Experimental Colitis Is Associated with Transcriptional Inhibition of Na\(^+\)/Ca\(^{2+}\) Exchanger Isoform 1 (NCX1) Expression by Interferon \(\gamma\) in the Renal Distal Convoluted Tubules*

Received for publication, October 3, 2014, and in revised form, January 26, 2015. Published, JBC Papers in Press, February 2, 2015, DOI 10.1074/jbc.M114.616516

Vijayababu M. Radhakrishnan‡, Pawel Kojs†, Rajalakshmy Ramalingam‡, Monica T. Midura-Kiela‡, Peter Angeli§, Pawel R. Kiela†,‡, and Fayez K. Ghishan‡†,2

From the Departments of ‡Pediatrics, Steele Children’s Research Center and §Immunobiology, University of Arizona Health Sciences Center, Tucson, Arizona 85724, and †Zanvyl Krieger School of Arts and Sciences Johns Hopkins University, Baltimore, Maryland 21218

Background: Defective renal Ca\(^{2+}\) reabsorption contributes to impaired systemic Ca\(^{2+}\) homeostasis and loss of bone density in IBD.

Results: During colitis, IFN\(\gamma\) represses NCX1 expression and activity in a Stat1-dependent manner.

Conclusion: IFN\(\gamma\) contributes to the reduced renal Ca\(^{2+}\) reabsorption during colitis by repressing basolateral NCX1.

Significance: IFN\(\gamma\) inhibits renal NCX1 to contribute to negative systemic Ca\(^{2+}\) balance and increased bone resorption in IBD patients.

NCX1 is a Na\(^+\)/Ca\(^{2+}\) exchanger, which is believed to provide a key route for basolateral Ca\(^{2+}\) efflux in the renal epithelia, thus contributing to renal Ca\(^{2+}\) reabsorption. Altered mineral homeostasis, including intestinal and renal Ca\(^{2+}\) transport may represent a significant component of the pathophysiology of the bone mineral density loss associated with Inflammatory Bowel Diseases (IBD). The objective of our research was to investigate the effects of TNBS and DSS colitis and related inflammatory mediators on renal Ncx1 expression. Colitis was associated with decreased renal Ncx1 expression, as examined by real-time RT-PCR, Western blotting, and immunofluorescence. In mIMCD3 cells, IFNy significantly reduced Ncx1 mRNA and protein expression. Similar effects were observed in cells transiently transfected with a reporter construct bearing the promoter region of the kidney-specific Ncx1 gene. This inhibitory effect of IFN\(\gamma\) is mediated by STAT1 recruitment to the proximal promoter region of Ncx1. Further in vivo study with Stat1\(^{-/-}\) mice confirmed that STAT1 is indeed required for the IFN\(\gamma\) mediated Ncx1 gene regulation. These results strongly support the hypothesis that impaired renal Ca\(^{2+}\) handling occurs in experimental colitis. Negative regulation of NCX1-mediated renal Ca\(^{2+}\) absorption by IFNy may significantly contribute to the altered Ca\(^{2+}\) homeostasis in IBD patients and to IBD-associated loss of bone mineral density.

The physiological levels of Ca\(^{2+}\) are tightly controlled by the concerted actions of different processes including intestinal Ca\(^{2+}\) absorption, Ca\(^{2+}\) reabsorption in the kidney, and exchange of Ca\(^{2+}\) from bone (1), all of which are controlled by 1,25(OH)\(_2\)D\(_3\) (2). Systemic imbalance of Ca\(^{2+}\) homeostasis, including disturbed renal and intestinal Ca\(^{2+}\) (re)absorption, has been hypothesized to contribute to the pathophysiology of bone loss associated with chronic inflammatory disorders, and especially with inflammatory bowel diseases (IBD) (3, 4). Indeed, increased urinary Ca\(^{2+}\) excretion has been described in IBD patients and in mouse models of colitis (5, 6). Renal epithelial Ca\(^{2+}\) reabsorption occurs primarily in distal convoluted tubules (DCT) and connecting tubules (CNT) via an active transcellular pathway. It depends on the concerted action of Ca\(^{2+}\)-transporting proteins within the apical and basolateral plasma membranes of the epithelial cells (7). In the epithelial cells of distal nephron, apical Ca\(^{2+}\) entry is thought to be mediated by the epithelial Ca\(^{2+}\) channel TRPV5 (previously known as ECaC1 or CaT2) (8) and then extruded at the basolateral side by Ca\(^{2+}\)-ATPase (PMCA) and Na\(^+/Ca^{2+}\) exchanger NCX1. Cytoplasmic calbindin D\(_{28K}\) serves as a cytoplasmic Ca\(^{2+}\) sensor and buffer which helps maintain the gradient for Ca\(^{2+}\) entry by keeping the intracellular concentration of free Ca\(^{2+}\) low and constant (9).

We have recently proposed a model where proinflammatory cytokines associated with active colitis, act to decrease the expression and activity of Klotho thereby leading to endocytosis of TRPV5 followed by UBR4-dependent ubiquitination and degradation, finally resulting in urinary Ca\(^{2+}\) wasting with bone loss (6, 10). However, impaired basolateral Ca\(^{2+}\) extrusion, which would amplify the effects of reduced TRPV5-dependent apical Ca\(^{2+}\) transport, has not been investigated.

*This work was supported, in whole or in part, by National Institutes of Health Grant R37DK033209 (to F. K. G.).
1 Both senior authors contributed equally to this work.
2 To whom correspondence should be addressed: Dept. of Pediatrics, Steele Children’s Research Center, University of Arizona Health Sciences Center; 1501 N. Campbell Ave, Tucson, Arizona 85724. Fax: 520-626-4141; E-mail: fghishan@peds.arizona.edu.

