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Hypoxia and Cytoplasmic Alkalinization Upregulate Growth Hormone Expression in Lymphocytes

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Abstract

We report here that culture of lymphoid cells under hypoxic conditions showed an increase in both luciferase expression from a GH-promoter luciferase construct and the levels of lymphocyte GH. The effect was mimicked by treatment of cells with cobalt chloride consistent with a specific oxygen-sensing mechanism. We identified a putative hypoxia response element (HRE) in the GH promoter at the region −176 bp to −172 bp that contains a copy of the hypoxia-inducible factor-1 (Hif-1) binding motif (5′-ACGTG-3′). The results also showed that culture of primary rat spleen cells with different doses of TMA induced a dose-dependent increase in lymphocyte GH by Western blot analysis. Greater levels of GH are induced in T cell-enriched populations compared to B cell-enriched populations after treatment with $CoCl_2$ or TMA. Our results suggest that the stressful cellular conditions likely to occur at sites of inflammation or tumor growth may induce the synthesis of lymphocyte GH.

Keywords

growth hormone; lymphocytes; hypoxia

1. Introduction

Growth hormone (GH) has been shown to initiate a broad range of biological effects on cellular growth and metabolism [1]. The biological effects are induced after GH binding to surface receptors (GHR) leading to the activation of numerous signaling pathways and alterations in cytosolic and nuclear activities [2;3]. In addition, internalization of the GH/GHR complex targets the GH to the nucleus [4] and the mitochondria [5]. Both full-length GHR and the alternatively spliced rodent GH-binding protein can be found in the nucleus where they influence proliferation [6]. Thus, it is apparent that GH and/or its receptor may locate intracellularly, and function in proliferation and perhaps by other unknown pathways to influence homeostasis.

It is also apparent that non-pituitary sites and cell types possess the ability to produce GH. Thus, the brain [7], mammary gland [8], placenta [9], skin [10], ovary [11], and cells of the immune system [12] produce GH. Although there are numerous reports that different immune cell lines, including both T and B cells as well as primary lymphoid cells can

produce GH, the mechanisms involved in regulating its production have remained more elusive [13]. There is a general consensus that mitogens enhance the levels of leukocyte GH while the effects of GH-releasing hormone (GHRH) [14-16] and insulin-like growth factor-1 (IGF-1) [17-19] are more controversial. In another study, it was shown that lymphocyte GH can be upregulated by IL-12 which increased T-helper-1 (TH1) IFNγ production along with a modest increase in T-helper-2 (TH2) IL-10 production [20]. Further, the same authors showed that the production of lymphocyte GH could be blocked by physiologic concentrations of norepinephrine or cortisol [20]. Our most recent results in rodent spleen cells by mass spectrometry and Western analysis have shown that different molecular weight isoforms of GH of approximately 100, 65, and 48 kDa can be detected in primary mouse spleen T and B cells [21]. In the same study, we showed that the GH isoforms could be induced by oxidative stress and that the larger molecular weight isoform appeared to reside primarily in the cytoplasm whereas the lower molecular weight isoform was primarily detected in the nucleus [21]. Nothing is known, however, about the potential synthesis of lymphocyte GH at sites of inflammation and tumor growth.

