Regulation of GTP-binding Protein (Gα$_s$) Expression in Human Myometrial Cells

A ROLE FOR TUMOR NECROSIS FACTOR IN MODULATING Gα$_s$ PROMOTER ACETYLATION BY TRANSCRIPTIONAL COMPLEXES*

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Background: The GTP-binding protein Gα$_s$ 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 Gα$_s$ promoter.

Conclusion: Promoter acetylation is important in governing expression of Gα$_s$.

Significance: TSA-induced myometrial relaxation can be overcome by TNF through repression of Gα$_s$ promoter activity.

The onset of parturition is associated with a number of proinflammatory mediators that are themselves regulated by the nuclear factor κB (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 proquiescent Gα$_s$ gene in human myometrial cells following stimulation with the proinflammatory cytokine TNF. In the 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 histone deacetylase inhibitor trichostatin A, an effect that in turn was abolished by the NF-κB inhibitor N^4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine. This result suggested a role for TNF in regulating Gα$_s$ 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 Gα$_s$ promoter is occupied by cAMP-response element-binding protein (CREB), Egr-1, and Sp1 transcription factors and that CREB-binding protein (CBP) transcriptional complexes form within this region where they induce histone acetylation, resulting in increased Gα$_s$ expression. TNF, acting via NF-κB, did not change the levels of CREB, Sp1, or Egr-1 binding to the Gα$_s$ promoter, but it induced a significant reduction in the level of CBP. This was associated with increased lev-

els of histone deacetylase-1 and surprisingly an increase in H4K8 acetylation. The latter is discussed herein.

During pregnancy, the uterus adopts a relative state of myometrial smooth muscle inactivity termed quiescence. At the onset of labor, 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 of gestation (severely preterm) is increasing (3, 69) with those infants having elevated risks of major long term mental and physical handicaps (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 labor are associated with a number of proinflammatory 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 κB (NF-κB)[4] (Refs. 5–22; for reviews, see Refs. 23 and 24).

Although 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 (Refs. 28–31; for a review, see Ref. 32). Of significance, however, is

[4] The abbreviations used are: NF-κB, nuclear factor κB; TSA, trichostatin A; HDAC, histone deacetylase; QNZ, N^4-[2-(4-phenoxyphenyl)ethyl]-4,6-quinazolinediamine; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; luc, luciferase; REST, repressor element 1-silencing transcription factor.

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 recognized 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 favor 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, which consists of 150 bp of DNA wrapped around a core histone octamer (two each of H2A, H2B, H3, and H4; Ref. 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 Gα_s. Indeed, signaling through Gα_s is thought to facilitate promotion of myometrial quiescence, and Gα_s protein levels have been shown to be reduced at the onset of labor (41, 42). There are, however, only a limited number of reports describing the transcriptional control of the Gα_s gene. The Gα_s promoter itself is located on chromosome 20, is a highly GC-rich segment of DNA, and therefore contains many binding sites for transcription factors that bind GC-containing DNA sequences, including those for CREB, Egr, and Sp families (48, 50, 51, 70). Our previous studies have suggested that the promoter region of the Gα_s 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 Gα_s promoter in a manner that utilized competition for the co-activator and histone acetyltransferase CBP (22). In our view, because levels of CBP are elevated in pregnant human myometrium but decline in laboring samples (52), 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 N^4-[2-(4-phenoxyphenyl)-ethyl]-4,6-quinazolinediamine (QNZ) prevented the effects of TNF. Utilizing the chromatin immunoprecipitation (ChIP) assay, we showed that the native Gα_s 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, UK. This study received approval from the Rotherham Local Research Ethics Committee (REC Reference Number 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 Ltd., Staffordshire, UK) as described by Chapman *et al.* (22). The Gα_s-luciferase (Gα_s-luc) reporter 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, OR), 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 h with 10 ng/ml TNF. For experiments investigating the role of CBP, 200 ng of RcRSV-HA-CBP or 200 ng of RcRSV-NRC-MCS was used. Transfections using histone deacetylase-1 (HDAC-1) utilized 0.5, 1.0, and 1.5 μg of pcDNA-HDAC-1 or 0.5, 1.0, and 1.5 μg of pcDNA3 to normalize the amount of DNA. Luciferase assays were performed three times in triplicate as described previously (22), and data were collected using a Berthold Sirius luminometer (Geneflow Ltd.).

Immunofluorescence—Myometrial cells were cultured in a 24-well plate and upon reaching 80% confluence were stimulated with 10 ng/ml TNF for 24 h. The cells were washed in PBS and fixed in 1% (v/v) formaldehyde overnight at 4 °C. Cells were washed in PBS three times for 5 min on a rocking platform and permeabilized with PBS containing 1% (w/v) BSA and 0.1% (v/v) Triton X-100 with three 5-min 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 h at 37 °C. The antibody was removed with three 5-min washes in PBS, and the goat anti-rabbit IgG secondary antibody was added to the wells at a dilution of 1:200 (in PBS containing 1% (w/v) BSA) for 1 h at 37 °C. The unbound antibody was washed with three 5-min PBS washes, and the nuclear counterstain DAPI was added to the wells at a 1:20 dilution in PBS.

ChIP Assay—The ChIP assay was performed using the Magna-ChIP assay kit provided by Millipore (catalog number 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 h. Cells were then fixed for 10 min using 37.5% formaldehyde added directly to the medium. This reaction was subsequently quenched by adding 2 ml of 1 M glycine made up in water. Finally, medium was removed, and cells were washed twice in ice-cold PBS (pH 7.4) supplemented with protease inhibitor mixture (Millipore). Cells were removed from each flask in the PBS:protease inhibitor mixture using a cell scraper. Appropriate samples were then pooled into control or stimulated groups, and the cell suspension was clarified by centrifugation at 4 °C at 800 × g for 5 min.

The cell pellet was resuspended in 500 μl of Magna-ChIP lysis buffer supplemented with protease inhibitor mixture. Essentially, a two-step lysis was undertaken to reduce nonspecific chromatin-IgG interactions. In the first lysis step, cells were opened, nuclei were obtained, and cells were then incubated in lysis buffer on ice for 15 min and vortexed briefly every 5 min. This suspension was clarified by centrifugation at 800 × g for 5 min at 4 °C. For the second lysis step, which opened the nuclei, the supernatant was discarded, the pellet was resuspended in 500 μl of Magna-ChIP nuclear lysis buffer, and the sample was subjected to ultrasonic cavitation on high power for three 10-s exposures to lyse the nuclei and fragment the chromatin. Chromatin fragments with an average size of ~500 bp were obtained.

