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Evidence That $G\alpha_q$-Coupled Receptor-Induced Interleukin-6 mRNA in Vascular Smooth Muscle Cells Involves the Nuclear Factor of Activated T Cells

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ABSTRACT

The immunosuppressant cyclosporin A inhibits transcription mediated by the nuclear factor of activated T-cells (NFAT), a key regulator of cytokine gene expression in lymphocytes that integrates phospholipase C signaling. NFAT is also expressed in vascular smooth muscle cells, but the genes it regulates there are unknown. Here we show that $G\alpha_q$-coupled P2Y nucleotide receptor signaling in rat vascular smooth muscle cells increases NFAT-mediated luciferase reporter expression. It also induces interleukin (IL)-6 gene expression but not other cytokine mRNAs including IL-1, IL-2, IL-3, IL-4, IL-10, γ-interferon, tumor necrosis factor-α, or tumor necrosis factor-β. IL-6 mRNA induction by UTP is more rapid and transient then that caused by IL-1β stimulation and is partially blocked by cyclosporin A or by expression of a *trans*-dominant NFAT inhibitor. Expression of recombinant NFATc1 markedly augments IL-6 mRNA induction by these and other agonists, which is partially attributable to NFAT-regulated paracrine mediators. However, *trans*-dominant NFκB inhibitors strongly interfere with IL-6 mRNA induction both by IL-1β and by UTP, which synergistically evoke IL-6 mRNA expression. These findings suggest that NFAT is among the cofactors involved in NFκB-dependent IL-6 gene induction by Ca^{2+}-mobilizing receptors in vascular smooth muscle cells.

Cardiovascular toxicity is a prominent side effect of cyclosporin A (CsA) or FK506 immunosuppressive therapy. The mechanisms underlying these drug side effects are unknown, but they occur at doses that are necessary for effective immunosuppression (Sander and Victor, 1995). This is consistent with the hypothesis that both immunosuppression and toxicity are due to a common mechanism of drug action. Inhibition of transcription mediated by the nuclear factor (NF) of activated T-cells (NFAT) is widely thought to account for immunosuppression caused by CsA and FK506 (Ho et al., 1996). The Rel-related NFAT family of transcription factors are produced from five known genes[1] and are best understood as regulators of immune cell cytokine gene expression (Rao et al., 1997). CsA and FK506 inhibit the phosphatase calcineurin, which mediates Ca^{2+}-dependent cytoplasmic to nuclear NFAT translocation after activation of phospholipase C (PLC) (Flanagan et al., 1991; Clipstone and Crabtree, 1992). In nuclei, NFAT assembles with other transcription factors on composite gene enhancer sites, the best known of which are the activator protein-1 (AP-1) basic leucine zipper proteins (e.g., c-fos and c-jun). Such NFAT partners are activated through parallel stimulation of protein kinase C and/or the mitogen-activated protein kinase cascades (Jain et al., 1992; Northrop et al., 1993). Thus, NFAT transcription reflects integration of multiple signaling pathways downstream of PLC-coupled receptors, such as those regulating responsiveness to foreign antigens.

Although initially considered a lymphocyte-specific transcription factor, the five NFAT genes are expressed differentially within most tissues, and no single NFAT isoform is restricted to thymus, spleen, or peripheral blood lymphocytes (Hoey et al., 1995). Conceivably, the cardiovascular toxicity associated with immunosuppressive drug therapy might be related to disruption of NFAT-mediated transcription in nonlymphoid cells. Recent work has established that NFAT-mediated transcription can be induced by signaling from

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[1] NFAT nomenclature is unsettled. Synonyms of the five known genes are: 1) NFATc1, NFAT2, NFATc; 2) NFATc2, NFAT1, NFATp; 3) NFATc3, NFAT4, NFATx; 4) NFATc4, NFAT3; and 5) NFAT5.

ABBREVIATIONS: CsA, cyclosporin A; NFAT, nuclear factor of activated T-cells; PLC, phospholipase C; PDGF, platelet derived growth factor; IL, interleukin; VSMC, vascular smooth muscle cell; AP-1, activator protein-1; LTR, long terminal repeat; IRES, internal ribosome entry site; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALU, arbitrary light units; CREB, cAMP response element binding protein; AP-1, activation protein-1; RPA, ribonuclease protection assay.

Ca^{2+}-mobilizing $G\alpha_q$ protein-coupled and growth factor receptors (Boss et al., 1996, 1998a,b). NFAT is thus positioned as an effector target for signaling directed by diverse families of mitogenic hormones, autacoids, and growth factors in growing numbers of cell types. These include vascular endothelial cells and vascular smooth muscle cells (VSMCs) and also cardiac myocytes (Cockerill et al., 1995; Boss et al., 1996, 1998a,b; Molkentin et al., 1998; Armesilla et al., 1999). The endogenous genes that NFAT regulates within VSMCs are currently unknown. The following data provide several lines of evidence that implicate NFAT in the pathways regulating immediate-early interleukin (IL)-6 gene expression in rat aortic VSMCs after stimulation with mitogenic factors.

Materials and Methods

Cell Culture. A continuous line of rat thoracic VSMCs (Gunther et al., 1982) obtained from R. W. Alexander (Emory University) were maintained in Dulbecco's modified Eagle's medium with 3.7 mg/ml $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated calf serum in a 37°C CO_2 incubator with 5% CO_2. After reaching confluence, cells were cultured in serum-free media for 16 to 24 h before experimental treatments and used between eight and 25 passages after primary explant. During this period, the cells maintain uniform growth and response characteristics. Phoenix retroviral producer cells (no. SD3443, American Type Culture Collection, Rockville, MD) were grown like VSMCs, except that 10% fetal bovine serum was used instead of calf serum. Stock solutions of CsA, a gift from Sandoz Pharmaceutical Co. (East Hanover, NJ), were prepared in 0.025% ethanol, 0.001% Tween-80 in phosphate-buffered saline. Nucleotide agonists were purchased from Sigma (St. Louis, MO), and platelet-derived growth factor (PDGF)-BB and IL-1β were purchased from Calbiochem, Inc. (San Diego, CA).