The abbreviations used are: IBD, inflammatory bowel disease; DCT, distal convoluted tubule; CNT, connecting tubule; FECA\(_{2+}\), fractional excretion of Ca\(^{2+}\); RACE, Rapid Amplification of cDNA Ends; TSS, transcription start sites; TBB, TATA box-binding protein; TNBS, trinitrobenzene sulfonic acid; DSS, dextran sodium sulfate.
Na\(^+\)/Ca\(^{2+}\) exchange is a major mechanism for cellular calcium extrusion by DCT and CNT (11, 12). The sodium-calcium exchanger 1 (Na\(^+\)/Ca\(^{2+}\) exchanger 1 or NCX1) is a basolateral plasma membrane transport protein that belongs to solute carrier family 8 (SLCA1) and plays a critical role in maintaining intracellular Na\(^+\) and Ca\(^{2+}\) homeostasis. There are three mammalian NCX isofoms: NCX1 is widely expressed in the heart, kidney, brain, blood vessels, and other organs; expression of NCX2 and NCX3 is limited mainly to the brain and skeletal muscle. Generally, NCX1 mediates Ca\(^{2+}\) efflux in nonpolarized cells as well as in polarized epithelia. Ncx1 mRNA is down-regulated in vitamin D deficiency (in 1α-OHase\(^{-/-}\) mice) and corrected by 1,25(OH)\(_2\)D\(_3\) supplementation (13). Huybers et al. (14) showed that experimental Crohn’s-like ileitis developed spontaneously by TNF\(_{\text{ARE}}\) mice is associated with reduced expression of renal Ncx1, although the cytokine(s) involved and mechanisms responsible for this regulation have not been described. This decrease was accompanied by decreased serum 1,25(OH)\(_2\)D\(_3\) levels and reduced trabecular and cortical bone thickness (14).

Characterization of transcriptional regulation of Ncx1 gene expression is complicated due to multiple-tissue-specific variants of Ncx1 resulting from alternative promoter usage (H1, K1, and Br1) and alternative splicing, which also differ in their ionic and kinase-dependent regulation (15–18). The mouse kidney-specific promoter has not been characterized previously. In this study, we map the mouse kidney-specific promoter and the related transcription start site. We also show that renal Ncx1 mRNA expression is reduced in two models of experimental colitis. We further show that IFN\(\gamma\) down-regulates Ncx1 expression in vivo and in vitro via a STAT1-dependent mechanism. Collectively, our data provide another layer of complexity which explains the mechanism of defective renal Ca\(^{2+}\) reabsorption in IBD, and its potential contribution to systemic Ca\(^{2+}\) imbalance and the associated loss of bone mineral density.

**EXPERIMENTAL PROCEDURES**

**Experimental Colitis—**TNBS colitis was induced in mice (129S1 background) by intracolonic single dose administration of trinitrobenzene sulfonic acid (TNBS, Sigma Aldrich) (2.5 mg/mouse dissolved in 50% ethanol). Control mice received 50% ethanol in PBS enema. All mice were sacrificed 5 days later. DSS colitis (129S1 background) was induced by 3% (w/v) dextran sodium sulfate (DSS; molecular weight 40 to 50 kDa; Affymetrix) added to the drinking water for 7 days, after which the mice were sacrificed and the tissues collected for analysis. Both TNBS and DSS are widely used chemically induced models of intestinal inflammation (19). In both models of colitis, the body weight changes were monitored daily after the induction of colitis. The mice were sacrificed when they lost 15–20% of their initial body weight. Colonic inflammation was assessed based on histological changes as well as levels of proinflammatory cytokines. The animal use protocol was reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee. Tissue samples including kidneys and colons were harvested for histology (H&E staining), as well as protein and RNA isolation. Since the distal convoluted tubules (DCT) and the connecting tubules (CNT) play a major role in Ca\(^{2+}\) reabsorption, we used P\(_{\text{TRPV5}}\)-EGFP transgenic mice (C57Bl/6), which express eGFP under the control of a 3.6-kb fragment of the mouse TRPV5 promoter in the epithelium of the late DCT, CNT, and the initial part of the cortical collecting duct, for the NCX1 functional assay. These mice were generously provided by Drs Jeppe Praetorius and Marlene Vind Hofmeister (Aarhus University, Denmark) (20). STAT1\(^{-/-}\) (129S6/SvEv) mice were purchased from Taconic Farms (Hudson, NY). All qPCR reactions were performed using pre-designed Taqman assay primer/probe sets (Life Technologies, CA), and ran on a Bio-Rad CFX96 real-time PCR detection system. Cycling parameters were determined and resulting data were analyzed by using the comparative Ct method as a means of relative quantification, normalized to an endogenous reference (TATA box-binding protein; TBP) and relative to a calibrator (normalized Ct value obtained from control mice) and expressed as 2\(^{-\Delta\Delta\text{Ct}}\) (Applied Biosystems User Bulletin #2: Rev B “Relative Quantification of Gene Expression”). Serum and tissue levels of mouse interferon \(\gamma\) (mIFN\(\gamma\)) were analyzed using an ELISA kit (eBioscience). The assay was performed according to manufacturer’s instructions.

**\(^{45}\text{Ca}^{2+}\) Uptake Assay—**The P\(_{\text{TRPV5}}\)-EGFP transgenic mice were injected with PBS or recombinant mIFN\(\gamma\) (50,000 units/mouse) intraperitoneal for 3 days. On the fourth day, the kidneys were collected, minced, and digested with RPMI 1640 medium containing 0.1% collagenase and 5% FBS. Tubule cells were stained with a nylon mesh and washed two times with RPMI 1640 medium containing 10% FBS plus penicillin and streptomycin. GFP\(^+\) cells were sorted using FACaria cell sorter and were used for the NCX1 activity as described earlier (21). Briefly, the cells were washed twice with washing buffer (10 mM MOPS (pH 7.4), 140 mM NaCl). Cells were then loaded with Na\(^+\) by incubation with 10 mM MOPS (pH 7.4), 140 mM NaCl, 1 mM MgCl\(_2\), in the presence of 0.4 mM ouabain, and 25 mM nystatin for 10 min at room temperature. The nystatin was removed from the cells by two washes with wash buffer containing 0.4 mM ouabain. Uptake was initiated by resuspending the cell pellet in assay buffer containing 10 mM MOPS (pH 7.4), 140 mM KCl (or NaCl as blank), 25 mM Ca\(_{\text{L}}\), 0.4 mM ouabain, and 5 \(\mu\)Ci/ml \(^{45}\text{Ca}^{2+}\). After 10 min of incubation, the reaction was terminated by adding 1 ml of ice-cold stop buffer (140 mM KCl and 1 mM EGTA). Cells were washed twice with stop buffer, and cell pellets were then lysed by freeze/thaw in 1N NaOH at 80 °C for 20 min. Aliquots were subjected to scintillation counting and protein assay.

**Fractional Urinary Ca\(^{2+}\) Excretion—**Plasma (P) and urine (U) creatinine (Cr) and Ca\(^{2+}\) were analyzed by commercial assays (Abcam, Cambridge, MA; and BioVision, Milpitas, CA, respectively) from the samples collected at the time of sacrifice. Fractional excretion of Ca\(^{2+}\) (FECa\(^{2+}\)) was calculated as described earlier (6).