Immunoprecipitation of Chromatin-bound Complexes—The fragmented chromatin was separated into 50-μl aliquots (~1 × 10^6 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 (Millipore, catalog number 07–645), anti-CREB (Cell Signaling Technology, catalog number 9192), anti-acetylated histone H3 (H3K9; Millipore, catalog number 06-599), anti-acetylated histone H4 (H4K8; Cell Signaling Technologies, catalog number 2594), anti-CBP and anti-Egr-1 (Santa Cruz Biotechnology Inc., sc-369 and sc-588, respectively), and nonspecific rabbit IgG (Abcam, catalog number ab46450). To this, a 20-μl bed volume of pre-prepared Dynabeads (Invitrogen) preadsorbed with 20 μg/ml sonicated salmon sperm DNA and 1 mg/ml BSA in Tris-EDTA buffer (pH 8.0) was 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 were washed to remove nonspecific binding using alternative high salt:low salt and LiCl washes in accordance with the manufacturer's instruc-

tions. 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′-GACGACCCC-AATTCAAATCG-3′; antisense, 5′-TCAGGCTCGGGGAAT-TTCC-3′) as a positive control for the ChIP assay, giving a product size of ~300 bp (22). As a positive control for promoter occupancy by CBP, PCR on the immunoprecipitated DNA was also carried out using primers flanking the CXCL9 promoter (56). The following primer sequences were used: CXCL9 sense, 5′-TTCCACATCCAGGTAGCAACTTTG-3′; CXCL9 antisense, 5′-TGTTGGAGTGAAGTCCGAGAATGT-3′. These primers gave the expected product size of 86 bp. Reaction conditions for both controls were those originally described in Saccani *et al.* (57): one cycle of denaturation at 94 °C for 3 min and 36 cycles of 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min followed by a final elongation at 72 °C for 10 min. 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 Gα_s promoter made its amplification by PCR difficult. Consequently, to analyze promoter occupancy in the Gα_s promoter (−837 to −618; Ref. 48) (70), the following primers were used: Gα_s-distal-3 sense, 5′-TTGGTTCGTGCT-CGCCTCGC-3′; Gα_s-distal-3 antisense, 5′-CAGTGACGAC-CCCTCGCACG-3′. The following protocol was also utilized to facilitate PCR from the GC-rich −837 to −618 region of the Gα_s promoter. Each 25-μl reaction consisting of 5× Hercules buffer, 25 mM dNTPs, 0.25 μl of ChIP DNA, 100 pmol of Gα_s-distal-3 sense, 100 pmol of Gα_s-distal-3 antisense, 0.25 μl of Hercules DNA polymerase (note that the manufacturer, Agilent, did not provide a value for the number of enzyme units/μl), and 1 μl of DMSO. Reaction conditions were as follows: one cycle of denaturation at 98 °C for 3 min and 38 cycles of 98 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s followed by a final elongation at 72 °C for 5 min. Samples were then resolved using 1.5% Tris acetate agarose gel electrophoresis, and a product size of ~219 bp (−837 to −618 bp of the Gα_s promoter) was obtained.

Band Intensity Analysis of Amplified, Immunoprecipitated DNA—In this study, end point PCR was used to give semiquantitative data on enrichment levels of these respective transcription factors on each promoter. These analyses were undertaken using the SynGene (Cambridge, UK) GBox Chemi-16 gel documentation system to capture images. Subsequent band quantification was undertaken using GeneTools Version 4 quantification software (SynGene). In all cases, the value ascribed to nonspecific 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 antiserum was obtained from Cell Signaling Technology (catalog number 3045) and detects transfected levels of RelA NF-κB only when residue Lys-310 is acetylated. Gβ (Santa Cruz Biotechnology Inc., sc-378) was utilized to ensure equal well loading as detailed in Chapman *et al.* (22). After incubation with an appropriate secondary antiserum (125 ng of goat anti-rabbit HRP conjugates; Dako, catalog number P0448), membranes were then washed in PBS with Tween 20 and developed according to the EZ-ECL detection protocol supplied by Geneflow Ltd.

