The Proinflammatory Cytokine, Interleukin-6, Up-regulates Calcium-sensing Receptor Gene Transcription via Stat1/3 and Sp1/3*

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Increased expression of the calcium-sensing receptor (CASR), which controls blood calcium homeostasis, leads to a decrease in the extracellular calcium set-point, thereby reducing parathyroid hormone secretion and renal calcium reabsorption and increasing calcitonin secretion resulting in reduced circulating calcium levels. Critically ill patients with elevated proinflammatory cytokine levels commonly have hypocalcemia, although the mechanism is not known. After intraperitoneal injection of interleukin (IL)-6 in the rat, circulating levels of parathyroid hormone, 1,25-dihydroxyvitamin D, and calcium fell within hours and remained low at 24 h. Expression of CASR (mRNA and protein) increased within hours in parathyroid, thyroid, and kidney and remained elevated at 24 h. The CASR gene has two promoters (P1 and P2) yielding transcripts having alternative 5′-untranslated regions but encoding the same receptor protein. Activities of P1 and P2 promoter/luciferase reporter constructs were stimulated ~2–3-fold by IL-6 in proximal tubule HKC cells and TT thyroid C-cells. Studies with P1 deleted and mutated promoter-reporter and Stat1 and/or Stat3 dominant-negative constructs showed that a Stat1/3 element downstream of the P1 start site accounted for the IL-6 induction. There are no Stat elements in the P2 promoter, but Sp1/3 elements are clustered at the transcription start site. A series of transfection experiments showed that Stat1/3 together with Sp1/3 was critical for IL-6 responsiveness of P2. By oligonucleotide precipitation assay, IL-6 rapidly promoted a complex containing both Sp1/3 and Stat1/3 on the Sp1/3 elements. In conclusion, Stat1/3 directly controls promoter P1, and the Stats indirectly regulate promoter P2 via Sp1/3 in response to IL-6. By this mechanism, the cytokine likely contributes to altered extracellular calcium homeostasis.

The calcium-sensing receptor (CASR) is expressed in the parathyroid hormone (PTH) producing chief cells of the parathyroid gland, the calcitonin-producing C-cells of the thyroid, and the cells lining the kidney tubule. The CASR, a plasma membrane G protein-coupled receptor, senses small changes in circulating calcium concentration and modulates intracellular pathways that alter PTH and calcitonin secretion or renal cation handling, thereby playing an essential role in blood mineral ion homeostasis. The relationship between extracellular ionized calcium and PTH concentrations is represented by an inverse sigmoidal curve. The activity and/or expression level of the CASR dictates the extracellular calcium set point (defined as the extracellular calcium concentration at which PTH secretion from the parathyroid gland or calcium reabsorption across the kidney tubule is half-maximal). Increases in extracellular calcium directly stimulate calcitonin secretion.

The importance of the CASR in orchestrating the endocrine control of blood calcium concentrations has been underscored by the identification of naturally occurring mutations in the CASR gene that cause human disease. Inactivating mutations result in hypercalcemia, and activating mutations result in hypocalcemia (1–3).

Hypocalcemia is common in critically ill patients, especially those with sepsis and major burn injury (4), and in nonacutely ill patients undergoing surgery (5). The mechanisms underlying the hypocalcemia are not known. Several factors may be involved, including decreased secretion of PTH and resistance to the action of PTH in kidney and bone. The metabolism and function of vitamin D are impaired. Calcitonin precursors are increased in the circulation of critically ill patients with sepsis and could contribute to the hypocalcemia (6, 7).

Several studies of critically ill patients have shown that serum interleukin-6 (IL-6) levels increase within hours of severe burns and infection and can rise to very high levels (8, 9). In these patients the serum IL-6 levels are even more elevated than those of other proinflammatory cytokines, like interleukin-1β (IL-1β) (10), and are inversely related to the serum calcium concentrations (6) and correlate with a poor prognosis (11–16). Carlstedt et al. (17) have shown that in vitro incubating isolated bovine parathyroid cells with IL-6 decreases PTH secretion at clinically...
relevant concentrations. Murphey et al. (18) found that \textit{in vivo} parathyroid CASR levels were up-regulated in a sheep model of burn injury in which circulating cytokine levels would be anticipated to be increased. However, further analyses are required to more fully understand the mechanisms linking the raised IL-6 levels to altered calcium metabolism.

Previously, we investigated the hypothesis that cytokines such as IL-1\(\beta\) increase CASR expression in those tissues important for the control of systemic calcium homeostasis thereby leading to hypocalcemia and hypoparathyroidism. We showed \textit{in vivo} in the rat that parathyroid, thyroid, and kidney CASR mRNA and protein increased after intraperitoneal injection of IL-1\(\beta\) (19). This was associated with decreased circulating PTH, 1,25-dihydroxyvitamin D (1,25(OH)\(_2\)D), and calcium. The CASR gene has two promoters (P1 and P2) yielding alternative transcripts containing either exon 1A or exon 1B 5'-untranslated region sequences that splice to exon 2 containing the ATG translation start codon (20–22). We demonstrated that both the CASR gene promoters have functional \(\kappa\)B elements that mediate up-regulation by IL-1\(\beta\) (19).