**Western Blotting—**Tissue samples or mIMCD3 cells were homogenized in RIPA buffer containing protease and phosphatase inhibitor cocktails and sonicated to shear DNA. The cleared lysates were separated by SDS-polyacrylamide gel electrophoresis (TGX gels, Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with Tris-buffered saline (TBS) containing 0.05% Tween-20 (Sigma-
IFNγ Inhibits Renal NCX1 during Colitis

Aldrich) and 5% w/v dry milk powder (Bio-Rad). The was followed by incubation with one of the following antibodies: NCX1 rabbit polyclonal (sc-30304, Santa Cruz Biotechnology), anti-STAT1 rabbit polyclonal (sc-346, Santa Cruz), anti-GFP mouse monoclonal (sc-9996, Santa Cruz Biotechnology), anti-pTyr201-STAT1 rabbit polyclonal (9167, Cell Signaling) or anti-GAPDH mouse monoclonal antibody (MA5-15738, Pierce) in 2.5% blocking buffer overnight at 4 °C. They were then washed with TBS containing 0.05% Tween-20, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling) at room temperature for 1 h and developed using Super Signal West Pico HRP substrate (Pierce).

Immunofluorescence—Kidneys were harvested and fixed in 10% neutral buffered formalin (Fisher Scientific). Fixed tissues were embedded in paraffin, and cut into 5-μm-thick tissue sections. After deparaffinization and rehydration, antigen retrieval was performed by heating the slides in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). After washing in phosphate-buffered saline (PBS), the slides were blocked for 1 h in 10% normal chicken serum in PBS containing Tween 20 (0.1%), (PBST). Next, sections were incubated with primary antibodies directed against NCX1 (AB3516P, 1:100 dilution, rabbit polyclonal, Millipore) and NCCT (sc-21554, 1:50 dilution, goat polyclonal, Santa Cruz Biotechnology) in PBST containing 3% BSA, overnight at 4 °C. After three washes in PBST, the slides were incubated with the chicken-anti-goat and anti-rabbit antibodies conjugated with Alexa Fluor (6 μg/ml in PBST, Invitrogen). After three washes with PBST, slides were mounted with a drop of mounting medium with DAPI (Molecular Probes, Life Technologies). The slides were visualized under the microscope (EVOS FL Auto, Life Technologies).

Identification of Mouse Renal-specific Ncx1 Gene Promoter—The RNA ligase-dependent rapid amplification of cDNA ends (5′-RLM-RACE) kit (FirstChoice RLM-RACE, Invitrogen) was employed to identify the mouse renal Ncx1 transcriptional start site and the gene promoter region. 10 μg of total RNA isolated from mouse kidneys was treated with calf alkaline phosphatase followed by tobacco pyrophosphatase, reverse transcribed using a gene-specific primer (5′-AAAGATGGTCTTGGGGTTC-3′), and subsequently tailed with terminal deoxynucleotidyl transferase using a 5′-CAAG-3′ adapter. The products were run on 1.5% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen). The primers amplifying the promoter region were: −379 forward 5′-GGGAGACGAGAGACAGTGG- TC-3′, −379 forward 5′-GGGTACCCCGAGGAAGTAGTGG- AGTTGCCTTCAAGATCT-3′, −110 forward 5′-GGGTACCAAAATTCCACTGCATTG-3′ and reverse primer 5′-GCCTCGAG- TGCTCTCCAGATCTGATG-3′. All amplicons were first cloned into the pGEM-T cloning vector (Promega), then restriction-digested with KpnI and Xhol and ligated into the corresponding site within the multiple cloning sites in the pGL3-basic vector (Promega). Both pGEM-T and pGL3 plasmids with inserts were fully sequenced and verified. The transfections of mIMCD3 cells with the reporter constructs were carried out in 24-well plates using the TransIT-T1 (Mirus) transfection agent and a luciferase assay was performed with a luciferase assay kit (Promega), and a tube luminometer (FB12, Zylux).

Analysis of the mouse kidney specific promoter sequence revealed the existence of a single potential binding site for STAT1 at position −385 to −397 relative to the TSS. Therefore, we constructed plasmid with a mutant STAT response element by site-directed mutagenesis. The mutant sequence for STAT1 at position −397 relative to the TSS is changed from 5′-TTCTGGGAA-3′ to 5′-CCTTGGGAA-3′. The mutant PCR products were amplified by using two different sets of primers, Primer set I (forward, 5′-GGGTACCCCGAGGAAGTAGTGGTC-CTTC-3′; reverse, 5′-ATTTCCAGGGTTCTTCTGCGCC-3′) and primer set II (forward, 5′-CTTGGGAGGAGGACCCTCGGAAATTT-3′; reverse, 5′-GCTCTAGACGCCCTCAGATCTGATG-3′). The two amplified products were mixed and amplified with forward primer of set I and reverse primer of set II. The final product was ligated to pGEM-T vector and sequenced. Subsequently, the insert was digested with KpnI/Xhol and ligated to pGL3-basic vector. Reporter assays were performed as described above.

Chromatin Immunoprecipitation (ChIP)—The chromatin immunoprecipitation assay was performed using the SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads, Cell Signaling Technology) according to the manufacturer’s protocol. Chromatin from IFNγ-treated and untreated mIMCD3 cells were immunoprecipitated with normal rabbit IgG (negative control) or anti-STAT1 antibodies (sc-346X, rabbit polyclonal, Santa Cruz Biotechnology). Stat1 binding to Ncx1 promoter DNA was quantified by real-time PCR using primers (forward, 5′-CTCACCCTGTCAGGCTCATGT-3′; reverse, 5′-ACTCGTCTATGGCTGTCTCT-3′) designed to amplify a 233-bp region spanning the putative STAT1 cis-element of the Ncx1 promoter using SYBR green. Chromatin incubated with beads alone or bead plus normal rabbit IgG was used to control for nonspecific binding. The products were run on 1.5% agarose gel to confirm the specificity of PCR amplification.