Myometrial Contractility—The effects of TNF were tested in a model of myometrial contractility at 10 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 and 5% CO_2 and allowed to contract spontaneously. Following equilibration, separate concentration-response curves for both TSA and the NF-κB inhibitor QNZ (59) were generated. The ability of QNZ to modulate the contractile response was carried out by preincubating tissue $\pm$ 10 or 30 ng/ml TNF for 1 h prior to cumulative additions of TSA. Data were analyzed by calculating activity integrals for contractions over a 30-min period, and concentration-effect curves were 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 $\pm$ S.E. (error bars). All data analyses were conducted using GraphPad Prism Version 5.02 (GraphPad Software, San Diego, CA). Data from transfection experiments and pharmacological studies were compared using one-way analysis of variance and analyzed further using Tukey's multiple comparison posttest. 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 proinflammatory cytokines, including TNF, are associated with the onset of both normal and preterm birth (23, 24). Taken together with data highlighting the reduction of Gα_s protein levels in term and preterm laboring myometrium, we speculate that the localized 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. First, cumulative addition of TSA was seen to relax spontaneously contracting, isolated myometrial strips (Fig. 1*A, red line; n* = 6). Significantly, whereas TNF had little effect on TSA-induced myometrial relaxation at 10 ng/ml (Fig. 1*A, blue line*), it did, however, greatly reduce the TSA-induced relaxation at a concentration of 30 ng/ml (Fig. 1*A, green line*). In support of 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 3.9×10^{-8} M
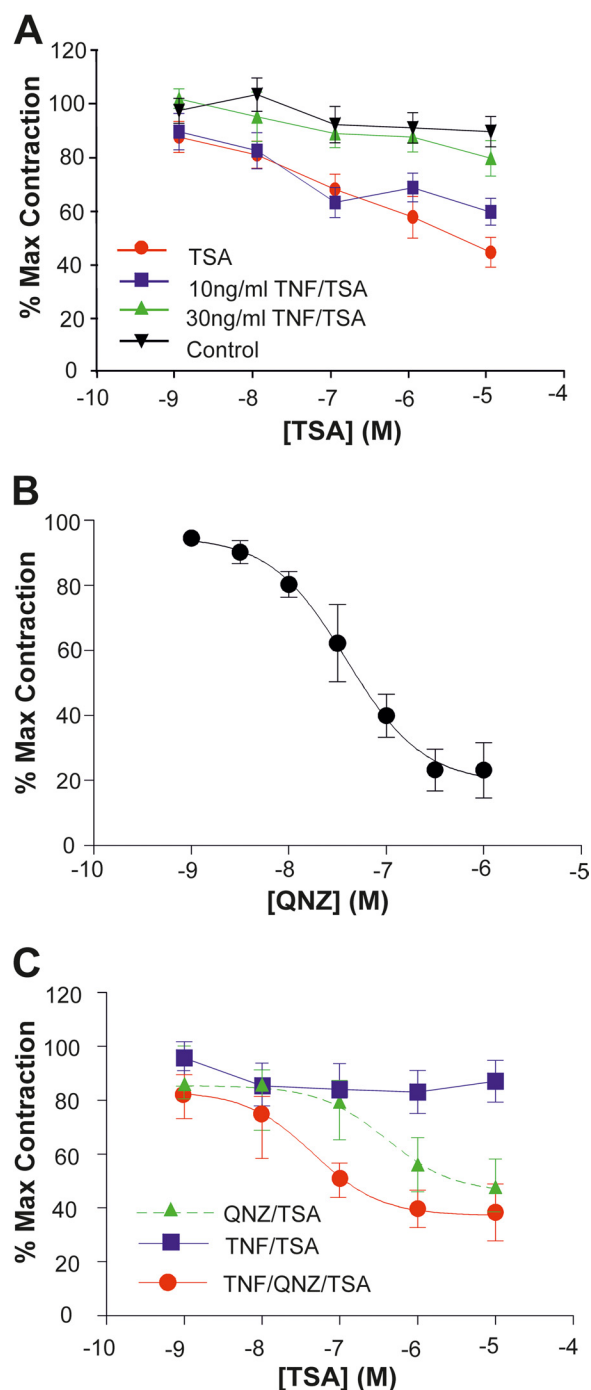
FIGURE 1. **TNF, TSA, and QNZ all effect myometrial contraction.** Isolated myometrial strips were prepared as described under "Materials and Methods." *A*, myometrial tissue strips (*n* = 6 for each observation) were incubated in either 10 or 30 ng/ml TNF for 1 h followed by the cumulative addition of 10^{-9}–10^{-5} M TSA to the bath. Data were analyzed using an unpaired, two-tailed *t* test, and results are expressed as the mean $\pm$ S.E. (*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 that 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 an IC$_{50}$ of 3.9×10^{-8} M. *C*, cumulative addition of TSA to myometrial tissues preincubated with QNZ (50 nM) and TNF (30 ng/ml) produced a leftward shift in the response compared with QNZ alone.
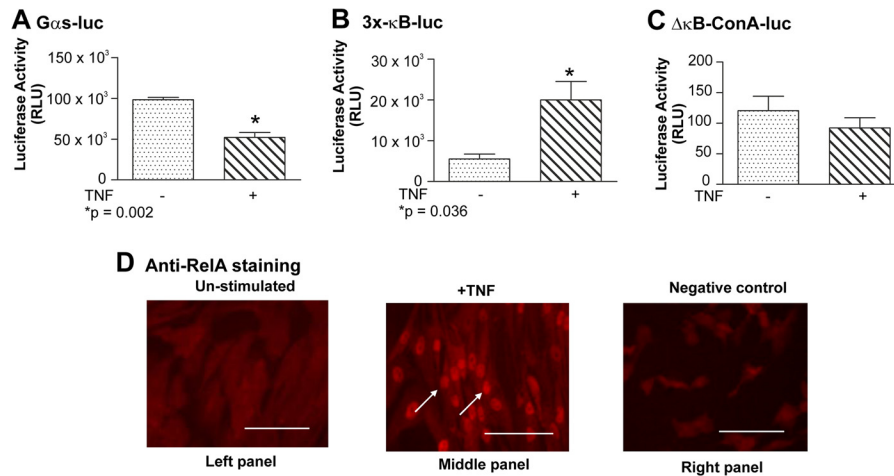
FIGURE 2. TNF represses Gα_s expression but induces NF-κB activity in primary myometrial myocytes. Primary myometrial cultures were transfected with 200 ng of either Gα_s-luc (*A*), 3x-κB-ConA-luc (NF-κB-responsive) (*B*), or $\Delta\kappa$B-ConA-luc (NF-κB-unresponsive) (*C*). After 24 h, cells were stimulated with TNF (10 ng/ml) for 24 h. Promoter activity was quantified using a Berthold Sirius tube luminometer. All experiments were performed three times in triplicate. Data were analyzed using a paired, two-tailed *t* test, and results are expressed as the mean ± S.E. (*error bars*); $p < 0.05$ was considered statistically significant. As expected, TNF repressed Gα_s 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 localization in primary myometrial myocytes (*D, white arrows*; compare *middle panel* (TNF-stimulated) with *left panel* (un-stimulated) and *right panel* (negative control); *scale bar*, 100 μm).

($n = 6$), suggesting a role for NF-κB in this contractile response (Fig. 1*B*).

Finally, preincubation of myometrial strips in 50 nM QNZ (a concentration close to the IC$_{50}$) followed by addition of TSA also produced relaxation (Fig. 1*C, 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. 1*C, 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 Gα_s Promoter but Induces NF-κB Activity—A decline in both the levels of mRNA and Gα_s protein within human myometrium has been shown to accompany the transition from quiescence to labor (22, 42). Although the full details of this mechanism remain unclear, we have previously described that the TNF-induced repression of a Gα_s-luc reporter vector transfected into primary myometrial myocytes is mediated by the NF-κB RelA subunit competing for limiting quantities of the key transcription cofactor CBP (22). Consequently, control experiments were undertaken to determine whether the myometrial cell cultures used in this study behaved in a similar manner. Briefly, TNF was seen to repress the Gα_s-luc reporter (Fig. 2*A*) but to induce transcription from the NF-κB-sensitive 3x-κB-luc vector (Fig. 2*B*). No activity was seen when cells harboring the ConA-luc vector (which is not NF-κB-sensitive) were exposed to TNF (Fig. 2*C*). Because we wished to investigate the regulation of the Gα_s promoter in the presence of activated NF-κB RelA, we confirmed as expected that TNF induced nuclear localization of RelA in primary myometrial myocytes (compare diffuse staining in unstimulated or nonspecific IgG-stained cells (Fig. 2*D, left* and *right panels*) with TNF-stimulated cells (Fig. 2*D, middle panel*)).

