and Band Intensity Analyses