RESULTS

Assessment of Colitis—Both TNBS and DSS administered mice experienced progressive weight loss and the colon showed typical gross signs of inflammation, i.e. increased wet weight, increased weight/length ratio, and colonic wall thickening (data not shown). Histological examination of the colon, revealed hyperplasia, loss of crypts, focal ulcerations, and loss of epithelial cells, those were consistent with other studies with these
models from our laboratory (6, 22–25). Colonic expression of proinflammatory cytokines, IFNγ, TNF, and IL-1β mRNA were elevated in both models of experimental colitis. Significant elevation of IL-17 expression was documented in DSS- but not in TNBS-treated mice (Fig. 1). Serum level of IFNγ was elevated in both models of experimental colitis. Significant elevation of IFNγ by the observation that even after injection of recombinant IFNγ into mice, as demonstrated by Western blotting. The effect of mIFNγ on NCX1 expression was also reproduced at the protein level in a time-dependent fashion (Fig. 4B). The mIFNγ signaling activation was abundant as evidenced by the increased levels of pTyr701-STAT1 expression both in vitro and in vivo (Fig. 4, B and D). The GFP+ renal distal tubule epithelial cells from the mIFNγ-injected PTNPS-EGFP transgenic mice were flow

Previous immunohistochemistry studies indicated that NCX1 expression in the epithelial cells is not limited to the basolateral membrane, but is also abundant in other intracellular compartments (28–30). We confirmed these observations and showed that consistent with NCX1 mRNA and protein expression, there was a dramatic decrease in NCX1-specific immunofluorescence in the DCT of colitic mice, as compared with healthy controls (Fig. 2C). IFNγ Down-regulates NCX1 Expression and Activity in Vitro and in Vivo—As IFNγ and other proinflammatory cytokines are typically up-regulated in colitis, including our experimental models, we tested their effects on NCX1 expression in cultured murine IMCD3 cells in vitro. This cell line expresses NCx1 mRNA and protein at levels easily detectable by both qPCR and Western blotting. We tested the effects of IFNγ, TNF, and IL1β individually (Fig. 3A) and in combination (data not shown) on NCx1 mRNA expression and showed that of the three major cytokines, IFNγ (100 units/ml) most profoundly reduced NCx1 transcript (Fig. 3A). Similar cytokine effects were observed on the cloned mouse renal Ncx1 gene promoter (Fig. 3B). Use of cytovoxin (combination of IFNγ, TNF, and IL1β) had a similar effect to IFNγ used individually (data not shown). Time course analysis of the effects of mIFNγ in mIMCD3 cells showed a significant and progressive decrease in the Ncx1 mRNA level by qPCR starting from 12 h (Fig. 4A). The effect of mIFNγ on NCX1 expression was also reproduced at the protein level in a time-dependent fashion (Fig. 4B). Similarly, mIFNγ injected PTNPS-EGFP transgenic mice showed significantly decreased expression of NCX1 both at the level of mRNA and protein, in the renal lysates (Fig. 4, C and D). The GFP+ renal distal tubule epithelial cells from the mIFNγ-injected PTNPS-EGFP transgenic mice were flow
sorted and used for the NCX1 functional Ca\(^{2+}\) uptake assay. Consistent with decreased Ncx1 mRNA and protein expression, the GFP\(^+\) cells from mIFN\(\gamma\)-injected mice showed a nearly 70% reduction in NCX1-mediated Na\(^+\)-dependent Ca\(^{2+}\) uptake (Fig. 4E). Furthermore, compared with PBS-injected controls, exogenous IFN\(\gamma\) significantly reduced renal Ca\(^{2+}\) reabsorption in vivo, as indicated by increased fractional urinary Ca\(^{2+}\) excretion (FECa\(^{2+}\)) (Fig. 4F).

Mapping of the Renal Ncx1 Transcription Start Site—Originally characterized and cloned as a cardiac Na\(^+\)/Ca\(^{2+}\) exchanger, NCX1 is also highly expressed in the brain and kidney, and at lower levels in almost all other tissues. The presence of different non-coding initiating exons is a consequence of three independent promoters selectively driving and regulating expression of Ncx1 in different tissues: one specific to heart, one to kidney, and one for other tissues. The Ncx1 gene transcript in mammals has been shown to undergo alternative splicing of six exons: A, B, C, D, E, and F (31). Exons A and B were shown to be mutually exclusive and the other four exons were cassette-type exons. Renal tissues specifically express exons B, C, D, E, F (31). The mouse renal Ncx1 alternative promoter and transcription start sites (TSSs) were evaluated by 5’ Rapid Amplification of cDNA Ends (RACE) using total RNA from mouse kidneys. After reverse transcription using primers provided with the RACE-PCR kit, the initial PCR reaction was performed using a reverse gene specific primer complementary to a sequence located in Exon C and the forward upstream primer provided by the kit. The products of this reaction were then subjected to nested PCR and yielded a 600-bp product representative of the predominant transcript present in the renal tissue (Fig. 5A). Following sequencing of this product, our findings were largely consistent with other species (rabbit, rat, and cat).
IFN\(\gamma\) Inhibits Renal NCX1 during Colitis

In response to mIFN\(\gamma\), −269 nt and −379 nt constructs did not show any change in the promoter activity, whereas −703 nt promoter was significantly inhibited (Fig. 5B), thus suggesting that an IFN\(\gamma\)-dependent repressive cis-element was positioned between −380 and −703 nt relative to the TSS. Interestingly, alignment of the previously characterized rat renal NCx1 gene promoter showed high homology to mouse promoter and showed similar response to IFN\(\gamma\) in transiently transfected mIMCD3 cells (data not shown). Further analysis of the −380/−703 nt promoter region showed a putative STAT1 response element present at position −387/−397 nt (TTCCTGGGAA), which bears a strong similarity to the γ interferon-activated site (GAS, 5′-TTCN(2–4)GAA-3′) known to bind STAT1 homodimers in response to IFN\(\gamma\) stimulation (33). This site was conserved in the rat renal NCx1 gene promoter. To further identify the requirement for this sequence, we eliminated this putative GAS element via site-directed mutagenesis. Contrary to the wild-type −703 nt promoter, the same construct with mutated GAS showed no decrease in response in transiently transfected and mIFN\(\gamma\)-treated mIMCD3 cells (Fig. 5C), thus demonstrating the requirement of an intact GAS site at −387/−397 nt relative to the TSS in IFN\(\gamma\)-driven inhibition of the mouse renal NCx1 promoter activity.

STAT1 Binds Directly to the NCx1 Promoter—To confirm IFN\(\gamma\)-inducible recruitment of STAT1 to the mouse NCx1 promoter in vivo (in mIMCD3 cells), we performed a quantitative ChIP assay with primers designed to span the promoter region of interest and amplify 233 bp (−279 to −511 nt). To visualize the specificity of amplification, Fig. 5D (left panel) depicts a representative DNA gel with 233-bp amplicon from the no-antibody controls (negligible background), anti-STAT1 ChIP, and input (2%) controls. The visibly increased recruitment of STAT1 to the NCx1 gene promoter was further quantified using SYBRGreen qPCR and showed at least 10-fold increase in STAT1/NCx1 promoter association in mIFN\(\gamma\)-treated mIMCD3 cells (Fig. 5D, right panel).