Plasmid Constructions. The retroviral expression vector pTJ66 is a multistep derivative of the retroviral vector pTJM9 (Boss et al., 1998a), constructed as follows. The neomycin resistance cassette was excised from pTJM9 by digestion with *Bcl*I and *Bst*B I and replaced with a multicloning site (*Sfi*I, *Xho*I, *Sfi*I) formed by the oligonucleotides 5′GATCAGGCCTTG-TAGGCCTAGGCTCGAGGCCTACAAGGCCTT and 5′CGAAGGCCTTG-TAGGCCTCGAGCCTAGGCCTACAAGGCCT. The resulting vector, pTJM11, was treated with *Bam*HI, *Hin*dIII, and the Klenow polymerase, to discard an internal cytomegalovirus promoter, and recircularized to create pCL2. pCL2 thus has two adapter-dependent universal cloning sites downstream of the single 5′-long terminal repeat (LTR) promoter (5′ *Sfi*I/*Sfi*I; 3′ *Bst*XI/*Bst*XI), which received NFATc1 (in the *Sfi*I sites) and an internal ribosome entry site (IRES):Zeo:eGFP cassette (in the *Bst*XI sites) by the following strategy. A shuttle expression vector termed pTJM2 was first constructed, which is derived from the pCDM8 expression vector (Invitrogen, Inc., San Diego, CA) by replacing the *supF* gene with a β-lactamase cassette. A zeocin resistance/eGFP fusion protein, in which the eGFP-coding sequence is at the carboxyl end, was cloned into *Hin*dIII/*Not*I-digested pTJM2 using a three-piece ligation strategy to create pKA11. This was accomplished by preparing 1) an *Eco*RI/*Not*I restriction fragment from pEGFP-N1 (Clontech, Inc., Palo Alto, CA), a zeocin resistance cDNA coding *Hin*dIII/*Eco*RI fragment created by polymerase chain reaction using the primers 5′ GCGAAGCTTCCATGGCCAAGTTGACCAGTG and 5′ CGCGAATTCGAAGGTCCTGCTCCTCGGC and the template pCMV-ShBle::ADH (a kind gift from T. Jaffredo, Centre National de la Recherche Scientifique, Toulouse, France; Gautier et al., 1996), and 3) *Hin*dIII/*Not*I-digested pTJM2. Subsequently, pIRES-ZGFP was created by fusing an *Msc*I/*Not*I fragment from pKA11 into the *Msc*I/*Not*I sites in pCITE4a+ (Novagen, Inc., Madison, WI), placing Zeo:eGFP downstream of an IRES sequence. Finally, pTJ66 was created by removing a *Pvu*II/*Not*I fragment from pIRES-ZGFP, blunting with Klenow, ligating with *Bst*XI adapters, and cloning into *Bst*XI-digested pCL2. The NFATc1 expression vector, termed pTJ67, was created by removing the human NFATc1 coding sequence from pSH107c (a gift from S. Ho and G. Crabtree, Stanford University, Palo Alto, CA; Ho et al., 1995), using *Avr*II and *Hin*dIII, blunting with Klenow, ligation with *Sfi*I adapters (formed with the oligonucleotides 5′CTAGGCCTACA and 5′AGGCCTAG), and inserting into *Sfi*I-digested pTJ66. Zeocin treatment (100 μg/ml; over 4–5 days) of cells infected with retrovirus prepared from this vector ensures expression in >99% of the cells in culture. To create the Zeo:eGFP:VIVIT expression shuttle plasmid pTJ80, a synthetic linker was cloned into the *Bsr*GI and *Not*I sites in the 3′ end of Zeo:eGFP in pKA11 using the oligonucleotides 5′GTACATGGCCGG-CCCCCACCCCGTGATCGTGATCACCGGCCCCCACGAGGAGTAAGC and 5′GGCCGCTTACTCCTCGTGGGGGCCGGTGATCACGATCACG-GGGTGGGGGCCGGCCAT, which create a coding sequence for the terminal peptide (MAGPHP**VIVIT**GPHEE) in-frame with the eGFP-coding sequence. A *Hin*dIII-*Not*I fragment from pTJ80 was then subcloned into the retroviral vector pCL2 for expression from the 5′LTR promoter, creating pTJ85. The control for this vector is pTJ84, which involved subcloning the *Hin*dIII-*Not*I Zeo:eGFP cassette from pKA11 into pCL2. *Trans*-dominant NFκB inhibitors used here are in the LZRS retroviral vector and were received as a gift from P. Khavari (Stanford University; Seitz et al., 1998).

Retroviral Production and VSMC Infection. The protocols describing transient, helper-virus free production of nonreplicating recombinant retroviruses and VSMC infection have been described in detail (Boss et al., 1998a), except that the Phoenix-Ampho producer cell line, rather than the Bing-CAK8 cells, were used here. The retroviral NFAT-specific luciferase reporter vector pKA7, which encodes a luciferase gene driven by a minimal promoter and a triple repeat of the upstream NFAT enhancer, both derived from the human IL-2 gene, has been described (Boss et al., 1998a). Luciferase expression from this vector is strictly dependent on NFAT (Boss et al., 1998a).

Luciferase Reporter Assays. Confluent VSMCs in serum-free medium grown on 24 multiwell tissue culture plates were stimulated with agonists for 5 h unless shown otherwise at 37°C in a 5% CO_2 incubator. The medium was aspirated, and luciferase activity was measured using a Turner Designs 20e luminometer (Sunnyvale, CA) as described previously (Takeuchi et al., 1993).