In this study, we postulated that IL-6, by up-regulating CASR expression in parathyroid, thyroid C-cell, and kidney tubule, reducing PTH secretion and renal calcium reabsorption, and increasing calcitonin secretion, contributes to altered calcium homeostasis. Here we have demonstrated that IL-6 stimulates CASR expression \textit{in vivo} and defined, for the first time, the mechanisms whereby the cytokine up-regulates CASR gene promoter activity. The resulting increases in CASR expression could be contributing to the hypocalcemia of critically ill patients.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant IL-6 was from Sigma. The human medullary thyroid carcinoma TT cell line was from the American Type Culture Collection (Manassas, VA) and the human proximal kidney tubule cells (HKC-8) were a gift of Dr. Martin Hewison, Cedars-Sinai Medical Center, Los Angeles. Dulbecco's modified Earle's medium (DMEM), Ham's F-12 (F-12) medium, fetal bovine serum (FBS), and antibiotics were from Life Technologies, Rockville, MD), human Sp1DN (dominant-negative construct) (pCI-neo-HA-MCS-Sp1(622–788)) (23), human Stat1\(\Delta\)N (dominant-negative construct) (pCI-neo-HA-MCS-Stat1\(\Delta\)N) (23), human Stat3\(\gamma\) (dominant-negative construct) (pCI-neo-HA-MCS-Stat3\(\gamma\)) (23), and the human Sp1\(\Delta\)N plasmid (pCMV6-X6 (catalog number SC101137; Origene Technologies, Rockville, MD), human PTH (Rat Intact PTH Elisa kit, Immunoreagents, San Clemente, CA), and 1,25(OH)\(_2\)D\(_3\) (immunoreactivity and radioimmunoassay kit, IDS Ltd., Bolton, UK). 1,25(OH)\(_2\)D\(_3\) (immunoreactivity and radioimmunoassay kit, IDS Ltd., Bolton, UK).

**RNA Extraction**—Total RNA was prepared from cells or tissues using TRIzol (Invitrogen) according to the manufacturer's instructions.

**Ribonuclease Protection Assay of Rat CASR mRNA**—For the CASR riboprobe, a 232-bp fragment of a rat CASR cDNA (24) was PCR-amplified (forward primer, 5'-ACCTTGAAGTGGTGGCCTCA-3' (in exon 3), and reverse primer, 5'-GGAAATGGTGGCCGGAGAAGATT-3' (in exon 4)) and cloned into PCR2.1 vector. For the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe that protects a 316-nucleotide transcript, the pTRI-GAPDH Rat vector (AM7432: Ambion Inc., Austin, TX) was used. After linearization of the vectors, the antisense probes were \textit{in vitro} transcribed with T7 polymerase incorporating [\(\alpha\)-\(\text{\textsuperscript{32}}\)P]UTP using a MAXI script kit, and the gel-purified riboprobes were used with a ribonuclease protection analysis II kit (Ambion Inc.). Each probe (2.5 \(\times\) 10\(^{5}\) cpn) was hybridized overnight with 2–25 \(\mu\)g of total RNA followed by digestion with a ribonuclease A:T1 mix (25). Protected frag-

### TABLE 1

| Oligonucleotides used for CASR gene promoter deletion constructs |
|---------------------------------------------------------------|
| Each forward (F) primer is named according to the first CASR gene nucleotide relative to the transcription start site (+1) of either promoter P1 or P2. The common reverse primer anneals to the sequence immediately upstream of the ATG in exon 2. Restriction enzyme recognition sites (ACGGCT, MluI; AAGCTT, HindIII) are in boldface type. Nucleotides in lowercase type are those added to promote efficient restriction enzyme digestion. |

| CASR P1 | F(−938) 5'-agtcAGGCTGACCTTTGATCCACGCTGTTATCTC-3' |
| CASR P2 | F(−701) 5'-agtcAGGCTGACCTTTGATCCACGCTGTTATCTC-3' |
| CASR P1 and P2 | Reverse 5'-agtcAGGCGTCTCTTGCTCTTGCGGCC-3' |

| CASR P1 | F(−341) 5'-agtcAGGCGTCTCTTGCTCTTGCGGCC-3' |
| CASR P2 | F(−382) 5'-agtcAGGCGTCTCTTGCTCTTGCGGCC-3' |
| CASR P1 and P2 | Reverse 5'-agtcAGGCGTCTCTTGCTCTTGCGGCC-3' |

**Animals and Experimental Procedures**—Normal male Sprague-Dawley rats (Charles River Laboratories, Inc., St. Constant, Quebec, Canada), weighing 180–200 g when received, were fed a standard rodent chow (Ralston Purina Co., LaSalle, Quebec, Canada) containing 1.01% calcium, 0.74% phosphorus, and 3.3 IU vitamin D\(_3\)/g. All animal experiments were carried out in compliance with, and were approved by, the Institutional Animal Care and Use Committee. Rats were injected intraperitoneally at 24, 16, 12, 8, or 4 h before death, with either vehicle (propylene glycol, 0.2 ml/100 g body weight) or 0.75 \(\mu\)g IL-6/100 g body weight. Blood was obtained by cardiac puncture, and the serum was separated and stored at −20°C. The rats were anesthetized with pentobarbital; the kidneys were taken, and the parathyroid and thyroid glands were microdissected separately and quickly frozen. Sera were analyzed for calcium and magnesium (Sigma kits), PTH (Rat Intact PTH Elisa kit, Immunoreagents, San Clemente, CA), and 1,25(OH)\(_2\)D\(_3\) (immunoreactivity and radioimmunoassay kit, IDS Ltd., Bolton, UK).
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### Table 2
Oligonucleotides used for EMSA and/or oligo pulldown experiments

| Consensus   | Oligonucleotides          | Sequences                        |
|-------------|---------------------------|----------------------------------|
| Spl         | 5'-ATTCTGATCCTGGGGGCGCAGC-3' |
| CASR promoters |                           |                                  |
| P1 Stat1-WT  | 5'-CCCTGATTTGAGGAAAAGGAG-3' |
| 1A Stat1-WT# | 5'-TTATTTTGTCTGAAATTTCCCAGAA-3' |
| 1A Stat1-Mut# | 5'-TTATTTTGTCTGAAATTAAACAAGAATGG-3' |
| P2 Sp1-WT*   | 5'-GCCAACCGCGCGCCGACCGGCGGAGTCCGGGCGGCGGCGGCGGAA-3' |
| P2 Sp1-Mut*  | 5'-GGCCACCGCGCGCCGACCGGCGGAGTCCGGGCGGCGGCGGCGGAA-3' |
| CgA-CRE-WTb  | 5'-GATTCACACCTTGACGTCAATTTCAGATC-3' |

* For oligo pulldown experiments, a biotin moiety was present at the 5' end.
* Cyclic AMP-response element sequence of the human chromogranin A gene is shown.