STAT1 Is Required for Down-regulation of NCx1 by IFN\(\gamma\) in Vivo—Signaling through STAT1 transcription factor is critical and specific for the biological functions of IFN\(\gamma\) (34). To verify our promoter construct and ChIP data in mice, and to further implicate STAT1-mediated inhibition of NCx1 gene expression by IFN\(\gamma\), we utilized Stat1\(−/−\) mice. Wild-type (WT) and Stat1\(1−/−\) mice on the same genetic background (129S6/SvEv-Tim1Rds>) were injected with PBS or recombinant mIFN\(\gamma\) (50,000 units/mouse) intraperitoneal daily for 3 days. On the fourth day, kidneys were collected and analyzed for pTyr701-STAT1, total STAT1, and NCX1 protein and mRNA expression. Consistent with the proposed key role of STAT1 in IFN\(\gamma\)-mediated inhibition of NCx1 gene transcription, no change in renal NCx1 mRNA and protein expression was observed in IFN\(\gamma\)-injected Stat1\(−/−\) mice (Fig. 6A). In WT mice, exogenous IFN\(\gamma\) strongly stimulated STAT1 protein expression in the kidney (Fig. 6B), suggesting that a sensitization mechanism, previously reported in macrophages (35), also exists in the kidneys. This was accompanied by strong expression of pTyr701-STAT1 and, as anticipated, a significant decrease in NCX1 protein expression (Fig. 6B).

FIGURE 3. A, effects of inflammatory cytokines on gene expression of NCx1 in mIMCD3 cells. Cells were treated with individual cytokines (100 units/ml of IFN\(\gamma\), 10 ng/ml of TNFa, and 2 ng/ml of IL1β) for 24 h, and the impact on the NCx1 mRNA expression was examined by real-time RT-PCR. NCx1 expression was normalized to that of TATA-box binding protein (TBP) relative to a calibrator (normalized Ct value obtained from untreated cells) and expressed as 2\(^{−ΔΔct}\) (*, p < 0.05 IFN\(\gamma\) versus control; ANOVA followed by Fisher test; n = 3). B, effects of individual cytokines on the NCx1 promoter activity in mIMCD3 cells transiently transfected with pGL3-703 NCx1 promoter construct. 24 h post-transfection, cells were treated for 24 h with indicated cytokines. Luciferase activity (RLU) was normalized to mg protein and expressed relative to the activity of the pGL3-703 construct in PBS-treated cells (mean ± S.E., *, p < 0.05 versus PBS, ANOVA followed by Fisher test; n = 3).

(17, 31, 32), and showed that mouse kidney NCx1 transcript variant starts with exon B (variant 1.3). 5′-RACE analysis also showed a splicing region in between exon B and exon C, which is separated by a 21.5 kb intronic region. The ATG translation start site in exon C was identified 29-bp downstream of the 5′ splicing site.

IFN\(\gamma\) Inhibits Renal NCx1 Promoter Activity—Based on the available public databases and analysis of 5′-RACE products, we had identified the TSS of NCx1 at position −375 nt relative to the start codon. Therefore, we cloned genomic fragments extending from positions −110, −269, −379, −703 to +1 nt relative to TSS into a pGL3-basic luciferase reporter vector, and we examined transcriptional activity following transient transfection into mIMCD3 cells. As shown in Fig. 5B, the transfected −269, −379, −703 nt NCx1 promoter constructs demonstrated basal activity more than 15-fold greater than baseline (promotereless pGL3 basic); the −110 nt promoter was insufficient to drive luciferase expression in mIMCD3 cells. Similar to the endogenous NCx1 gene in mIMCD3 cells, mIFN\(\gamma\) was most potent among the three tested cytokines in inhibiting the activity of the longest cloned promoter fragment (−703 nt; Fig. 5B).
**DISCUSSION**

IBD patients are at higher risk of developing metabolic bone disease and low bone mineral density (BMD) than healthy subjects. Patients with inflammatory bowel disease have a more than 40% greater incidence rate of bone fracture than that of the general population (36–38). The prevalence of osteopenia and osteoporosis in IBD ranges from 22 to 77% and from 17 to 41%, respectively (37, 39, 40). The etiology of low bone mass in IBD patients is likely multifactorial, including corticosteroid use, decreased physical activity and muscle strength, poor nutritional status, and malabsorption (particularly affecting vitamin D, vitamin K, and Ca²⁺ homeostasis), as well as inflammation-associated inflammatory soluble mediators. The latter, such as IL-1β, IL-6, TNF, affect the bone directly to alter the modeling and remodeling processes (3).

Bone is highly dynamic and it undergoes constant remodeling throughout life. The remodeling involves coupled resorption of existing bone and the formation of new bone. The supply of calcium and inorganic phosphate available for bone formation is a result of highly regulated and complex homeostatic mechanism involving paracellular and transcellular intestinal and renal (re)absorption. In the intestine, diminished Ca²⁺ supply is believed to be the result of vitamin D₃ insufficiency, avoidance of milk and dairy food consumption and relatively common lactose intolerance in IBD patients (41), and the direct effect of inflammatory cytokines on the expression of the key components of transcellular Ca²⁺ absorption, TRPV6, calbindin D₉K, and calcium pump PMCA1b (14). The latter report also described decreased expression of renal calbindin-D₂₈K and NCX1, although the consequences or mechanisms of this phenomenon were not described.

Our recent work has demonstrated that, consistent with the clinical data (5), experimental murine colitis is also associated with increased urinary fractional Ca²⁺ excretion and decreased bone mineral density (6). We have postulated that IBD-associated TNF and IFNγ lead to transcriptional inhibition of renal