The −837 to −618 Region of the Endogenous Gα_s Promoter Is Occupied by CREB, Egr-1, and Sp1 and Recruits CBP—The Gα_s promoter originally utilized in the Gα_s-luc vector represents the region from −790 to the transcription start site (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 Gα_s promoter, region −837 to −618, regulated expression (illustrated in Fig. 3*A*).

The native Gα_s promoter is a GC-rich, TATA-less regulatory region (48). Using the TransFac algorithm (70), 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 transcriptionally competent euchromatin (50). As such, it was decided to examine Sp1 in this context. Egr1 was chosen because it is a potent GC-rich DNA-binding zinc finger transcription factor. Moreover, it has been shown to compete with NF-κB RelA for DNA binding, and it binds to CBP (28, 60). We decided to investigate CREB because 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 Gα_s regulation (22, 45, 52). Consequently, to determine whether these factors were present on the endogenous Gα_s promoter, we isolated primary myometrial smooth muscle cells as detailed above and cultured them until confluent. The ChIP assay was then utilized to determine whether these factors were present on the distal Gα_s promoter fragment. Fig. 4, *A–C*, demonstrate that CREB, Sp1, and Egr-1 occupied the −837 to −618 aspect of the endogenous Gα_s promoter, respectively. Positive controls for this experiment included occupation of the IκBα promoter by RelA NF-κB (Fig. 4*D*). Other endogenous Sp and Egr family members were not detected in Western analyses prior to the ChIP study (data not shown).
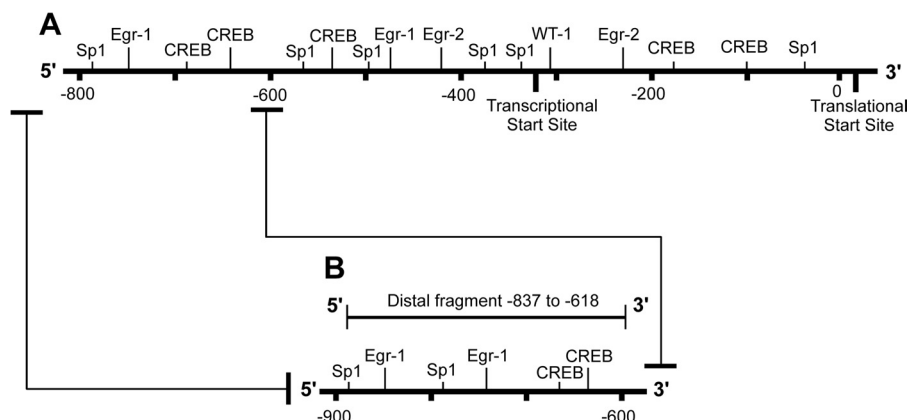
FIGURE 3. **Schematic illustration of the Gα_s promoter highlighting the −837 to −618 region.** The Gα_s promoter originally utilized in the Gα_s-luc vector represents the −790 to the transcription start site (*A*). In terms of experimental analyses, this study focused on the more distal aspect of this promoter (−837 to −618; *B*), and this was the target for use in the ChIP assays herein unless stated otherwise.

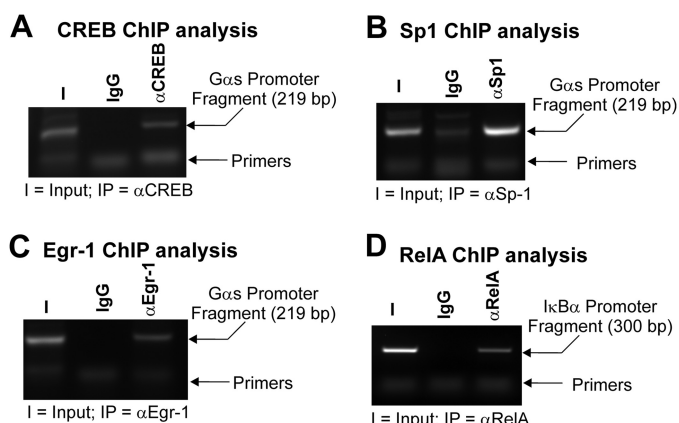FIGURE 4. **The −837 to −618 Gα_s promoter fragment is occupied by CREB, Sp1, and Egr-1.** Primary cultures of myometrial cells were subjected to the ChIP assay. It was clear that CREB (*A*), Sp1 (*B*), and Egr-1 (*C*) occupied the endogenous distal Gα_s promoter. TNF-induced RelA occupancy of the IκBα promoter illustrated that the ChIP assay was functioning correctly (*D*). *I*, input; *IP*, immunoprecipitating antibody.

FIGURE 5. **Exogenous CBP induces Gα_s-luc activity, whereas endogenous CBP is recruited to the −837 to −618 region of the native Gα_s promoter.** Primary myometrial cultures were transfected with 200 ng of Gα_s-luc and 200, 400, or 500 ng of CBP. Cells were harvested after 24 h, and luciferase activity was quantitated. All experiments were performed three times in triplicate. Data were compared using one-way analysis of variance and analyzed further using Tukey's multiple comparison test, and results are expressed as the mean ± S.E. (*error bars*); $p < 0.05$ was considered statistically significant. CBP strongly induced Gα_s expression at all doses (*A*; $p = 0.001$). Using the ChIP assay on cultures of primary myometrial myocytes, it was shown that endogenous CBP also occupied the endogenous Gα_s promoter (*B*, *upper panel*). CBP occupancy of the CXCL9 promoter served as the positive control (*B*, *lower panel*). *I*, input; *IP*, immunoprecipitate.