Western Blot Analysis. Whole cell extracts of VSMCs were collected and resolved by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide minigels (Bio-Rad, Inc. Richmond, CA) and then probed with an anti-NFATc1 monoclonal antibody purchased from ABR, Inc. (Boulder, CO) as described previously (Boss et al., 1998a).

RNase Protection Assays (RPA). VSMCs were grown to confluence in 35-mm-diameter wells on tissue culture plates. The Trizol reagent (Life Technologies/BRL, Inc., Gaithersburg, MD) was added after stimulation, and the samples were frozen at −20°C until processed to extract total RNA. RNA aliquots (2.5–10 μg) were lyophilized and resuspended in hybridization buffer supplied with the RiboQuant RPA kit (Pharmingen, Inc., San Diego, CA). A mixture of [^{32}P]UTP-labeled riboprobes was synthesized using the rCK-1 multiprobe template set (Pharmingen), according to the manufacturer's directions. These riboprobes detect mRNAs encoding the cytokines IL-1α, IL-1β, tumor necrosis factor (TNF)-β, IL-3, IL-4, IL-5, IL-6, IL-10, TNFα, IL-2, γ-interferon, and the constitutively expressed L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. After hybridization and RNase digestion as recommended by the manufacturer, the samples were resolved by electrophoresis through 5% polyacrylamide/5 M urea gels, which were exposed to storage phosphor screens (Molecular Dynamics, Inc., Sunnyvale, CA). A volume integration protocol was used to quantify hybridization signals using the ImageQuant software (Molecular Dynamics). To control for variation in sample processing, IL-6 mRNA hybridization signals (measured as volumes) were divided by the hybridization volume of the ribosomal L32 mRNA within each sample.

Data Analysis. Statistical significance was calculated using a two-tailed paired t test, using routines in Prism v3.0 (GraphPad Software, Inc., San Diego, CA).

Results

P2Y Receptors Induce NFAT-Mediated Transcription in VSMCs. To determine whether $G\alpha_q$-coupled P2Y receptors induce NFAT-mediated transcription in VSMCs, cultured rat VSMCs with a stable NFAT-specific luciferase reporter transgene were stimulated with various extracellular nucleotides. Previous studies have established that this reporter provides reliable measure for NFAT activation (Boss et al., 1998a). As shown in Fig. 1, the nucleotides UDP and UTP evoke luciferase responses that are ~10-fold over basal levels, wherein UDP (EC_{50} = 1.2 ± 0.2 μM, mean ± S.E.M., n = 6) is slightly more potent than UTP (EC_{50} = 4.7 ± 0.1 μM). The response to ATP is weak by comparison and occurs only at the highest tested concentrations, whereas ADP has no significant effect on this NFAT-mediated transcriptional response. This agonist potency series is identical with that for nucleotide-stimulated NFκB transcription in these cells (Abbott et al., 2000). Given this, and also measures of extracellular UTP catabolism, and reverse transcriptase-polymerase chain reaction studies (Abbott et al., 2000), the simplest explanation is that gene expression responses to UTP in this preparation most likely reflect activation of the UDP-preferring $G\alpha_q$-coupled $P2Y_6$ nucleotide receptor subtype (Abbott et al., 2000).

Differential Induction of IL-6 mRNA Expression by UTP and IL-1β. A multiplex ribonuclease protection assay was used to screen for cytokine mRNAs that are induced in VSMCs stimulated with 100 μM UTP. Responses were compared with those of maximally effective concentrations of the cytokine IL-1β, which does not activate PLC and was chosen initially as a positive control for a known IL-6 mRNA inducer. Of the 11 cytokine mRNAs surveyed, NFAT is involved in immune cell induction of IL-2, IL-3, IL-4, γ-interferon, and TNFα (Rao et al., 1997). However, as shown in Fig. 2, both agonists stimulate only IL-6 gene expression in VSMCs but by different magnitudes and kinetics. The response to a bolus of UTP is robust but transient, with a peak effect in this series of experiments of 24 ± 5-fold over basal levels after 1 h of treatment (n = 3), which returns to basal levels within 4 h. In contrast, IL-6 mRNA induction by IL-1β is weaker but sustained, with a maximal effect of 8 ± 2-fold over basal levels (n = 3) occurring 2 h after treatment.

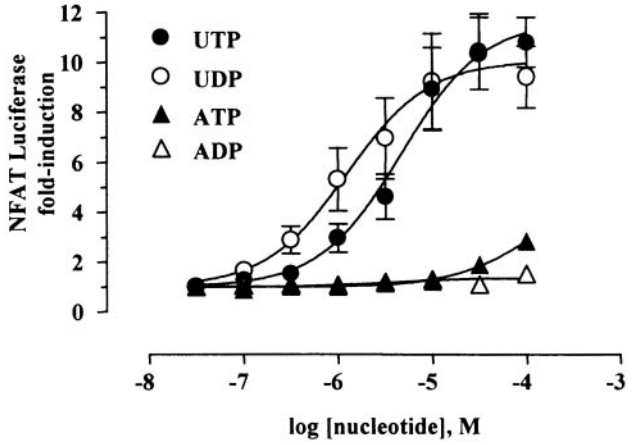

Fig. 1. Pharmacological characterization of P2Y receptor-mediated NFAT-specific transcription in VSMCs. A stable VSMC cell line infected with an NFAT-specific luciferase reporter was stimulated with the indicated concentrations of nucleotides, and luciferase activity was measured 5 h later. Each point represents the mean ± S.E.M. of six individual experiments performed in duplicate, expressed as the fold response over basal levels of activity in each experiment.