Conditions of hypoxia and extracellular acidic pH have been shown to predominate at sites of inflammation and in the tumor microenvironment [22;23]. Hypoxia caused by the increased metabolic demand of cells, reduction in metabolic substrates and competition by pathogens appears to activate a signaling network involving the hypoxia-inducible factor (HIF) [23]. Under conditions of hypoxia, prolyl hydroxylases are inhibited and HIF-1α can translocate to the nucleus where it binds HIF-1β and promoter regions called hypoxic-response elements (HRE) of target genes [22]. HIF-regulated genes function to decrease mitochondrial oxygen consumption, manage the metabolic shift to anaerobic glycolysis and balance the decreased cellular pH owing to increased lactic acid production. This optimization of cell energetics and homeostasis for survival and function during hypoxia has important influences on immunity. For cells involved in innate immunity, hypoxia amplifies the NF-κβ pathway by increasing the expression and signaling of toll-like receptors (TLRs), and stimulating phagocytosis and leukocyte recruitment [24]. For cells involved in adaptive immunity, HIF-1α induces a shift from a type 1 helper T-cell (Th1) phenotype, which enhances functions of macrophages and cytotoxic T cells, to a type 1 helper T-cell (Th2) phenotype, which inhibits Th1-mediated microbicidal actions [25]. Hypoxia-induced signaling pathways also stimulate the differentiation and proliferation of regulatory T cells and increase extracellular levels of adenosine, which protects tissues by restraining effector functions of T cells [26]. In general, hypoxia amplifies the activity of innate immune cells while suppressing the response of recruited cells of the adaptive immune response [23]. HIF also triggers an adaptation strategy that leads to induction of specific genes dedicated to pH homeostasis. The gene products include the Na^+/H^+ exchanger (NHE) that extrudes protons from the cytoplasm at the expense of the Na^+ gradient and the monocarboxylate transporter (MCT) that evacuates lactic acid [22;27]. Since we already knew that lymphocyte GH could be induced under oxidizing conditions, which exist at sites of inflammation [21], we also wanted to determine whether there was an association between lymphocyte GH production at sites of inflammation and/or tumor growth where conditions of hypoxia and acidity also predominate. Together, our data show that lymphocyte GH is induced during hypoxic/acidic conditions and, therefore, may play a role in inflammation and/or tumor immunity.

2. Materials and methods

2.1 Cell culture conditions

The mouse EL4 T lymphoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI medium supplemented with 10% fetal calf serum and penicillin, streptomycin, and mycostatin (100 U/ml). Cell viability was monitored by trypan blue exclusion. For hypoxia conditions, cells at a concentration of 5 ×

10^5 cells/ml were cultured in a humidified incubator (Forma Scientific, Marietta, Ohio) with 2% oxygen, 5% carbon dioxide, 93% nitrogen (v/v). Control cultures were incubated under normoxic condition (21% oxygen, 5% carbon dioxide, 74% nitrogen). Male (4-6 wks old) Sprague-Dawley rats were obtained from Harlan (Prattville, AL). Following sacrifice, spleens were removed and teased into single cell suspensions at 10^7 cells/ml in RPMI supplemented with 10% fetal bovine serum plus penicillin, streptomycin and mycostatin (100 U/ml). Nylon adherent (B cells) and nylon nonadherent (T cells) lymphocytes were enriched by passage through nylon wool columns as previously described [21]. T cell enriched populations and B-cell enriched populations were judged to be 83% and 68% pure, respectively, as determined by specific mitogen responsiveness and immunofluorescence [28]. The EL4 cell line and primary rat spleen cells were routinely treated and cultured for approximately 18 h prior to centrifugation and preparation of cell extracts for Western blot analysis or luciferase assays. For pH measurements, cells were cultured overnight with TMA, hypotonically lysed, centrifuged, and the cytoplasmic pH determined with a pH probe. All animal manipulations were conducted according to the guidelines and requirements of the University of Alabama at Birmingham Animal Welfare Committee.