Our previous work demonstrated that when CBP and the Gα_s-luc reporter vectors were co-transfected into primary myometrial cells expression of exogenous CBP was shown to enhance activity of the Gα_s promoter (Fig. 4*A* and 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 Gα_s promoter, those factors identified in Fig. 3, *A–C*, 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 whether CBP was also present within the −837 to −618 section of the native Gα_s 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 Gα_s promoter in native chromatin derived from myometrial myocytes (Fig. 5*B*, *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 able to see CXCL9 promoter occupancy by CBP (Fig. 5*B*, *lower panel*).
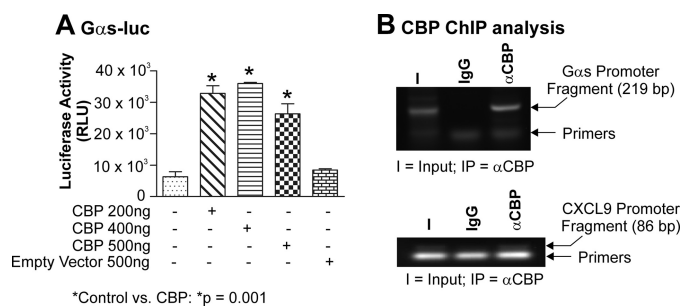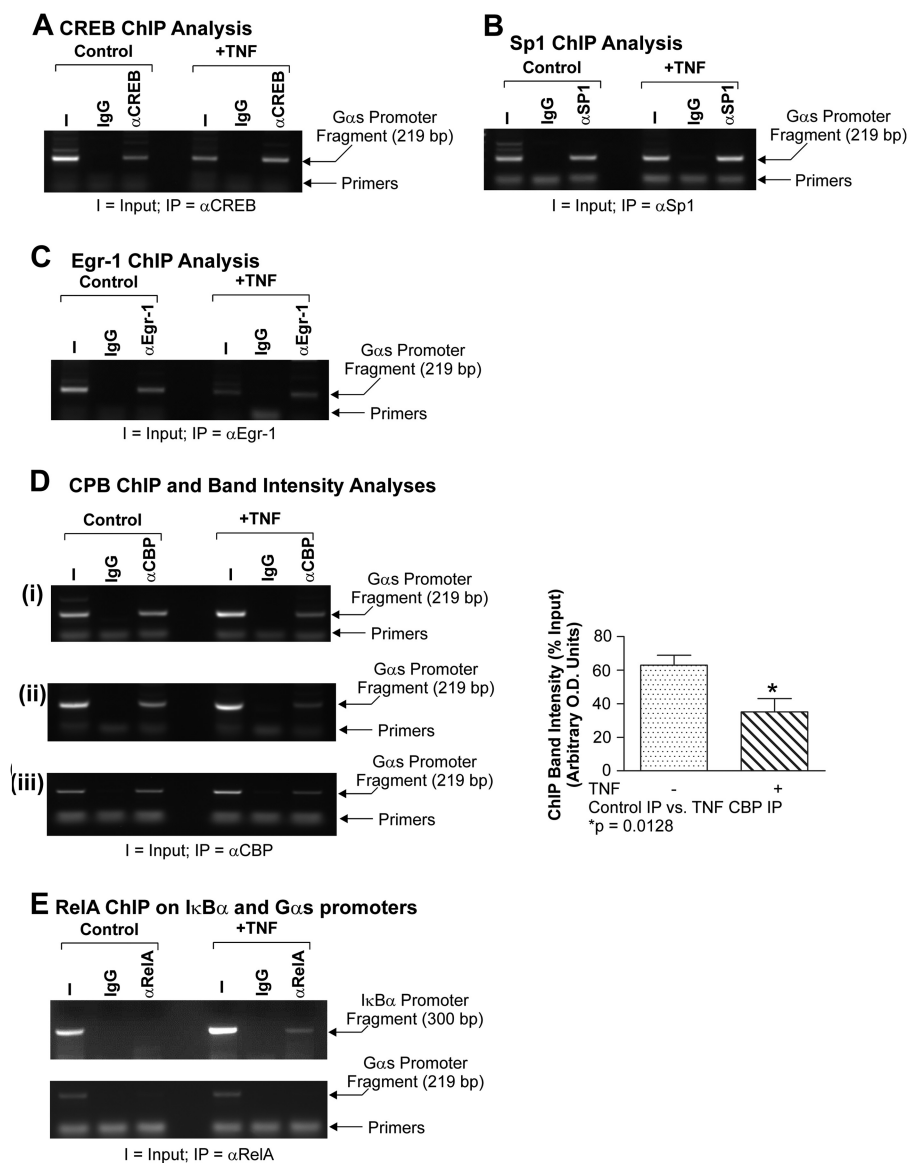
TNF Does Not Reduce Gα_s Promoter Occupancy by CREB, Sp1, or Egr-1 but Does Reduce Binding by CBP—TNF has been shown to repress a Gα_s promoter construct transfected into myometrial cells (22). The factors involved in regulating the natural Gα_s promoter within its native context, however, have not been defined. In the present study, we used the ChIP assay on chromatin extracted from TNF-stimulated primary myometrial myocytes to determine whether there were any changes in the level of Gα_s 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. 6*A*), Egr-1 (Fig. 6*B*), or Sp1 (Fig. 6*C*) on the −837 to −618 portion of the native Gα_s promoter when the myometrial cells were exposed to TNF.

As stated above, CBP is not thought to possess intrinsic DNA binding ability but instead relies on recruitment to the promoter through interactions with promoter-bound ancillary factors, including those identified herein. Expression of exogenous CBP alone was seen to enhance expression from the Gα_s-luc reporter vector (Fig. 5*A*; discussed above). Moreover, we previously demonstrated that expression of exogenous CBP could

FIGURE 6. TNF does not reduce Gα$_s$ 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 h and subjected to the ChIP assay with the antisera depicted. All experiments were performed three times. Data were compared using a paired, two-tailed *t* test, and results are expressed as the mean ± S.E. (*error bars*); $p < 0.05$ 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 Gα$_s$ promoter (*D, panels i–iii*; $p = 0.0128$). 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 Gα$_s$ promoter (*E, lower panel*). *I*, input; *IP*, immunoprecipitate.

relieve TNF-induced repression of the Gα$_s$-luc promoter vector (22). That observation suggested that Gα$_s$ repression occurred because CBP was removed or lost from the transcription complex based on the Gα$_s$ promoter. To test this notion further, we undertook a series of ChIP analyses on TNF-stimulated primary myometrial myocytes to determine whether the level of CBP occupying the Gα$_s$ promoter was reduced when cells were exposed to TNF. Fig. 6*D* clearly illustrates that the level of CBP present within the −837 to −618 region of the endogenous Gα$_s$ promoter is reduced ~2-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. 6*E, upper panel*). Moreover, we have previously demonstrated that RelA does not bind to the Gα$_s$ promoter, and consequently, that

observation was utilized in this study to confirm that the effects observed herein were specific because once again there was no binding of RelA to the endogenous Gα$_s$ promoter (Fig. 6*E, lower panel*).