#### FIGURE 1.
Serum PTH, 1,25(OH)2D, and calcium levels are decreased by IL-6. Rats were injected intra-peritoneally with IL-6 and sacrificed at the times shown, and serum PTH, 1,25(OH)2D, and calcium levels were determined as described above. Each value is the mean ± S.E. (n = 3). The asterisks indicate a significant difference (p < 0.05) from the time 0 value.

#### A
![Graph showing the decrease of PTH levels](#)

#### B
![Graph showing the decrease of 1,25(OH)2D levels](#)

#### C
![Graph showing the decrease of calcium levels](#)

#### Western Blot Analysis of the CASR—Tissues or cells were lysed in triple detergent buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% NaN3, 0.1% SDS, 1 mM EDTA, 100 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1% Nonidet P-40, 0.5% sodium deoxycholate) for 5 min at 0°C. The lysates were spun at 12,000 × g for 2 min at 4°C, and the supernatants were stored at −80°C. Aliquots were electrophoresed through 4–12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes.

### Notes
- Nuclei were prepared from 10 to 20 nuclei of HKC in PBS carrier alone. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4°C, and lysed with Nonidet P-40 buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.2% Nonidet P-40). After 8 min on ice, nuclei were pelleted at 8000 g for 2 min at 4°C, and the supernatants were stored at −80°C. Western blot analysis of the CASR.
- Nuclei run-on transcription assays—Relative transcription rates were measured using a nuclear run-on assay (26). Nuclei were prepared from 10 to 20 × 106 HKC or TT cells incubated with either 5 ng/ml IL-6 or 1% bovine serum albumin in PBS carrier alone. Cells were scraped into ice-cold PBS, pH 7.4, pelleted at 4°C, and lysed with Nonidet P-40 buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.2% Nonidet P-40). After 8 min on ice, nuclei were pelleted at 8000 g for 2 min at 4°C, and the supernatants were stored at −80°C. Western blot analysis of the CASR.
- Oligonucleotides were resolved on 6% acrylamide denaturing gels and exposed to x-ray film.

#### References
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Induction of parathyroid, thyroid, and kidney CASR mRNA by IL-6.

Rats were injected intraperitoneally with IL-6, sacrificed at the times shown, and CASR and GAPDH mRNA levels of parathyroid gland (A), thyroid gland (B), and kidney (C) were measured by ribonuclease protection assay as described under “Experimental Procedures.” Autoradiographs of representative ribonuclease protection analysis signals are shown for each time point. * = undigested probe. CASR and GAPDH mRNA levels were assessed by densitometry, and each value is the mean ± S.E. (n = 3). Asterisks indicate a significant difference (p < 0.05) from the time 0 value. Injection of vehicle was without effect (data not shown).

For induction of thyroid and kidney CASR protein by IL-6, rats were injected intraperitoneally with IL-6 and sacrificed at the times shown, and thyroid and kidney tissues were harvested and extracts made and subjected to CASR immunoblot analysis as described under “Experimental Procedures.” For each time point, representative samples (from two different rats) are shown. D, thyroid, immunoblot; E, densitometric analysis; F, kidney, immunoblot. The lane with asterisk is one in which the blot was developed with the CASR antibody preadsorbed with the peptide against which it was raised. G, densitometric analysis. E and G, values are mean ± S.E. (n = 3). Asterisks indicate a significant difference (p < 0.05) from the time 0 value.
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**FIGURE 3. CASR gene transcription is induced by IL-6.** Nuclear run-on assays were performed as described under "Experimental Procedures" on nuclei of human thyroid C-cells (TT) (A) and human kidney proximal tubule (HKC) (B) cells cultured with and without IL-6 (10 ng/ml) for 8 h. Autoradiographs of representative experiments repeated three times are shown. Densitometry was performed and relative transcription rates calculated taking that for CASR exon 1A as 100%. For the experiment shown, the RNA transcripts were labeled with biotin-16-UTP. Similar results were obtained with [35S]UTP-labeled transcripts.

immunoblotting was carried out as described above with the antibody preadsorbed for 1 h with the peptide (10 μg/ml) against which it was raised. Antibody-antigen complexes were detected by chemiluminescence using the Lumi-glo kit (Invitrogen). Duplicate membranes were probed with a β-tubulin antibody as control.

**Human CASR Gene Promoter Constructs**—The construction of the P1-luciferase reporter plasmid (designated here as P1(-1438)) containing the P1 promoter, exon 1A, and the 5′ part of exon 2 to nucleotide −1 (nucleotide +1 is the A of the ATG initiation codon), upstream of the luciferase reporter gene in pGL3 basic, has been described previously (P1-WT; 22). The deletion constructs, P1(-938), P1(-701), P1(-382), and P1(-194) were prepared using standard techniques (see Table 1 for primer sequences). A P1(-1438)StatMut construct in which a Stat element in exon 1A is mutated was prepared using standard techniques (see Table 1 for primer sequences). A P1(-1438)StatMut construct in which a Stat element in exon 1A is mutated was prepared using standard techniques (see Table 1 for primer sequences). The overlapping fragments were generated by the overlap extension site-specific mutagenesis method (29) using P2(-1438) as template. The primers used were as follows: forward primer 5'-CTTTAATGCAGTATTCCA and reverse primer 5'-TTTCCCCAGCTTCTTCTTCTTCTTTCCG-3' with a HindIII site in boldface type, RM (5'-TCCCGGCCTTCCGGAGCTTCTTTCCG-3' with a HindIII site in boldface type), and R2 (5'-TGCAAGCTTGGTTTCTGCCGTCCTCCAGGGC-3' with HindIII site in boldface type). The overlapping fragments were denatured and amplified with primers F1 and R2 to generate product 3 that was cleaved with HindIII and cloned into the HindIII-digested P2(-459) plasmid to create P2(-459)Sp1Mut.