Ectopic NFATc1 Expression Augments NFAT-Mediated Transcription. Responses were measured in NFAT-luciferase reporter VSMCs to determine whether overexpression of the NFATc1 isoform enhances NFAT-mediated transcription. Using retroviral vectors, NFATc1 is expressed from a bicistronic mRNA coexpressing the visible selection marker Zeo:eGFP, providing a strategy to ensure that all cells express the recombinant NFATc1 mRNA (Fig. 3A), which was confirmed by microscopic inspection and fluorescence-activated cell sorting (data not shown). Control cells were infected and treated in parallel with retrovirus prepared from the parental vector, pTJ66, which only expresses Zeo:eGFP. Western blot analysis using an NFATc1-specific antibody shows substantially higher expression of recombinant NFATc1 over levels of endogenous NFATc1 (Fig. 3B). This results in enhanced basal NFAT-specific luciferase transcription, which is ~10-fold greater in cells expressing NFATc1 [1.69 ± 0.12 arbitrary light units (ALU)] compared with control cells (0.12 ±.0.01 ALU). Agonist-stimulated NFAT-specific luciferase transcription is also enhanced in cells expressing recombinant NFATc1, although the responses are not strictly proportional to its effect on basal transcription. For example, compared with responses in control cells, maximal activation by phorbolmyristyl acetate plus ionomycin is enhanced only 2-fold in NFATc1 cells, whereas those to PDGF and angiotensin II are 10-fold greater in NFATc1 cells.

Effects of CsA and Ectopic NFAT Expression on IL-6 Induction. Using both the NFATc1 overexpression cell line and its control cell line, we tested how inhibiting calcineurin using CsA (1 μM) affects IL-6 mRNA induction after a 1-h treatment with various classes of agonists (Fig. 4). This series of experiments was performed in parallel, and responses in both cell lines are normalized to the maximal effect of UTP in control cells observed within each experiment. By normalizing the data in this way, we illustrate both the differential efficacy of the various agonists and how ectopic NFATc1 expression dramatically augments IL-6 mRNA responses to these agonists. In control cells, CsA significantly inhibits basal, UTP-, and PDGF-BB-stimulated IL-6 mRNA expression (Fig. 4A). For unclear reasons, unlike the effects of other agonists, we found substantial interassay variability in the maximal effect associated with angiotensin II treatment relative to that for UTP, thus explaining the error bars in those data. However, within each individual experiment, CsA also significantly attenuates the angiotensin II response. In contrast to the effects of these mitogens, the response to IL-1β in control cells is CsA insensitive.

Ectopic NFATc1 expression strongly enhances both basal and agonist-stimulated IL-6 mRNA induction, and a greater fraction of these responses are inhibited by CsA than in control cells, including that for IL-1β (Fig. 4B). For example, UTP is consistently the strongest inducer of the IL-6 mRNA in both control and NFATc1 cells. Recombinant NFATc1 expression enhances the UTP response an additional 10-fold

over that seen in control cells, and the degree of CsA inhibition increases from 45% in control cells to 88% in NFATc1 cells. Similarly, the relative response to IL-1β is enhanced by NFATc1 expression, and unlike in control cells, CsA inhibits a significant component of the IL-1β response in cells expressing NFATc1. Interestingly, the response to angiotensin II is only slightly enhanced by NFATc1 expression, but this also remains CsA sensitive.

Effects of a *Trans*-Dominant NFAT Inhibitor. A recently described peptide inhibitor of NFAT, termed VIVIT, was fused to the carboxyl terminus of the Zeo:eGFP module and expressed in VSMCs from a retroviral vector. Zeo:eGFP without this epitope was expressed in parallel cells as a control. The VIVIT peptide mimics a purported calcineurin-docking site on the NFAT surface. Thus, unlike CsA, VIVIT inhibits NFAT dephosphorylation and nuclear translocation without affecting calcineurin catalytic activity (Aramburu et al., 1999). Fluorescence-activated cell sorting analysis of the eGFP signal confirmed that between 90 and 95% of the cells in culture express the fusion protein after infection with the retrovirus (data not shown). Zeo:eGFP:VIVIT expression inhibited NFAT-specific luciferase activity by ~67% (Fig. 5A), which approaches the 80% level of inhibition routinely caused by CsA (Boss et al., 1998a). UTP-stimulated IL-6 mRNA induction in VSMCs expressing Zeo:eGFP:VIVIT is 55 ± 15% (mean ± S.E., $n = 4$) of the level induced in parallel treated control cells expressing Zeo:eGFP (Fig. 5, B and C). This inhibitory effect is similar to that caused by CsA (see Fig. 4A).

NFATc1 Expression Enhances IL-1α and IL-1β mRNA Expression in VSMCs. In control VSMCs, IL-1α and IL-1β mRNAs are undetectable under basal conditions and are not induced by either IL-1β or UTP. In contrast, we noticed that the IL-1α and IL-1β mRNAs are slightly detectable over background hybridization in VSMCs that overexpress recombinant NFATc1. Treatment for 1 h with UTP, IL-1β, and PDGF-BB enhances the expression of each mRNA (Fig. 6). Furthermore, CsA treatment attenuates induction of the IL-1β mRNA to a greater degree than it affects the IL-1α mRNA.