Gα$_s$-luc Promoter Activity Is Repressed by HDAC-1 But Induced by the HDAC Inhibitor TSA—As discussed above, histone acetylation is generally associated with transcription of a given promoter, whereas reciprocal histone deacetylation generally alters chromatin structure in a manner such that it becomes refractory to transcription, thereby silencing a given gene. In terms of myometrial function, there are increasing data that suggest that deacetylation of myometrial genes may be of importance during quiescence. Indeed, both Phillips *et al.* (45) and Karolczak-Bayatti *et al.* (47) have reported the binding of HDAC-1 to two other GC-rich control regions, namely the

human chorionic gonadotropin/luteinizing hormone 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 Gα_s-luc promoter construct. Importantly, when monolayers of primary myometrial smooth muscle cells were co-transfected with Gα_s-luc and increasing amounts of HDAC-1, HDAC-1 caused a dose-dependent repression of the Gα_s-luc promoter construct (Fig. 7A). Interestingly, the effect was observed with 500 ng of HDAC-1 plasmid DNA but reached 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 Gα_s-luc vector (Fig. 6A) were not merely due to promoter squelching, we also used the ChIP assay to immunoprecipitate the endogenous Gα_s promoter using anti-HDAC-1 antiserum. Cultures of primary myometrial cells were treated with TNF or not stimulated at all. As seen in Fig. 7B, TNF treatment caused an association of HDAC-1 with the endogenous Gα_s promoter, supporting the notion that HDAC-1 is recruited to this region in the presence of inflammatory mediators.

Because we observed that exogenous HDAC-1 could repress activity of the Gα_s promoter, the obvious corollary was that the HDAC inhibitor TSA should induce activity of the Gα_s-luc construct. To test this notion, primary myometrial smooth muscle cells transfected with the Gα_s-luc vector were exposed to TSA (330 nM). Fig. 7C clearly demonstrates that TSA can induce expression from the Gα_s-luc promoter construct. Moreover, the TNF-induced repression of Gα_s 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 (labor-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 recognizing 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 localization 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 the TNF effect). Equal loading of samples was confirmed by probing for Gβ protein (Fig. 7E).

TNF Increases Histone H4K8 Acetylation on the Gα_s Promoter but Not That of Histone H3K9—It has been well documented that CBP is a potent histone acetyltransferase targeting a variety of intracellular factors, including histones H3 and H4 (38). Acetylation of both H3 and H4 can be associated with both transcriptional activation and repression of a number of genes as they modify the inherent promoter architecture, thereby enhancing the accessibility of the promoter to RNA polymerase II (Refs. 65 and 66 and references therein). At present, however, there are no data describing whether the Gα_s promoter is acetylated. Because we have identified CBP occupancy of the −837 to −618 region of the native Gα_s promoter (Fig. 5B) and found that the level of CBP occupying that region of the Gα_s promoter is reduced in the presence of TNF (Fig. 4D), we wanted to determine (i) whether the Gα_s promoter was acetylated and (ii) whether there were changes in the acetylation status of either histone H3 or H4 within the Gα_s distal promoter fragment in primary myometrial myocytes upon exposure to TNF. Once again, myocytes were stimulated with TNFα for 24 h, and further ChIP analyses were performed for pan-acetylated lysine residues, acetylated histone H3, or acetylated histone H4 within the endogenous distal Gα_s fragment.