**Cell Culture**—HKC cells were grown in DMEM supplemented with 10% FBS. TT cells were cultured in RPMI 1640 medium with 10% FBS and 5% horse serum. All maintenance media contained 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. For mitogen-activated protein kinase (MAPK) inhibitor studies, cells were grown in 6-well plates to 75–80% confluence and cotransfected with either pGL3 or P1 or P2 promoter constructs and the Renilla luciferase vector (internal control). Twelve to 15 h after transfection, cells were serum-starved for 8 h, then stimulated or not with 10 ng/ml IL-6, and harvested 9 h later. Ten μM of the MAPK inhibitor U0126 (Promega, Madison, WI) or 0.1% DMSO vehicle were added to the medium 30 min prior to stimulation. After cell lysis, supernatants were assayed for luciferase activity.

**Transfection and Reporter Assay**—For transient transfection, cells were trypsinized, plated in 6-well dishes in DMEM, 10% FBS (1–4 × 10^5 cells per well), and incubated overnight. The next day, cells were transfected with 30 μg/well of Superfect reagent with 1 μg of CASR promoter construct and 0.5 μg of Renilla luciferase construct per well. The following day, cells were serum-starved in DMEM overnight and cultured with or without cytokine for 10 h. The cells were washed in PBS and lysed in 250 μl of passive lysis buffer (Promega) on ice. The lysates were vortexed for 30 s and supernatants collected by centrifugation (12,000 rpm, 20 min, 4 °C). Luciferase activity was measured in a Fluostar Optima luminometer (BMG Labtech) using 45 μl of cell lysate and D-Luciferin. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Nuclear Extracts of HKC Cells**—Cells were stimulated with IL-6 (10 ng/ml) for 30 min, washed, scraped into 1 ml of PBS, and centrifuged at 1500 x g for 10 min at 4 °C. Cell pellets were processed in a loose Dounce tissue homogenizer in 2 volumes of buffer (25 mM Tris, pH 7.9, 0.3 mM DTT, 420 mM NaCl, 10 mM EDTA, 0.5 mM PMSF, and protease inhibitors). Nuclear pellets were obtained by centrifugation (25,000 x g for 20 min at 4 °C), resuspended in 20 mM HEPES, pH 7.9, 25% glycerol,
significant difference.

test. A

line was then determined using Dunnett’s multiple comparison analysis of variance. The significance of differences from base

from the

buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT, 0.1% Nonidet P-40) with phosphatase inhibitors and protease inhibitors. Cell debris was removed by centrifugation (2 × 5 min, 10,000 × g, 4 °C). Cell extracts were incubated with 1 μg of biotinylated double-stranded oligonucleotides corresponding to the wild-type or mutated CASR P1 Stat1/3 or CASR P2 Sp1 elements (see Table 2 for sequences) for 16 h. DNA-protein complexes (1 μg of biotinylated oligo and 700 μg of protein) were added to Catch and Release Spin Columns (Upstate, Charlottesville, VA) with anti-biotin antibody (1 μg, Abcam, Cambridge, MA) and 10 μl of antibody capture affinity ligand with 1× wash buffer in a 500-μl total volume. The screw-cap columns were rotated at 4 °C for 30 min and then centrifuged (2000 × g; 30 s). Columns were washed three times and eluted with denaturing buffer, and eluates were subjected to immunoblotting with either Stat1 or Stat3 or Sp1 or Sp3 antibodies.

Statistics—Data are expressed as mean ± S.E. The results from the in vivo IL-6 response studies were initially subjected to analysis of variance. The significance of differences from base line was then determined using Dunnett’s multiple comparison test. For the nuclear run-on assays and luciferase transfection assays, comparisons were performed by Student’s t test. A p value of <0.05 was taken to indicate a statistically significant difference.

RESULTS

Interleukin-6 Decreases Serum PTH, 1,25(OH)_2D, and Calcium Levels in Vivo—To examine the effect of IL-6 on extracellular calcium homeostasis, the cytokine was administered to rats, and circulating PTH, 1,25(OH)_2D, and calcium levels were monitored over a 24-h period. After a single intraperitoneal injection of IL-6 in rats, serum PTH, 1,25(OH)_2D, and calcium levels were significantly decreased at 8, 16, and 24 h (Fig. 1).

Interleukin-6 Up-regulates Parathyroid, Thyroid, and Kidney CASR mRNA Levels in Vivo—To assess whether alterations in circulating PTH, 1,25(OH)_2D and calcium levels brought about by IL-6 could be due to altered CASR expression in tissues important for regulation of extracellular calcium homeostasis, we measured CASR mRNA levels by ribonuclease protection assay throughout the 24-h period. After the injection of IL-6 in rats, parathyroid, thyroid, and kidney CASR mRNA levels rose significantly above basal level to peak at 16 h, and the levels were still elevated at 24 h (Fig. 2, A–C). The peak values relative to basal levels were 4.0-fold (parathyroid and thyroid) and 3.5-fold (kidney). Injection of vehicle had no effect on CASR mRNA levels (data not shown).