---

**FIGURE 4. A–F, effects of IFNγ on cellular levels of Ncx1 mRNA and protein.** A. Ncx1 mRNA expression as determined by real-time RT-PCR in mIMCD3 cells treated with PBS or 100 units/ml of mIFNγ for 0, 12, 24, 48, and 72 h (mean ± S.D. of three independent experiments). Ncx1 expression was normalized to that of TATA-box binding protein (TBP) relative to a calibrator (normalized Ct value obtained from untreated healthy mice) and expressed as 2⁻¹⁻⁰⁻⁰⁻¹ (*, p < 0.05 treated versus control, Student’s t test). B. Representative Western blot showing NCX1, pTyr⁷⁰⁷ STAT1 and GAPDH (as loading control) in mIFNγ-treated mIMCD3 cells. C. Ncx1 mRNA expression of in the kidneys of mIFNγ injected PTRPV5-EGFP transgenic mice was quantified by real-time RT-PCR as described for panel A. D. Representative Western blot analysis of NCX1, pTyr⁷⁰⁷ STAT1, GFP, and GAPDH proteins in renal lysates of PBS- and mIFNγ-injected PTRPV5-EGFP transgenic mice. E. NCX1-mediated Ca²⁺ uptake in GFP⁺ epithelial cells of the renal DCT isolated from mIFNγ-injected PTRPV5-EGFP transgenic mice. PBS or mIFNγ were injected intraperitoneal daily for 3 days in PTRPV5-EGFP transgenic mice, which express GFP expression under the control of the TRPV5 promoter (C57Bl/6 background). GFP⁺ cells were flow-sorted using FACAria and used for the NCX1 activity assay as described under “Experimental Procedures.” Data from three independent experiments are presented as Na⁺-dependent Ca²⁺ uptake relative to PBS-injected mice (*, p < 0.05, Student’s t test). F. Fractional urinary Ca²⁺ excretion (FECa²⁺) in IFNγ-injected mice. Mean ± S.D., *, p < 0.05; Student’s t test.
FIGURE 5. A, identification of the transcription initiation site (+1) of the mouse renal Ncx1. Representative DNA gel of the 5'-RLM-RACE product with mouse kidney RNA. −TAP refers to negative control lacking Tobacco Acid Pyrophosphatase (TAP) used for removing the capping nucleoside. B, luciferase reporter gene activity with mouse kidney specific Ncx1 promoter constructs: pGL3-Basic, pGL3–110, pGL3–269, pGL3–379, and pGL3–703 (promoter length relative to the identified transcription start site) were transiently transfected into mIMCD3 cells treated with PBS or 100 units/ml mIFNγ for 24 h. Luciferase activity (RLU) was normalized to mg protein and expressed relative to the activity of the promoterless PGL3-basic (pGL3) vector in PBS-treated cells (mean ± S.E., *, p < 0.05 PBS versus IFNγ, Student’s t test; n = 3). C, effects of IFNγ on the pGL-703 WT promoter, or construct with mutated core GAS element. Data calculated as above and normalized to the activity of pGL-703 WT promoter in PBS-treated cells (mean ± S.E., *, p < 0.05 PBS versus IFNγ, Student’s t test; n = 3). D, inducible STAT1 recruitment to Ncx1 gene promoter in IFNγ-treated mIMCD3 cells. ChIP assay was performed using a control rabbit IgG or an anti-STAT1 antibody as described under “Experimental Procedures.” Representative DNA gel and the summary of real-time PCR analysis are depicted in left and right panels, respectively. −Ab indicates control IgG, +Ab indicates anti-STAT1 antibody. Bar graph (mean ± S.E.) indicates an over 10-fold difference in STAT1 recruitment (*, p < 0.05 PBS versus IFNγ, Student’s t test; n = 3).
expression of Klotho (10), which leads to hyper-sialylation of the apical TRPV5 channel, its endocytosis, UBR4-mediated ubiquitination, and primarily proteasomal degradation (6), thus providing a compelling and comprehensive mechanism of impaired apical renal Ca\textsuperscript{2+}/H\textsuperscript{+} transport in IBD. Decreased expression of NCX1, a Na\textsuperscript{+}/Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger responsible for the basolateral Ca\textsuperscript{2+}/H\textsuperscript{+} extrusion in the renal DCT, as reported by Huybers et al. (14) in TNF\textsuperscript{ARE} mice, would add another mechanism further contributing to the diminished transcellular Ca\textsuperscript{2+} reabsorption in the kidney during IBD. Such a decrease would likely be independent of potentially altered vitamin D\textsubscript{3} metabolism, as renal Ncx1 gene expression is not affected by dietary Ca\textsuperscript{2+} or vitamin D\textsubscript{3} supply (42, 43).

TNF\textsuperscript{ARE} mice are considered a valuable model of Crohn’s-like ileitis. They bear an endogenous 69 bp deletion of the 3’-AU-rich region in the gene encoding TNF and, as a result, have high circulating levels of TNF protein. However, the development of intestinal inflammation in this model depends on Th1-type cytokines, such as IL-12 and IFN\textgamma\textsuperscript{2}, and TNF\textsuperscript{ARE} mice on IFN\textgamma\textsuperscript{-/-} background develop attenuated inflammation (44). Utilizing two alternative models of chemically induced colitis associated with significantly increased colonic IFN\textgamma\textsuperscript{2} production, we showed a similar decrease in renal expression of Ncx1 mRNA and protein. While in vivo neutralization of IFN\textgamma\textsuperscript{2} could not be used due to the anticipated confounding effect of reduced colitis, our in vitro studies with mIMCD3 cells showed that IFN\textgamma\textsuperscript{2} was more potent than TNF or IL-1\textbeta and sufficient to reduce the Ncx1 expression to the same extent as cytomix (all three cytokines combined). IFN\textgamma\textsuperscript{2} is abundantly produced by cultured colonic mucosal tissue and intestinal lamina propria mononuclear cells obtained from IBD patients (45, 46). Even in remission, CD8\textsuperscript{+} lymphocytes from IBD patients show an increased capacity for IFN\textgamma\textsuperscript{2} induction (47). However, circulating levels of IFN\textgamma\textsuperscript{2} are not associated with the disease activity or serum C-reactive protein levels and cannot be reliably measured due to a very short half-life of the cytokine, its rapid binding to heparan sulfate, and its quick degradation (26). In our studies, experimental colitis was associated only with a minor trend toward increased serum or renal tissue IFN\textgamma\textsuperscript{2} concentrations.

![Figure 6](https://example.com/figure6.png)

**FIGURE 6.** STAT1 is required to down-regulate the expression of NCX1 by IFN\textgamma\textsuperscript{2}. WT and Stat1\textsuperscript{-/-} mice (129S6/SvEv background) were injected with PBS or mIFN\textgamma through i.p for 3 days and kidneys were collected for RNA and protein extraction. A, real-time RT-PCR analysis. Ncx1 expression was normalized to that of TBP relative to a calibrator (normalized Ct value obtained from untreated WT or Stat1\textsuperscript{-/-} mice) and expressed as 2\textsuperscript{-ΔΔCt}; p < 0.05 treated versus control, Student’s t test; n = 5). B, representative Western blot analysis of NCX1, pTyr\textsuperscript{701}-STAT1, and GAPDH (loading control) in renal lysates of PBS and mIFN\textgamma injected WT and Stat1\textsuperscript{-/-} mice.
Moreover, in mice repeatedly injected with the recombinant cytokine, we were also unable to show an increased level of serum IFNγ. Despite this, we saw a dramatic increase in renal Stat1 and pTyr701-STAT1 expression in the kidney (Fig. 6b), thus confirming that circulating IFNγ reaches and exerts its signaling effects in the kidney. Most importantly, colitis or recombinant IFNγ injection resulted in decreased expression of NCX1 in the renal distal convoluted tubules, as demonstrated by Western blotting, qPCR, and immunofluorescence. We also confirmed decreased NCX1-mediated Na+-dependent Ca2+-uptake in DCT epithelial cells isolated from healthy or colitic mice.