NFATc1-Mediated Paracrine Effect on IL-6 mRNA Induction. To test the possibility that profound IL-6 mRNA induction in NFATc1 overexpression cells might involve paracrine effects, cells expressing recombinant NFATc1 were cocultured over monolayers of normal VSMCs using permeable culture chamber inserts. Cells were infected in parallel with the empty vector (pTJ66) for a control overlay. After the cells reached confluence over 4 to 5 days in culture, the

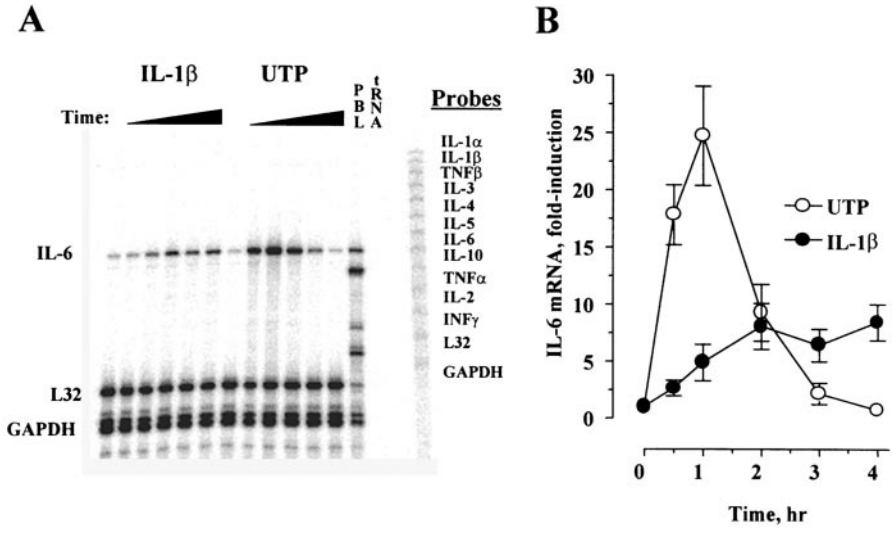

Fig. 2. Time courses for IL-6 mRNA induction in VSMCs by UTP and IL-1β. VSMCs were stimulated in parallel with either 100 μM UTP or 10 ng/ml IL-1β for the indicated times. Ribonuclease protection assays used a mixture of antisense [^{32}P]UTP-labeled riboprobes directed against the indicated cytokine mRNAs. A, a phosphorimage from a representative experiment. PBL, rat peripheral blood lymphocyte RNA as a positive hybridization control; tRNA, the probes were hybridized with yeast tRNA and digested. Only the IL-6, L32, and GAPDH mRNAs are detectable. INFγ, γ-interferon. B, quantified results from three independent experiments, expressed as the means ± S.E.M. fold-induction of IL-6 mRNA normalized by the L32 hybridization signal.

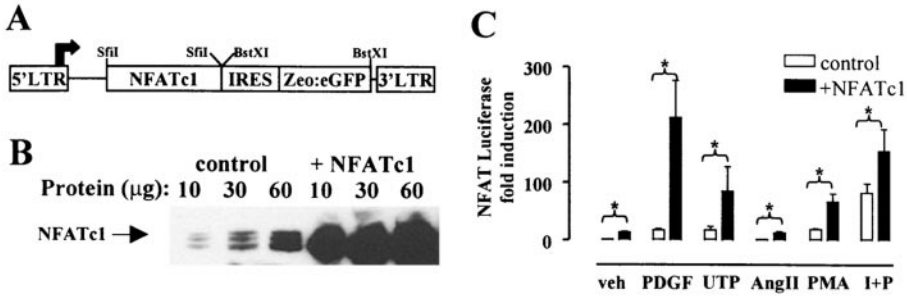

Fig. 3. Expression of recombinant human NFATc1 in VSMCs. A, schematic of the LTR region for retroviral expression vector pTJ67 depicting NFATc1 cDNA sequence, IRES, and Zeo:eGFP resistance marker. The remainder of the circular plasmid is not shown for clarity. B, representative Western blot using an anti-NFATc1 antibody of endogenous NFATc1 protein in control cells compared with level of recombinant protein expressed from pTJ67 infected cells. C, NFAT-specific transcriptional responses after 5 h of stimulation with various agonists. The data are normalized to the basal level of luciferase activity (means ± S.E. in ALU) in control cells (0.121 ± 0.002 ALU), to illustrate that NFATc1 expression enhances basal NFAT activity (1.69 ± 0.17 ALU). For reference, responses to PDGF were 2.14 ± 0.32 ALU and 25.6 ± 7.8 ALU in control and NFATc1 cells, respectively. PDGF, 30 ng/ml PDGF-BB; UTP, 100 μM UTP; AngII, 100 nM angiotensin II; PMA, 100 nM phorbolmyristyl acetate; I+P, 100 nM ionomycin plus 100 nM PMA; veh, vehicle. Each bar represents the mean ± S.E.M. of three independent experiments performed in duplicate. *, significant difference, paired t test ($P < .05$).

inserts were discarded and IL-6 mRNA induction was then measured in the bottom monolayers 1 h after UTP was added. As shown in Fig. 7, NFATc1 overlay cells have no effect on the IL-6 mRNA response to vehicle but sensitize UTP-stimulated IL-6 mRNA expression compared with control. We observed no contamination of the bottom monolayers by Zeo:eGFP-positive overlay cells. These results suggest that NFATc1-regulated paracrine factors may sensitize subsequent $G\alpha_q$-coupled receptor-stimulated IL-6 mRNA expression in VSMCs.