In our system, we did not observe any change in the levels of acetylated lysine within the −837 to −618 Gα_s 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 ∼2.5-fold in the Gα_s band intensity when ChIP was utilized for acetylated histone H4K8 (Fig. 8C), suggesting that this modification of the endogenous Gα_s 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 h (a similar time course utilized for the transfection and ChIP studies herein) remains unclear, although certainly at 24 h 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 is an inhibitor of deacetylation). It remains unclear which NF-κB dimers are responsible for the TNF-induced effect. Although 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 and p52-RelB heterodimers, that are not sensitive to IκBα inhibition (23, 32) and would therefore not be targeted by QNZ.
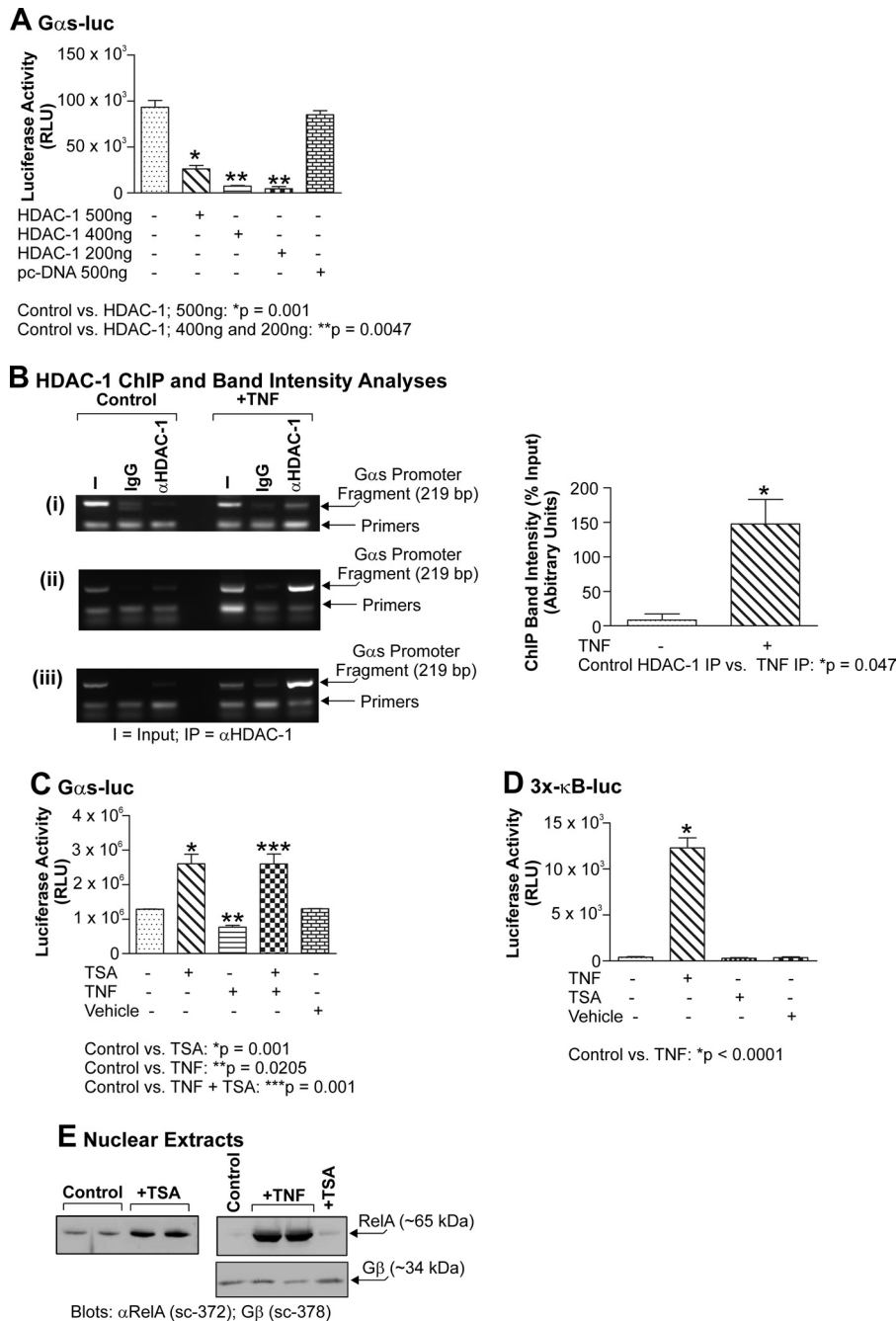
FIGURE 7. **Gα_s-luc activity is repressed by HDAC-1 but induced by TSA.** Primary myometrial cultures were transfected with 200 ng of Gα_s-luc and 200, 400, or 500 ng of HDAC-1. Cells were harvested after 24 h, and luciferase activity was quantitated. HDAC-1 strongly repressed Gα_s expression at all doses and maximally repressed Gα_s expression at 200 ng (*A*; $p = 0.0047$). Primary cultures of myometrial cells were stimulated with TNF (10 ng/ml) for 24 h 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. (*error bars*); $p < 0.05$ was considered statistically significant. TNF was seen to induce an increase in HDAC-1 occupancy of the endogenous Gα_s promoter (*B, panels i–iii*; $p = 0.047$). Primary myometrial cultures were transfected with 200 ng of Gα_s-luc and then 24 h later stimulated with TNF (10 ng/ml) for 24 h. TSA (330 nM) was then added to the culture medium for 24 h. Cells were harvested, and luciferase activity was quantitated. Data were compared using one-way analysis of variance and analyzed further using Tukey's multiple comparison test, and results are expressed as the mean ± S.E. (*error bars*); $p < 0.05$ was considered statistically significant. TSA was seen to activate Gα_s-luc activity and overcome TNF-induced Gα_s-luc repression (*C*; $p < 0.001$). Primary myometrial cultures were transfected with 200 ng of Gα_s-luc and then 24 h later stimulated with either TNF (10 ng/ml) for 24 h, TSA (330 nM) for 24 h, 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 h, TSA (330 nM) for 24 h, or TNF and then TSA. Nuclear extracts were subsequently prepared and probed for the expression of acetyl-RelA (Lys-310) and total RelA. No acetyl-RelA was detected in response to any stimulant (not shown). TSA, however, induced significant RelA nuclear localization, 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 ± S.E. (*error bars*). *RLU*, relative light units. *I*, input; *IP*, immunoprecipitate.

The Gα_s Promoter Is Bound by a Number of Transcription Factors—Our current hypothesis suggests that acetylation of the Gα_s promoter by CBP is a prerequisite for Gα_s expression in the human myometrium, a process that would facilitate uterine quiescence seen during pregnancy (22, 52). The corollary to this, therefore, is that inflammatory mediators, including TNF,
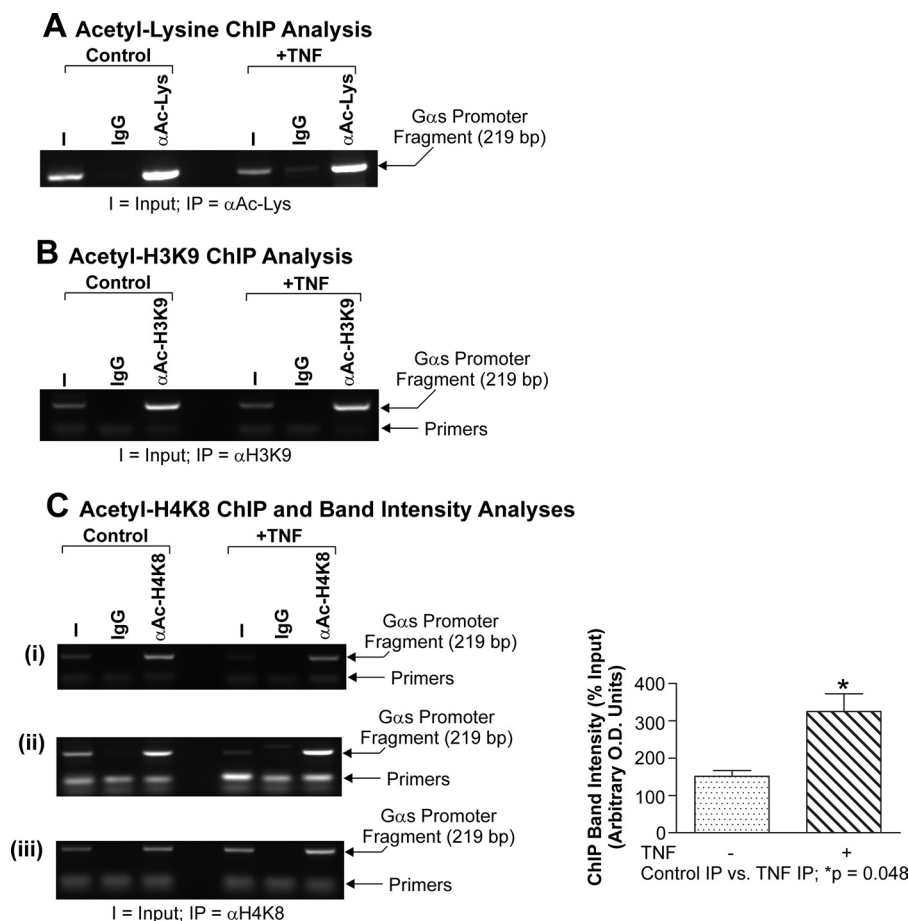
FIGURE 8. **TNF does not alter global levels of histone acetylation or histone H3K9 acetylation but does increase histone H4K8 acetylation in the −837 to −618 Gα$_s$ promoter fragment.** Primary cultures of myometrial cells were stimulated with TNF (10 ng/ml) for 24 h and subjected to the ChIP assay with the antisera depicted. Data were compared using a paired, two-tailed t test; $p < 0.05$ was considered statistically significant. All experiments were performed three times, and results are expressed as the mean $\pm$ S.E. (*error bars*). TNF did not alter the global levels of acetylated lysine-containing proteins (*A*) or acetylated histone H3K9 (*B*) within the endogenous Gα$_s$ promoter, but it induced an ~3-fold increase in acetylated histone H4K8 levels in the endogenous −837 to −618 Gα$_s$ promoter region (*C*, *panels i–iii*). *I*, input; *IP*, immunoprecipitate.