To address the molecular mechanism of transcriptional repression of Ncx1 by IFNγ in mice, we characterized the primary mouse renal transcript of Ncx1 as Ncx1.3, identified the transcription start site, cloned the kidney-specific mouse Ncx1 gene promoter, and identified the GAS element in the proximal promoter. The effects of IFNγ on Ncx1 promoter activity in vitro and gene transcription in vivo were dependent on the presence and inducible recruitment of Stat1 to the GAS element at position −387/−397 nt, a site also identified in silico in the previously characterized rat renal Ncx1 gene promoter. A human renal-specific NCX1 promoter has not been characterized at this time. Since IFNγ can activate additional signaling pathways and can regulate gene expression by STAT1-independent pathways (48), the importance of STAT1 in mediating the inhibitory effects of IFNγ was further demonstrated in vivo, whereby IFNγ did not affect renal Ncx1 expression in Stat1−/− mice.

The most frequent biological effect of the stimulation of the IFNγ/STAT1 pathway is transcriptional activation of a vast network of IFNγ-inducible genes, while transcriptional repression is a much less common phenomenon. Interestingly, and most likely related to the alternative promoter use and the transcriptional context, IFNγ may repress, as in the case of renal Ncx1 gene, or activate the expression of Ncx1, as described for the brain-specific promoter in microglia by Nagano et al. (49). These discrepancies will have to be addressed mechanistically in the future, ideally in the context of human NCX1 gene regulation.

In conclusion, our findings showed that renal NCX1 expression and function is down-regulated over the course of experimental colitis, and pointed to a dominant role for IFNγ, which inhibits Ncx1 gene transcription via a STAT1-dependent mechanism. Since NCX1 is one of the key transporters responsible for renal Ca2+ reabsorption and systemic Ca2+ homeostasis, our observations provide a new mechanism contributing to the reduced renal Ca2+ reabsorption in IBD, which together with reduced expression of KLOTHO (10) and TRPV5 (6), as well as the postulated reduced intestinal Ca2+ absorption (14), likely contributes to a negative systemic Ca2+ balance and increased bone resorption in IBD patients.

Acknowledgments—We thank Douglas W. Cromey (for assistance with confocal imaging), the Arizona Cancer Center/Arizona Research Laboratories (AZCC/ARL)-Division of Biotechnology Cytometry Core Facility, and the Cancer Center support grant (CCSG-CA 023074). We acknowledge the support of the University of Arizona Cytometry Core Facility and Paula Campbell for help with cell sorting and the University of Arizona Genetics Core for DNA sequencing support.

REFERENCES

1. Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2005) Calcium absorption across epithelia. Physiol. Rev. 85, 573–622
2. Brown, A. J., Dusso, A., and Slatopolsky, E. (1999) Vitamin D. Am. J. Physiol. 277, F157–F175
3. Ghishan, F. K., and Kiela, P. R. (2011) Advances in the understanding of mineral and bone metabolism in inflammatory bowel disease. Am. J. Physiol. Gastrointestinal and Liver Physiology 300, G191–G201
4. Tilg, H., Moschen, A. R., Kaser, A., Pines, A., and Dotan, I. (2008) Gut, inflammation and osteoporosis basic and clinical concepts. Gut 57, 684–694
5. Breuer, R. L., Gelzayd, E. A., and Kirnser, I. K. (1970) Urinary crystallloid excretion in patients with inflammatory bowel disease. Gut 11, 314–318
6. Radhakrishnan, V. M., Ramalingam, R., Larmonier, C. B., Thurston, R. D., Laubitz, D., Midura-Kiela, M. T., McFadden, R. M., Kuro-O, M., Kiela, P. R., and Ghishan, F. K. (2013) Post-translational loss of renal TRPV5 calcium channel expression, Ca(2++) wasting, and bone loss in experimental colitis. Gastroenterology 145, 613–624
7. Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2002) Molecular mechanism of active Ca2+ reabsorption in the distal nephron. Annu. Rev. Physiol. 64, 529–549
8. Hoenderop, J. G., van der Kemp, A. W., Hartog, A., van Os, C. H., Willems, P. H., and Bindels, R. J. (1999) The epithelial calcium channel, ECaC, is activated by hyperpolarization and regulated by cytosolic calcium. Biochem. Biophys. Res. Commun. 261, 488–492
9. Schwaller, B. (2010) Cytosolic Ca2+ buffers. Cold Spring Harbor Perspectives in Biology 2, a004051
10. Thurston, R. D., Larmonier, C. B., Majewski, P. M., Ramalingam, R., Midura-Kiela, M., Laubitz, D., Vandewalle, A., Besselens, D. G., Mühlbauer, M., Jobin, C., Kiela, P. R., and Ghishan, F. K. (2010) Tumor necrosis factor and interferon-γ down-regulate Klotho in mice with colitis. Gastroenterology 138, 1384–1394, 1394 e1381–e1382
11. Van Baal, J., Yu, A., Hartog, A., Fransen, J. A., Willems, P. H., Lytton, J., and Bindels, R. J. (1996) Localization and regulation by vitamin D of calcium transport proteins in rabbit cortical collecting system. Am. J. Physiol. 271, F985–F993
12. Peng, J. B., Shin, X. Z., Berger, U. V., Vassilev, P. M., Brown, E. M., and Hediger, M. A. (2000) A rat kidney-specific calcium transporter in the distal nephron. J. Biol. Chem. 275, 28186–28194
13. Hoenderop, J. G., Dardenne, O., Van Abel, M., Van der Kemp, A. W., Van Os, C. H., St-Arnaud, R., and Bindels, R. J. (2002) Modulation of renal Ca2+ transport protein genes by dietary Ca2+ and 1,25-dihydroxyvitamin D3 in 25-dihydroxyvitamin D3–1α-hydroxylase knockout mice. FASEB J. 16, 1398–1406
14. Huvers, S., Apostolaki, M., van der Eerden, B. C., Kollias, G., Naber, T. H., Bindels, R. J., and Hoenderop, J. G. (2008) Murine TNF(DeltaARE) Crohn’s disease model displays diminished expression of intestinal Ca2+ transporters. Inflammatory Bowel Diseases 14, 803–811
15. Müller, J. G., Isomatsu, K., Koushik, S. V., O’Quinn, M., Xu, L., Kappler, C. S., Hapke, E., Zile, M. R., Conway, S. J., and Menick, D. R. (2002) Cardiac-specific expression and hypertrophic upregulation of the feline Na+(Ca2+)+exchanger gene H1-promoter in a transgenic mouse model. Circulation Research 90, 158–164
16. Quednau, B. D., Nicoll, D. A., and Philipson, K. D. (1997) Tissue specificity and alternative splicing of the Na+(Ca2+)+exchanger isoforms NCX1, NCX2, and NCX3 in rat. Am. J. Physiol. 272, C1250–C1261
17. Barnes, K. V., Cheng, G., Dawson, M. M., and Menick, D. R. (1997) Cloning of cardiac, kidney, and brain promoters of the feline ncxl1 gene. J. Biol. Chem. 272, 11510–11517
18. Xu, L., Renaud, L., Müller, J. G., Baioc, C. F., Bonnema, D. D., Zhou, H., Kappler, C. S., Kubalak, S. W., Zile, M. R., Conway, S. J., and Menick, D. R. (2006) Regulation of Ncx1 expression. Identification of regulatory elements mediating cardiac-specific expression and up-regulation. J. Biol. Chem. 281, 34430–34440
19. Wirtz, S., Neufert, C., Weignann, B., and Neurath, M. F. (2007) Chemically induced mouse models of intestinal inflammation. Nature Protocols 2, 541–546
IFNγ Inhibits Renal NCX1 during Colitis