NFκB Is Necessary for IL-6 mRNA Induction in VSMCs. A recent study has shown that basal and angiotensin II-stimulated IL-6 mRNA induction in rat VSMCs is attenuated by a pharmacological inhibitor of IκB degrada-

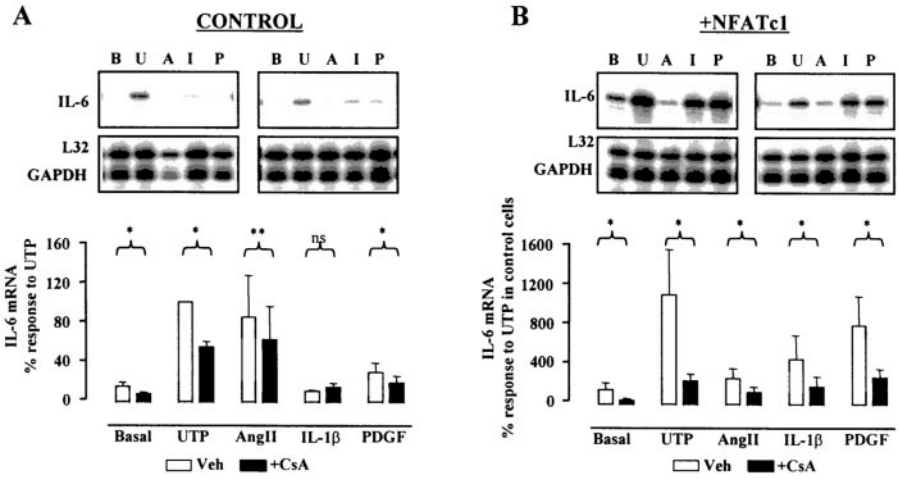

Fig. 4. Effect of CsA and ectopic NFATc1 on agonist-stimulated IL-6 mRNA induction in VSMCs. A, response in control cells. B, response in cells overexpressing NFATc1. Top panels, hybridization signals from an RPA from a representative experiment in which all treatments were performed in parallel for 1 h. Only the IL-6, L32, and GAPDH signals are shown. The concentrations of reagents used are 1 μM CsA, 100 μM UTP (U), 100 nM angiotensin II (A), 10 ng/ml IL-1β (I), and 30 ng/ml PDGF-BB (P). Bar graphs, quantified responses from four independent experiments in which each bar represents the mean ± S.E.M. of the L32-normalized IL-6 mRNA hybridization signal. AngII, angiotensin II; Veh, vehicle. All responses are expressed as percentages of the UTP response (taken as 100%) in control cells. Note the factor of 10 difference between the ordinate scales in the two graphs. *, statistically significant difference, paired *t* test ($P < .05$); ns, no significant difference; **, statistically significant difference, paired *t* test ($P < .05$). The statistical test asked whether the responses in the presence of CsA differ from agonist alone. The error bars reflect variation relative to UTP responses across each experiment.

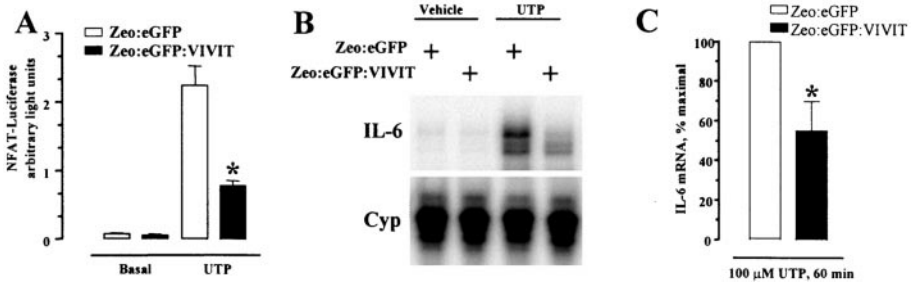

Fig. 5. UTP-stimulated IL-6 mRNA attenuation by a *trans*-dominant NFAT inhibitor. VSMC lines were established in parallel using a retrovirus expressing the Zeo:eGFP:VIVIT fusion protein or Zeo:eGFP as control. A, basal and 100 μM UTP-stimulated NFAT-specific luciferase reporter activity after 5 h of treatment, expressed as ALU, from duplicate determinations in two separate experiments. B, representative phosphorimage showing basal and UTP-stimulated IL-6 mRNA levels after 60 min. A cyclophilin mRNA (Cyp) was used as an internal standard. C, quantified data from four independent mRNA experiments, expressed as percentages of the UTP response in Zeo:eGFP cells within each experiment. *, significantly lower than Zeo:eGFP control cell response, $P < .05$, one-tailed paired *t* test.

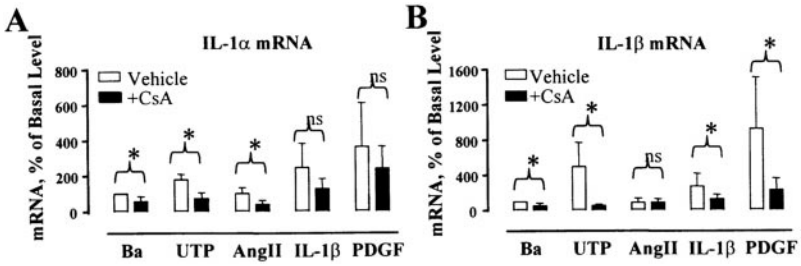

Fig. 6. Recombinant NFATc1 expression enhances IL-1α and IL-1β mRNA expression in VSMCs. These two mRNAs are not detectable above background signal in control cells but are increased ~2- to 3-fold over background in basal (Ba) samples of VSMCs that overexpress NFATc1. AngII, angiotensin II. Each bar represents the mean ± S.E. ($n = 4$) level of L32-normalized IL-1α (left) or IL-1β (right) hybridization signal, expressed as a percentage of the basal level in NFATc1 recombinant cells after a 1-h treatment with agonists. Thus, UTP stimulates IL-1α mRNA levels ~2-fold and IL-1β mRNA levels ~5-fold. *, significant affect of CsA, $P < .05$; ns, not significantly different.

tion, suggesting a crucial role for NFκB in the response to mitogens (Han et al., 1999). Furthermore, we recently reported that P2Y receptors stimulate transcription from a NFκB-specific retrovirus-based luciferase reporter but only to a level that is ~10% of that caused by IL-1β (Abbott et al., 2000). Expression in VSMCs of two independently acting *trans*-dominant NFκB inhibitors, Δ-SP-p50 (Logeat et al., 1991) or IκBα-M protein (Van Antwerp et al., 1996), can block up to 90% of the effect of either agonist on NFκB reporter gene activity (Abbott et al., 2000). IL-6 mRNA levels were measured after a 1-h treatment with either UTP or IL-1β in two cell lines expressing either of these inhibitors and compared with that in VSMCs infected with a negative control retrovirus. As shown in Fig. 8A, IL-6 mRNA induction by both agonists is almost completely abrogated in cells expressing the NFκB inhibitors, suggesting that NFκB is a necessary factor in each response.