Interleukin-6 Up-regulates Thyroid and Kidney CASR Protein Levels in Vivo—To evaluate whether the changes observed in CASR mRNA levels were reflected in corresponding changes in CASR protein levels, immunoblot analysis was conducted on extracts of rat thyroid and kidney. Under the conditions used,
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A

Relative luciferase activity

Fold increase

IL-6 +/-

1

3.2

2.6

2.8

1.9

1.5

Luciferase

PGL3

P1(-1438)

P1(-938)

P1(-701)

P1(-382)

P1(-194)

IL-6

B

Relative luciferase activity

Fold increase

IL-6 +/-

1

2.3

2.1

Luciferase

PGL3

P2(-459)

P2(-341)

IL-6

FIGURE 5. Responsiveness of parent and deletion CASR P1 and P2 promoter-reporter constructs to IL-6. Deletion constructs of the CASR P1 promoter (A, left side) or the CASR P2 promoter (B, left side) with appropriate 5'-untranslated region sequences were cloned upstream of the luciferase reporter gene in pGL3 basic as described under "Experimental Procedures." Constructs are numbered relative to the start sites (+1) of either the P1 or P2-driven transcripts. HKC cells were transfected with either the promoterless (pGL3) construct or the CASR P1 promoter (A, right side) or CASR P2 promoter (B, right side) constructs and stimulated (+) or not (−) with IL-6 as described under "Experimental Procedures." **, p < 0.01, ***, p < 0.001 luciferase activity relative to unstimulated control.

the CASR exists in both monomeric and dimeric forms. The monomeric core-glycosylated (immature) species is 140 kDa, and mature, fully glycosylated species is 160 kDa (Fig. 2, D and F). The slower mobility forms (∼300 kDa) are likely to be dimers (28). After injection of IL-6 in rats, thyroid and kidney CASR protein levels (the 140- and 160-kDa species related to β-tubulin values) rose ∼3-fold over basal levels by 24 h (Fig. 2, E and G). Injection of vehicle had no effect on CASR protein levels (data not shown). Parathyroid CASR protein levels were not assessed because of the insufficient amount of tissue.

Interleukin-6 Increases CASR Gene Transcription—To assess whether the changes in CASR mRNA levels reflected changes at the transcription level, nuclear run-on assays were performed on extracts of human TT cells and HKC cells cultured with and without IL-6 for 8 and 12 h. CASR gene exon 1A, exon 1B, and exon 2 transcripts were all stimulated ∼2-fold, and COX2 gene transcription ∼3-fold, in both cell types (Fig. 3; data not shown). GAPDH gene transcription was unaffected by IL-6. Therefore, IL-6 specifically stimulates endogenous CASR gene transcription.

Transcriptional Activities of Human CASR P1 and P2 Are Up-regulated by IL-6—To assess the effect of IL-6 on the transcriptional activity of specific CASR gene promoters, constructs were used in which human CASR P1 or P2 promoters drive transcription of a luciferase reporter gene (Fig. 4A). When transfected into HKC cells, the basal activities of P1 and P2 were 8- and 33-fold that of the promoterless control, respectively (Fig. 4, B and C). In the TT thyroid C-cells, the basal activities of P1 and P2 were 9- and 32-fold that of the control, respectively (Fig. 4D). The addition of IL-6 stimulated reporter activity of P1 and P2 constructs in a dose-dependent manner in HKC (Fig. 4, B and C) and TT cells (data not shown) with an ∼2-fold increase over basal at 10 ng/ml (Fig. 4, B–D). Thus, the proinflammatory cytokine stimulates the activity of both CASR gene promoters.

IL-6 Up-regulates the Activities of Parent and Deleted CASR P1 and P2 Promoter Constructs—To identify regions of the CASR promoters that confer responsiveness to IL-6, a series of deletion constructs of either the P1 promoter with exon 1A and exon 25'-UTR (Fig. 5A, left side) or the P2 promoter with exon 1B and exon 25'-UTR driving a luciferase reporter gene (Fig. 5B, left side) were prepared. The parent or deleted constructs were transfected into HKC (or TT cells) that were then stimulated or not with IL-6. For the P1 constructs, basal activity increased with constructs P1(−938) to P1(−194), relative to the parent P1(−1438) construct (Fig. 5A, right side), suggesting the presence of a repressor region between −1438 and −938. IL-6 stimulated the activity of all wild-type sequence constructs (1.5–3.2-fold). For the P2 constructs containing sequence upstream of the P2 transcription start site, IL-6 induced promoter activity 2.1–2.3-fold (Fig. 5B, right side).

Interleukin-6 Signaling and Gene Transcription—The IL-6 receptor (IL-6R) consists of an IL-6 binding α chain, gp80, and a signal transducer, gp130, which is shared among the receptors for the IL-6-related cytokine subfamily. Binding of IL-6 to its receptor activates JAK family members. Activated JAKs phosphorylate and activate STAT family members that dimerize and translocate to the nucleus and bind to specific STAT-response
elements thereby activating gene transcription (30, 31). Stat1 and Stat3 mediate IL-6 signaling. The JAKs can also couple to the Ras-Raf-MAPK pathway modifying the activity of a variety of transcription factors such as the STAT itself but also other regulators that act through their own cognate response elements. Other transcription factors like AP-1, serum-response factor (SRF), and Sp1/3 potentially can respond to signaling pathway factors activated by IL-6 and regulate gene expression (32).

FIGURE 6. Sequence of the human CASR gene promoter P1, exon 1A, promoter P2, exon 1B, and exon 2 up to the ATG initiation codon. TATA and CAAT homologies are boxed. Transcription start sites are marked by arrowheads, and exons are in boldface type. Nucleotides are numbered relative to the transcription start sites (+1) of each promoter. Potential response element homologies revealed by scanning with MatInspector are boxed.

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elements in P1 and/or P2 promoters and/or the corresponding 5'-untranslated regions (UTR) (Fig. 6). Of special note, in CASR promoter P1 there is a consensus Stat1 element in exon 1A and in CASR promoter P2 there are no Stat elements of any type. However, there are several Sp1 elements that cluster at the transcription start site.