20. Hofmeister, M. V., Füchtbauer, E. M., Fenton, R. A., and Praetorius, J. (2011) The TRPV5 promoter as a tool for generation of transgenic mouse models. Adv. Exp. Med. Biol. 704, 277–286

21. Qiu, Z., Nicoll, D. A., and Philipson, K. D. (2001) Helix packing of functionally important regions of the cardiac Na(+)-Ca(2+) exchanger. J. Biol. Chem. 276, 194–199

22. Billeray-Larmonier, C., Uno, J. K., Larmonier, N., Midura, M. T., Lipko, M. A., Janikashvili, N., Bai, A., Thurston, R., and Ghishan, F. K. (2009) Changes in mucosal homeostasis predispose NHE3 knockout mice to increased susceptibility to DSS-induced epithelial injury. Gastroenterology 137, 965–975, 975.e61–910

23. Navath, S., Rao, V., Woodford, R. M., Midura-Kiela, M. T., Abad, A. M., Alleti, R., Kiela, P. R., and Mash, E. A. (2012) Design, Synthesis, and Testing of a Molecular Truck for Colonic Delivery of 5-Aminosalicylic Acid. ACS Medicinal Chemistry Letters 3, 710–714

24. Kiela, P. R., Laubitz, D., Larmonier, C. B., Gobbi, P., Castaldo, P., Minelli, A., Salucci, S., Magi, S., Corcione, E., and Fenton, R. A., and Praetorius, J. (1998) Alternative promoters and cardiac muscle cell-specific expression of the Na(+)-Ca(2+) exchanger. J. Biol. Chem. 273, H217–H232

25. Qiu, Z., Nicoll, D. A., and Philipson, K. D. (2001) Helix packing of functionally important regions of the cardiac Na(+)-Ca(2+) exchanger complexed with GM1 ganglioside in nuclear membrane translocator. J. Biol. Chem. 276, 147, 123–134

26. Majewski, P. M., Thurston, R. D., Ramalingam, R., Kiela, P. R., and Ghishan, F. K. (2010) Cooperative role of NF-(1)B and poly(ADP-ribose) polymerase 1 (PARP-1) in the TNF-induced inhibition of PHEX expression in osteoblasts. J. Biol. Chem. 285, 34828–34838

27. Wu, G., Xie, X., Lu, Z. H., and Ledeen, R. W. (2009) Sodium–calcium exchanger complexed with GM1 ganglioside in nuclear membrane transfers calcium from nucleoplasm to endoplasmic reticulum. Proc. Natl. Acad. Sci. U. S. A. 106, 10829–10834

28. Nicholas, S. B., Yang, W., Lee, S. L., Zhu, H., Philipson, K. D., and Lytton, J. (1998) Alternative promoters and cardiac muscle cell-specific expression of the Na(+)-Ca(2+) exchanger gene. Am. J. Physiol. 274, H217–H232

29. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421

30. Hu, X., Herrero, C., Li, W. P., Antoniv, T. T., Falck-Pedersen, E., Koch, A. E., Woods, J. M., Haines, G. K., and Ishvakh, L. B. (2002) Sensitization of IFN-gamma Jak-STAT signaling during macrophage activation. Nature Immunology 3, 859–866

31. Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armaka, M., Apostolaki, M., Pizarro, T., Kotylarov, A., Forster, I., Flavell, R., Gaestel, M., Tischls, P., Cominelli, F., and Kollias, G. (2002) Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn’s-like inflammatory bowel disease. J. Exp. Med. 196, 1563–1574

32. Fais, S., Capobianchi, M. R., Silvestri, M., Mercuri, F., Pallone, F., and Dianzani, F. (1994) Interferon expression in Crohn’s disease patients: increased interferon-γ and -α mRNA in the intestinal lamina propria mononuclear cells. J. Interferon Res. 14, 235–238

33. Boggi, P., Castaldo, P., Minelli, A., Salucci, S., Magi, S., Corcione, E., and Amoroso, S. (2007) Mitochondrial localization of Na(+)+Ca(2+) exchangers NCX1–3 in neurons and astrocytes of adult rat brain in situ. Pharmacol. Res. 56, 556–565

34. Kofuji, P., Lederer, W. J., and Schulze, D. H. (1994) Mutually exclusive and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264, 1415–1421

35. Ramana, C. V., Gil, M. P., Schreiber, R. D., and Stark, G. R. (2002) Stat1-dependent and -independent pathways in IFN-γ-dependent signaling. Trends Immunol. 23, 96–101

36. Noguchi, M., Hiwatashi, N., Liu, Z., and Toyota, T. (1995) Enhanced interferon-γ- and α mRNA in the intestinal lamina propria mononuclear cells. J. Interferon Res. 14, 235–238

37. Nagano, T., Kawasaki, Y., Baba, A., Takemura, M., and Matsuda, T. (2004) Up-regulation of Na(+)-Ca(2+) exchange activity by interferon-γ in cultured rat microglia. J. Neurochem. 90, 784–791