which we have previously shown to serve as a potent repressor of the Gα$_s$ promoter (25), will exert their action through repression of the Gα$_s$ promoter.

In this study, to determine how TNF may repress expression from the Gα$_s$ promoter, we have been able to demonstrate for the first time that the −837 to −618 region of the endogenous Gα$_s$ 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. First, the size of the chromatin fragments we obtained after sonication was ~500 bp (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 Gα$_s$ fragment studied herein. For example, the ChIP is still specific for CREB, Egr-1, and Sp1, 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.

Second, to put this into the 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 Gα$_s$ region or whether they are restricted to this particular nucleosomal region. Such explanations, however, do not detract from our findings because they still support the thesis that these factors are binding and regulating the Gα$_s$ promoter.

We also demonstrated that exogenous histone deacetylase activity, namely HDAC-1, could repress the Gα$_s$-luc promoter, whereas TNF stimulation induced occupancy of the Gα$_s$ −837 to −618 fragment by endogenous HDAC-1. Furthermore, the HDAC inhibitor TSA had the opposite effect and induced Gα$_s$ promoter activity. Together, these observations support the view that deacetylation of the Gα$_s$ 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 Gα$_s$ Promoter Repression—The obvious question posed by our data is that if deacetylation is required to reduce or silence Gα$_s$ expression why should an increased recovery of acetylated histone H4 be observed in the

distal Gα$_s$ fragment in the presence of TNF. First, in terms of an explanation for this observation of increased H4K8 acetylation with TNFα, is that acetylation at H4K8 is required to stabilize the interaction between DNA and the repressor element 1-silencing transcription factor (REST) protein (for a review, see Ref. 65). This mechanism requires that the interaction between REST and DNA is stabilized by the binding of the ATP-dependent chromatin-remodeling 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 Gα$_s$ promoter region would facilitate the binding and stabilization of the REST-mSin3 complex to the distal Gα$_s$ 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; in the present study, we did not see such H3K9 deacetylation in the presence of TNF.

In terms of interaction with those factors in the distal Gα$_s$ promoter, REST has been shown to bind Sp1 and Egr-1. In both studies, bidirectional regulation of the specific target genes was noted; *i.e.* Sp1 could activate expression of sodium channels, whereas 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 that a similar mechanism may govern expression of the Gα$_s$ 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 KDAC8 modified the Hsp20 acetylation profile in such a manner that myometrial contractility was subsequently inhibited.

At present, the exact mechanism by which TNF influences acetylation and Gα$_s$ expression is unclear. TNF is a potent inducer of NF-κB activity; indeed, RelA NF-κB is documented to undergo CBP-mediated acetylation at Lys-218, -221, and -310 (63). Moreover, Dai *et al.* (64) subsequently reported that newer HDAC inhibitors, including suberoylanilide hydroxamic acid and MS-275, induced acetylation and nuclear localization of RelA. Dai *et al.* (64) suggested that this was necessary for NF-κB-induced antiapoptotic effects in certain leukemia cells. 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 and 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 localization without activating the NF-κB-sensitive 3x-κB-luc reporter. The significance of that observation remains unclear at present, but it may be due to a delay in IκBα resynthesis similar to that seen for pervanadate-induced NF-κB activation (2). Moreover, it would also suggest that in the context of myometrial function a cautious approach to using HDAC inhibitors as tocolytic (labor-stopping) drugs should be used. Briefly, there is a growing body of literature interested in understanding whether HDAC inhibitors may be of use clinically as tocolytic drugs (17, 49, 68). Although such studies do present convincing evidence that HDAC inhibitors such as TSA exert robust prorelaxant effects on isolated strips of human myometrium (Refs. 17, 49, and 68 and see Fig. 1), we must, however, also consider potential unwanted effects, including acetylation and subsequent activation of non-chromatin-based factors (*e.g.* NF-κB RelA) that could also promote labor such as those described by Chen *et al.* (63). Indeed, our data demonstrate that TNF can overcome TSA-mediated myometrial relaxation. If HDAC inhibitors have a positive effect on the activity of NF-κB, then utilizing them in the clinical setting may only serve to exacerbate those premature myometrial contractions that the drug was meant 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. Although TNF itself did not induce contraction, the NF-κB inhibitor QNZ did repress the TNF effect and sensitized the tissue to TSA, implying that the NF-κB family serves an important function in this modulatory role. Subsequent studies into the regulation of the proquiescent factor, the Gα$_s$ GTP-binding protein, demonstrated that the −837 to −618 region of the endogenous Gα$_s$ promoter is occupied by CREB, Egr-1, and Sp1 transcription factors. Moreover, CBP is also associated with this region: we believe CBP is required to acetylate the Gα$_s$ promoter, leading to Gα$_s$ expression. In the presence of TNF, although there was no discernible change in the level of CREB, Sp1, or Egr-1 bound to the Gα$_s$ 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 Gα$_s$-luc construct, whereas reciprocal activation was seen with exogenous CBP or the HDAC inhibitor TSA. Taken together, our data suggest that expression from the Gα$_s$ promoter is in part governed by protein acetylation.

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