The MAPK Pathway Does Not Markedly Affect the IL-6 Induction of the CASR P1 and P2 Promoters—To examine whether MAPK activity was important for either basal activity and/or IL-6-stimulated activity of the CASR promoters, the P1 and P2 parent and deleted constructs were transfected into HKC cells (or TT cells) that were stimulated or not with IL-6 in the absence or presence of the MAPK inhibitor U0126. For all the CASR P1 constructs (Fig. 7A, left side), in the presence of the inhibitor, basal promoter activity was 70–80% that in the absence of the inhibitor (Fig. 7B, right side). With respect to IL-6 stimulation, although the absolute activity level achieved for all constructs, P1 and P2, was slightly less in the presence than in the absence of the MAPK inhibitor, the fold stimulation was no different (Fig. 7, A and B). Overall, while the MAPK pathway appears to modestly affect basal activity of the CASR promoters, it does not influence their induction by IL-6.

Stat1 and Stat3 Regulate CASR P1 Activity via the Stat Element in Exon 1A—To further examine the role of STATs in mediating the IL-6 induction of the CASR P1 promoter, P1(−1438) constructs, either wild-type or mutated at the Stat1 element in exon 1A [P1(−1438)Stat1Mut], were transfected either alone or with a Stat1DN construct or a Stat3DN construct into HKC cells and the cells were stimulated or not with IL-6. For the wild-type construct, a 3.5-fold increase in activity over basal was stimulated by IL-6, and this increase was virtually abolished by coexpression of either Stat1DN or Stat3DN (Fig. 8). The induction of the P1(−1438)Stat1Mut by IL-6 was markedly reduced relative to that of the wild-type sequence construct and cotransfection with either Stat1DN or Stat3DN completely abolished the induction (Fig. 8). Therefore, the Stat1 element in exon 1A is very important for the IL-6 induction of promoter P1, and either Stat1 or Stat3 can serve to mediate the IL-6 signal.

Sp1 Is Critical for Basal and IL-6-stimulated CASR P2 Promoter Activity—To examine the role of Sp1 in the basal activity and IL-6 induction of the CASR P2 promoter, the P2(−459) or P2(−341) constructs (or the P2(−459)Sp1Mut construct in which the three Sp1 sites clustered at the transcription site were mutated) were transfected either alone or with an Sp1 expression vector or with an Sp1DN (dominant-negative) expression vector into HKC cells that were then stimulated or not with IL-6. Cotransfection of the Sp1 vector with either P2(−459) or P2(−341) led to increases in basal activity of ~30%, whereas cotransfection of Sp1DN vector with either P2(−459) or P2(−341) led to decreases in basal activity of ~30% (Fig. 9A). The activities of P2(−459) and P2(−341) were stimulated >2-fold by IL-6 either without or with coexpression of Sp1. The

**FIGURE 7. Effect of MAPK inhibition on basal activity and IL-6 up-regulation of the CASR P1 and P2 promoters.** CASR P1 (A) or P2 (B) promoter-reporter constructs were transfected into HKC cells with (+) or without (−) pretreatment with the MAPK pathway inhibitor, U0126 (10 μM), and the cells were stimulated (+) or not (−) with IL-6 as described under “Experimental Procedures.” *, p < 0.05; ***, p < 0.001 luciferase activity relative to IL-6 unstimulated control.
Interleukin-6 Up-regulates CASR Gene Transcription

Stimulation of these promoter constructs by IL-6 was virtually abolished by coexpression with Sp1DN (Fig. 9A). The P2(−459)Sp1Mut construct was without activity, and coexpression of either Sp1 or Sp1DN had no effect, and the construct was unresponsive to IL-6 (data not shown). Therefore, Sp1 is essential for basal activity and is critical for the IL-6 up-regulation of the CASR P2 promoter.

Stats Are Essential for the IL-6 Up-regulation of the CASR P2 Promoter—The P2 promoter has no consensus STAT elements; however, Stats could be mediating IL-6 effects by interacting with other transcription factors that do bind to response elements in the promoter. To examine the potential role of Stats in the IL-6 induction of the CASR P2 promoter, the wild-type P2(−459) or P2(−459)Sp1Mut constructs were transfected either alone or with Stat1DN or Stat3DN expression vectors into HKC cells that were then stimulated or not with IL-6. Cotransfection of any of the above dominant-negative constructs alone with wild-type P2(−459) led to a marked reduction in the fold stimulation with IL-6 (1.2–1.4-fold versus 2.5-fold) (Fig. 9B). The P2(−459)Sp1Mut was without activity under all conditions tested (Fig. 9B). Hence, Stat1 and Stat3 are critical for the IL-6 up-regulation of the CASR P2 promoter.

Protein-DNA Complexes Form on a Stat Element in Exon 1A—EMSAs were conducted with oligonucleotides representing a consensus Sp1 element or a cluster of putative Sp1 elements spanning a region just upstream of and just downstream of the transcription initiation site of the CASR gene P2 promoter and HKC nuclear extract. The protein-DNA complexes that formed with CASR gene Sp1 elements and consensus Sp1 element had similar electrophoretic mobilities, and they were shifted in a similar fashion by the addition of antibodies against either Sp1 or Sp3 (Fig. 11). The addition of unlabeled oligonucleotides representing the wild-type CASR P2 sequence reduced the intensity of the labeled consensus and CASR gene Sp1 element-protein complexes in a similar manner, whereas addition of an unlabeled mutant oligonucleotide (data not shown) or an oligonucleotide representing a cyclic AMP-binding protein response-element had no effect (Fig. 11). Complexes did not form on a putative Sp1 element further upstream of the transcription initiation site of the CASR gene P2 promoter (data not shown).

IL-6-dependent Formation of a Stat Complex on the CASR P1 Stat1/3 Element—To test whether endogenous Stat1 or Stat3 binds to the P1 (exon 1A) Stat1/3 element (−129 → −99), oligonucleotide precipitation (DNA pulldown) assays were performed. Extracts of HKC cells treated with IL-6 for varying times demonstrated increasing amounts of a complex comprising the CASR P1 Stat1/3 element and Stat1 or Stat3 (Fig. 12A). Formation of the complexes was rapid and peaked at 5 min (Fig. 12A) with no further increase at 20 min (data not shown). Neither Sp1 nor Sp3 was present in the complex (Fig. 12A). Virtually no complexes formed on mutated CASR gene oligonucleotides (Fig. 12B).