The data in Fig. 8A predict that NFκB-dependent and NFκB-independent mechanisms might operate synergistically to control IL-6 mRNA expression in response to the mitogen. To test this, IL-6 mRNA was measured at different times up to 4 h in VSMCs costimulated with UTP and IL-1β and compared with responses to each agonist alone, treated in parallel. Costimulation results in a markedly synergistic induction of the IL-6 mRNA during the period between 1 and 3 h after cotreatment with UTP and IL-1β (Fig. 8B). The earliest times after costimulation are insensitive to CsA treatment, but CsA suppresses responses between 2 and 4 h after costimulation (Fig. 8C).

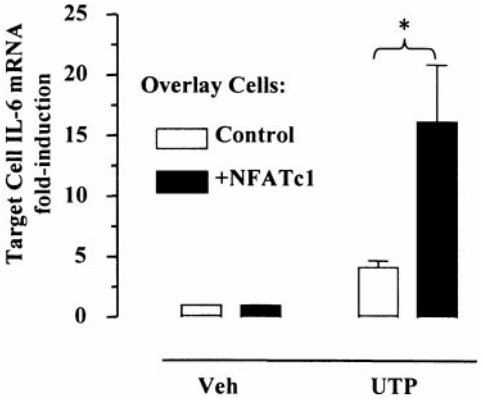

Fig. 7. NFATc1 expression sensitizes UTP-stimulated IL-6 mRNA expression by a paracrine mechanism. Cells expressing NFATc1 or control cells infected with pTJ66 virus (feeder cells) were cultured in permeable inserts over monolayers of normal VSMCs (recipient cells). After reaching confluence, the inserts were removed, and IL-6 mRNA levels were measured in the recipient cells after a 1-h treatment with 100 μM UTP. Veh, vehicle. Each bar represents the mean ± S.E. from four experiments, expressed as the fold induction over basal levels of L32-normalized IL-6 mRNA in recipient cells cocultured with control feeder cells. *, statistically significant affect of NFATc1 feeder cells ($P < .05$).

Discussion

The growing awareness that NFAT proteins are more widely distributed than originally thought has important implications. In lymphocytes, NFAT utilization is better coupled to more profound and sustained, rather than weaker, Ca^{2+} signals (Timmerman et al., 1996). In this way, NFAT is refractory to weaker cell activation because of ancillary physiological signals and more efficiently engaged in response to powerful stimuli such as a foreign antigen. Whether this discriminative capacity holds true in nonlymphoid cells is uncertain, and few conceivable physiological factors alone would seem capable of triggering VSMC activation to the degree that antigens can activate lymphocytes. Outside of immune cells, we speculate that NFAT may couple more subtle forms of signaling to gene regulation. The likelihood that differences might exist in modes of NFAT regulation between various cell phenotypes is evident in part by our finding that none of the known NFAT-regulated genes in other cells is induced by P2Y receptor activation in VSMCs. Obviously, higher orders of cellular controls must work along with NFAT to restrict expression of these several cytokines to different cellular contexts. Nevertheless, the finding begs the question of whether regulation of the heterologous NFAT enhancer used in our luciferase reporter, which is derived from the human IL-2 gene, fully represents VSMC-restricted properties of NFAT-mediated regulation. For this reason, and as a beginning to understand its physiological role, finding NFAT-regulated genes endogenous in VSMCs is important.

The present data provide several observations indicating a role for NFAT in immediate-early IL-6 gene expression in VSMCs in response to P2Y receptor signaling. They indicate that NFAT likely does not function as the sole transcription factor regulating IL-6 gene expression and are, instead, consistent with the hypothesis that NFAT coregulates IL-6 expression in a manner that is strictly dependent on coactivation by NFκB. Three experimental findings suggest a role for NFAT. The first two are inhibition by CsA and expression of the Zeo:eGFP:VIVIT protein, as two independently acting inhibitors of NFAT-mediated transcription. One acts by inhibition of calcineurin catalytic activity, whereas the latter

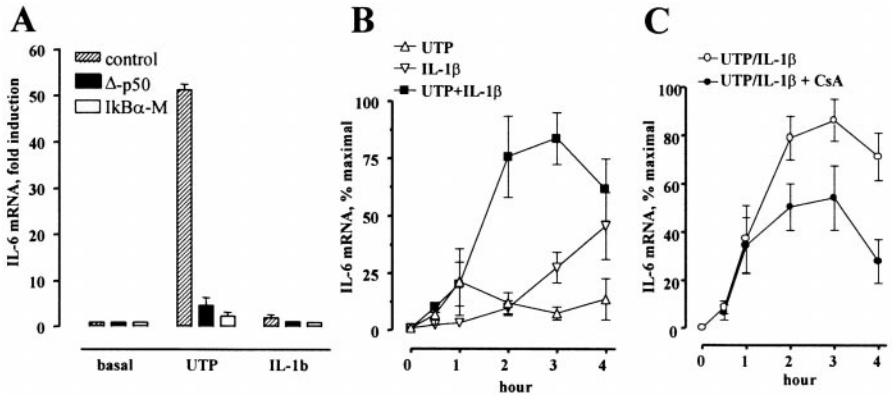

Fig. 8. NFκB is necessary for UTP- and IL-1β-stimulated IL-6 mRNA induction. Three VSMC lines were created by infection with a retrovirus that does not express a protein (control), a retrovirus expressing a mutant NFκB p50 protein (Δ-p50), or a nondegradable IκBα mutant (IκBα-M). L32-normalized IL-6 mRNA levels were measured by RPA after treating for 1 h with vehicle (basal), 100 mM UTP, or 10 ng/ml IL-1β. Each bar represents the mean ± range of responses from two experiments, where mRNA levels are expressed as the fold induction over basal levels in control cells.