IL-6-dependent Formation of an Sp-Stat Complex on the CASR P2 Sp1 Elements—To test whether endogenous Stat1 or Stat3 (with Sp1 or Sp3) is bound to the P2 Sp1/3 element (−19 → +34), oligonucleotide precipitation assays were performed. Extracts of HKC cells treated with IL-6 for varying
**FIGURE 9. Sp1 and STATS are critical for IL-6 up-regulation of the CASR P2 promoter.** A, CASR P2 promoter-reporter constructs, wild-type P2(-459) or P2(-341) were transfected into HKC cells, either alone (−) or with (+) a wild-type Sp1 construct or a dominant-negative Sp1 (Sp1DN) construct, and the cells were stimulated (+) or not (−) with IL-6 as described under “Experimental Procedures.” *, p < 0.05; ***, p < 0.001 luciferase activity relative to IL-6 unstimulated control. B, CASR P2 promoter-reporter constructs, either wild-type P2(−459) or P2(−459)/Sp1Mut (in which the Sp1 elements clustered at the transcription start site are mutated), were transfected into HKC cells, either alone (−) or with (+) either a dominant-negative Stat1 construct (Stat1DN) or a dominant-negative Stat3 construct (Stat3DN), and the cells were stimulated (+) or not (−) with IL-6 as described under “Experimental Procedures.” *, p < 0.05; ***, p < 0.001 luciferase activity relative to IL-6 unstimulated control.
Interleukin-6 Up-regulates CASR Gene Transcription

In this study we have investigated the mechanisms underlying the stimulation of CASR expression by the proinflammatory cytokine, IL-6. In vivo administration of IL-6 to rats led to falls in serum PTH, 1,25(OH)2D3, and calcium that were maintained over a 24-h period. We demonstrated that IL-6 up-regulates parathyroid, thyroid, and kidney CASR mRNA levels and for the thyroid and kidney (for which adequate amounts of tissue could be obtained) that the CASR protein levels were up-regulated. Human thyroid C-cell and kidney proximal tubule cell CASR gene transcription was increased by IL-6 in vitro. Levels of both CASR gene P1 and P2 promoter-driven transcripts were up-regulated.

In renal proximal tubule HKC and thyroid C-cell TT cells, IL-6 stimulated transcriptional activity of transfected P1 and P2 reporter gene constructs ~3- and >2-fold, respectively. IL-6 functions by binding the cell-surface IL-6 receptor (IL-6R) that consists of an IL-6-binding α chain (gp80) and a signal transducer, gp130, which is shared among the receptors for the IL-6-related cytokine subfamily. Binding of IL-6 to its receptor activates JAK family members. Activated JAKs phosphorylate and activate STAT family members that dimerize and translocate to the nucleus and bind to specific Stat-response elements thereby activating gene transcription (30, 31). There are several members of the STAT family with Stat1 and Stat3 being the ones that mediate IL-6 signaling. The JAKs can also couple to the MAPK pathway modifying the activity of a variety of transcription factors such as the STAT itself, but also other regulators that act through their own cognate response elements (32). Other transcription factors like AP-1, SRF, and Sp1/3 (34) respond to signaling pathway factors activated by IL-6 and regulate gene expression. For some genes the MAPK pathway is important for mediating the stimulatory effect of IL-6 either by activating Stats themselves and/or other transcriptional regulators. Indeed, scanning the CASR gene promoters with computer programs that predict response element sequences identified putative AP-1, SRE (responsive to SRF), and Sp1/3-response elements in addition to putative Stat response elements. The MAPK pathway could potentially affect the activities of all the transcriptional regulators that bind to these elements. However, in this present study use of the MAPK inhibitor, U0126, indicated that although activation of the MAPK pathway contributed modestly to basal activity of both P1 and P2 promoters, it was not involved in a major way in the IL-6 induction of either of them.

Of the several members of the STAT family, Stat1 and Stat3 are the ones that mediate IL-6 signaling. The present studies with deletion and mutated constructs of both CASR P1 and P2 promoters focused attention on a consensus Stat1/3 element in exon 1A (controlling P1) and Sp1/3 elements in promoter (exon 1A) Stat1/3 element and HKC nuclear extracts. Electrophoretic mobility assays with double-stranded oligonucleotides representing a putative CASR gene P1 promoter (exon 1A) Stat1/3 element and HKC nuclear extracts. Electrophoretic mobility assays with double-stranded oligonucleotides representing potential Stat1/3 elements in promoter (exon 1A) and a consensus Stat1/3 element (see Table 2) were conducted as described under “Experimental Procedures,” and antibodies (Ab) against Stat1 or Stat3 were added as indicated. Stat1/1, homodimeric; Stat1/3, heterodimeric; and Stat3/3, homodimeric complexes that formed on the CASR gene oligonucleotides are indicated.

In this present study use of the MAPK inhibitor, U0126, indicated that although activation of the MAPK pathway contributed modestly to basal activity of both P1 and P2 promoters, it was not involved in a major way in the IL-6 induction of either of them.

Of the several members of the STAT family, Stat1 and Stat3 are the ones that mediate IL-6 signaling. The present studies with deletion and mutated constructs of both CASR P1 and P2 promoters focused attention on a consensus Stat1/3 element in exon 1A (controlling P1) and Sp1/3 elements clustering at the transcription start site of promoter P2 as being critical for IL-6 up-regulation of the CASR gene.
The virtual complete loss of IL-6 inducibility with cotransfection of Stat1 and/or Stat3 dominant-negative constructs and the full-length P1 promoter construct confirmed the involvement of these Stats for the upstream promoter. The loss of IL-6 inducibility with cotransfection of an Sp1 dominant-negative and the full-length P2 promoter constructs confirmed the involvement of Sp1 for the downstream promoter. Interestingly, even though consensus Stat elements are not present in the P2 promoter, cotransfection of Stat1 or Stat3 dominant-negative constructs abolished the IL-6 inducibility of the P2 promoter.

In EMSAs with HKC nuclear extract, complexes comprising predominantly Stat3/3 homodimers and Stat1/3 heterodimers (and less Stat1/1 homodimers) formed on the Stat1 element in exon 1A. No complexes formed on another putative Stat1 element further upstream within promoter P1. The fact that Stats formed complexes on this particular element confirmed the indication from the transfected promoter-reporter experiments that the IL-6 up-regulation of the P1 promoter occurred through the exon 1A Stat element.

Stats were initially discovered as signaling molecules mediating interferon-γ action, and this cytokine is a potent Stat1 activator, and formation of Stat1/1 homodimers is favored on Stat elements in contrast to IL-6-promoting Stat3/3 homodimers as seen in the present study (35). Further evidence of the rapid induction (within minutes) by IL-6 of endogenous Stat1 and Stat3 interaction with the CASR P1 promoter Stat element was provided by an oligonucleotide precipitation (DNA pulldown) assay.

In EMSAs with HKC nuclear extract complexes comprising Sp1/1 homodimers, Sp1/3 heterodimers, and Sp3/3 homodimers formed on the cluster of Sp1 elements spanning the transcription start site of promoter P2. In conjunction with the transfected promoter-reporter experiments in which cotransfection of an Sp1 dominant-negative construct almost completely abolished the IL-6 induction of promoter

FIGURE 12. IL-6 induces binding of Stat1/3 to the CASR P1 (exon 1A) promoter and Sp1/3 and Stat1/3 to the CASR P2/exon 1B promoter. HKC cells were incubated with IL-6 (10 ng/ml) for the indicated times and cell extracts made. A, left, aliquots were subjected to biotinylated oligo pulldown assay with oligonucleotides containing the Stat1/3 element in exon 1A (CASR P1 promoter) (see Table 2) as described under “Experimental Procedures.” Precipitated complexes were subjected to immunoblotting (IP) with anti-Stat1 or Stat3 or Sp1 or Sp3 antibodies, and total cell extracts were monitored for Stat1 or Stat3 or Sp1 or Sp3 expression (Total). Autoradiographs of representative experiments are shown. A, right, relative densitometric values taking the peak intensity (at 5 min) as 100; mean ± S.E. (n = 3). B, oligo pulldown assays with P1 Stat wild-type (WT) oligo (left) and P1 Stat mutated (MUT) oligo (right). C, aliquots of the same HKC extracts were subjected to biotinylated oligo pulldown assay with oligonucleotides containing the Sp1/3 elements in the CASR P2 promoter/exon 1B (see Table 2) as described under “Experimental Procedures.” Left, precipitated complexes were subjected to immunoblotting (IP) with anti-Sp1 or Sp3 or Stat1 or Stat3 antibodies, and total cell extracts were monitored for Sp1 or Sp3 or Stat1 or Stat3 expression (Total). Autoradiographs of representative experiments are shown. Right, relative densitometric values taking the peak intensity (at 5 min) as 100; mean ± S.E. (n = 3). D, oligo pulldown assays with P2 Sp wild-type (WT) oligo (left) and P2 Sp mutated (MUT) oligo (right).
P2, this firmly established the importance of the Sp1 cluster in mediating the up-regulation by the cytokine. In addition, the Sp1 elements are key for the basal activity of the promoter.

For some genes that contain both Stat and Sp1 elements in their promoters, it appears that the effectiveness of the cytokine-mediated transcriptional activation is due to synergy between the particular Stat and Sp1 acting at their cognate response elements. For example, this occurs with Stat1 and Sp1 for interferon-γ stimulation of the intercellular adhesion molecule-1 gene (36) and with Stat3 and Sp1 for the CCAAT/enhancer binding protein δ gene (37). However, this is not the case with the CASR P2 promoter as it has no consensus Stat elements. However, the virtual abolition of the IL-6 induction of P2 promoter reporter constructs by Stat1 or Stat3 dominant-negative constructs established a key role for the Stats in the up-regulation of this promoter by IL-6. Again, use of the DNA pulldown assay was key in establishing the interaction of not only endogenous Sp1 and Sp3 but also Stat1 and Stat3 in a complex at the Sp1 element cluster in the CASR P2 promoter. A similar mechanism has been proposed for cytokine induction of other gene promoters that lack Stat elements but have Sp1 elements. For example, IL-6 stimulates transcriptional activation of the vascular endothelial growth factor gene via direct interaction between Stat3 and Sp1 (38), and Stat3 interaction with Sp1 mediates the up-regulation by leptin of the tissue inhibitor of metalloproteinase 1 gene (39).

In summary, our studies provide further insight into how the altered CASR expression that affects the endocrine control of blood calcium homeostasis may be achieved in metabolic alterations occurring in critically ill patients (and in other pathophysiological situations) where circulating proinflammatory cytokine levels are increased. In these situations, inflammation promotes local blood coagulation that although beneficial carries the risk of increased systemic coagulation. The mechanisms that we are uncovering may underlie a critical counter-regulatory system that minimizes the deleterious effects of calcium and cytokines in promoting intravascular coagulation and atherosclerosis during the inflammatory response.

Potentially, interventions that lessen the relative fall in serum calcium may be helpful as in critically ill patients a greater degree of hypocalcemia is associated with a worse prognosis. The present study provides the framework to explore whether the administration of calcilytics, small molecules that target and antagonize the CASR, would be beneficial in critically ill, hypocalcemic patients.

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