purportedly interferes by preventing calcineurin from interacting with NFAT (Aramburu et al., 1999). Each reduces IL-6 mRNA expression by ~50%, and the degree of IL-6 mRNA responsiveness unaffected by these inhibitors could reflect NFAT-independent mechanisms of mRNA induction. In the case of the VIVIT inhibitor, it is also possible that residual responses occur in a proportion of cells that express lower levels of fusion protein. However, quite possibly CsA- and VIVIT-resistant responsiveness could reflect NFAT-mediated transcription that occurs independently of calcineurin activation. For example, significant amounts of nuclear NFATc2 are present in these cells under basal, nonstimulated conditions and would be positioned to coactivate gene expression in an apparently Ca^{2+}-independent manner (Boss et al., 1998a). Given this, as a complementary approach to using inhibitors, we also show that overexpression of NFATc1 markedly sensitizes the IL-6 gene to subsequent agonist stimulation. Although this approach could have indirect effects on IL-6 mRNA, for example, through regulation of paracrine-sensitizing factors, it is notable that a substantially greater inhibition of agonist response in these cells is achieved by CsA treatment. Because the CsA was added just briefly before the agonist, and because the IL-6 mRNA levels were measured only after a 60-min treatment, this suggests a significant direct role for NFATc1 in the immediate-early phase of the response. Thus, the most likely site for this effect would be at the level of promoter activation.

Previously, only a few plausible connections have linked NFAT to the regulation of IL-6 gene expression. In mast cells, IL-6 protein expression is attenuated by CsA or FK506 and is also correlated with an increased NFATc1 protein expression that follows manipulation with a Vav transgene (Fruman et al., 1995; Williams and Coleman, 1995; Song et al., 1999). Reduced IL-6 production is also reported in chimera mice with a targeted thymocyte NFATc1 deletion (Yoshida et al., 1998). One contrary study has shown, through purported NFκB induction, that FK506 treatment actually increases murine IL-6 gene expression in nonlymphoid tissues (Muraoka et al., 1996). Whether this is related to nonoverlapping cellular activities of the two immunosuppressants is uncertain. Unlike CsA, FK506 promotes Ca^{2+} release from ryanodine-sensitive endoplasmic reticulum, presumably by inhibiting the interaction between FK-binding proteins and the ryanodine receptor (Bers et al., 1998).

Previous evidence that NFκB and other coactivators participate in IL-6 gene expression is considerably more substantial than that of a role for NFAT. Various stimuli, including cytokines, G-protein-coupled receptor agonists, growth factors, phorbol esters, and lipopolysaccharide can stimulate IL-6 mRNA expression in VSMCs and other cells. Several recent studies have implicated CREB, AP-1, and NFκB in the responses to mitogens (Kranzhofer et al., 1996; Beasley, 1997; Gaumond et al., 1997; Kozawa et al., 1997; Leung et al., 1998; Funakoshi et al., 1999; Han et al., 1999). In addition to expanding an understanding of how CsA affects IL-6 mRNA induction, the most notable surprise in our data compared with these studies is the relatively weaker immediate-early effect of IL-1β compared with UTP. The more robust response to UTP, synergistic effects during costimulation with a cytokine, and the finding that a *trans*-dominant NFκB inhibitor blocks induction together argue for complex mechanisms of IL-6 gene regulation by $G\alpha_q$-coupled receptor signaling. NFκB activation is an apparently common mechanism shared by both physiological stimuli, and yet P2Y receptors appear to evoke the IL-6 mRNA by using additional mechanisms. In this preparation, UTP is reported as a broadly effective inducer of transcription mediated by several coactivators including AP-1, CREB, serum response/ternary complex factors, and the NFAT protein (Abbott et al., 2000). IL-1β has little discernible effect on transcription mediated by these factors and, instead, rather selectively stimulates NFκB-mediated transcription, which it does substantially more effectively than P2Y receptor signaling and by apparently distinct mechanisms (Abbott et al., 2000). Notably, exploiting the retroviral luciferase reporter strategy, we have shown that CsA has no effect on transcription mediated by any of these other factors and, instead, selectively inhibits NFAT-mediated transcription in this preparation (Robida et al., 2000).

Reconciling this apparent complexity associated with $G\alpha_q$-coupled receptor-induced IL-6 gene expression will be important, but it is notable that neither NFAT nor any other coactivator alone accounts for the regulation of a leading prototype for an NFAT-responsive locus, which is the IL-2 gene in human T-cells. IL-2 gene expression depends on full occupancy of all of its enhancers by diverse groups of transcription factors, including NFκB, that are coactivated by a strong antigenic stimulus (Garrity et al., 1994). Numerous other examples of promoter function dependent on synergistic and coordinate assembly of multiple transcription factors have been described (Tjian and Maniatis, 1994). The present results predict that NFAT participates as a cofactor in NFκB-dependent activation of the IL-6 gene by mitogenic, Ca^{2+}-mobilizing stimuli and will prove an important frame of reference when this hypothesis is tested using other approaches. Other transcriptional coactivators are likely involved in addition to NFAT and NFkB, and studies are under way to dissect out this regulation using approaches that will build on the findings established in this report.

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