2.2 Preparation of GH promoter luciferase constructs and luciferase and β-galactosidase assays

Rat GH promoter luciferase plasmid DNA (pGL2-B luciferase vector, Promega) containing a fragment (−417 to +13 bp) of the GH promoter [29] was cotransfected by electroporation with the pCR3.1 vector (Invitrogen) containing G418 resistance at a ratio of 10:1. Cells were resuspended at 30×10^6 cells/ml in RPMI 1640 (no serum) + 10 mM dextrose, 0.1 mM dithiothreitol (DTT) containing plasmid DNA. A pulse of 400 mV and 960 μF was delivered to the cells in a 0.4 cm-cuvette using the Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA). After the pulse, the cells were maintained in growth medium. Twenty-four hours after transfection, the viable cells were recovered on ficoll-hypaque gradients, washed and then treated with G418 (500 μg/ml) for an additional 21 days before beginning an experiment. For analysis of the putative HIF-1 response element in the GH promoter, a fragment of the GH promoter (−183 bp to −162 bp) was cloned into the basic pGL2 vector utilizing MLV and HindIII restrictions sites. For experiments, the luciferase construct was transiently transfected along with the β-galactosidase construct into EL4 cells, and treated as described in the results. After 18h, the cells were harvested and extracts prepared for luciferase and β-galactosidase assays described below. To measure luciferase activity, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in 0.4 ml of lysis buffer [0.1 M KPO_4 (pH 7.9), 0.5 Triton X-100, and 1 mM DTT)] on ice for 15 min. Luciferase activity was determined as follows: A 75 × 12-mm polystyrene tube containing 100 μl of cellular extract was placed in an Optocomp I luminometer (MGM Instruments, Hamden, CT), 200 μl of assay buffer [100 mM tricine, 10 mM $MgSo_4$, 2 mM ethylene-diaminetetraacetic acid, 1 mM DTT, 2 mM ATP, and 0.1 mM luciferin (pH 7.8)] was injected, and peak luminescence was measured over a 10-S window after a 1-S delay. Protein concentration was used to normalize for variations in handling of replicate cultures and was determined by the Bio-Rad protein dye reagent. In one study, the β-Gal activity was used to normalize for variations in transfection efficiency and was determined by incubating 100 μl of cellular extract with 60 mM β-mercaptoethanol, and 1 mg/ml O-nitrophenyl-β-D-galacto-pyranoside in 0.1 M Na_2HPO_4 (pH 7.3) (total volume=300μl) at 37°C for 15 min. The reaction was stopped by the addition of 700μl of 0.1 M Na_2CO_3; absorbance at 410 nm was measured on a spectrophotometer. The luciferase activity was divided by the protein concentration or the β-Gal activity and the quotient was expressed as relative luciferase activity.

2.3 Western blot analysis

Rat spleen cells were pelleted by centrifugation and resuspended in Tris/Triton-X lysis buffer (1 mM Tris containing 0.1% Triton-X, 10 μg/ml leupeptin, 2 μg/ml of aprotinin, 1 mg/ml PMSF). The cell lysate mixture was incubated on ice for 45 min and then centrifuged for 15 min at 13,000 × g at 4°C. Protein concentration was determined with the Bio-Rad protein assay reagent. The lysate was snap frozen and stored at −70°C until analyzed by Western blotting. Extracts were thawed on ice and immediately denatured by boiling for 5 min in Laemmli SDS sample loading buffer, followed by SDS-PAGE with 8% polyacrylamide gels and transferred to Immunoblot PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Nonspecific binding sites were blocked by incubating the membranes in PBS (pH 7.4) with 0.1% Tween-20 and 10% skim milk for 1 h at 25°C. A polyclonal Ab specific for the detection of rat GH (T-20, sc-10365 from Santa Cruz Biotechnology, Santa Cruz, CA) was added according to the manufacturer's instructions and the membrane incubated with the antisera overnight at 4°C and washed in PBS containing 0.1% Tween-20. The membrane was then incubated 4 h with a 1:2000 dilution of affinity-purified rabbit anti-goat antisera, horseradish peroxidase conjugated (Bio-Rad Laboratories) and washed twice in PBS containing 0.1% Tween-20 and once in dH_2O. Immunoreactive proteins were visualized using the ECL Western blotting analysis system (Amersham Pharmacia Biotech Inc., Sunnyvale, CA). Film was scanned and analyzed using Scion Image Software (Scion Corp., Frederick, MD). Blotted membranes were stripped and reprobed with specific antibodies to actin at a 1:4000 dilution. Densitometric analysis is represented graphically as the triplicate mean ratio of GH/actin with error bars representing the standard error of the mean ($p<0.05$).

2.4 Chemicals and reagents

Goat GH antiserum (T-20, sc-10365) for detection of rat GH was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Monoclonal anti-β-actin Ab (A5441) was purchased from Sigma-Aldrich Corporation (St. Louis, MO). All other chemicals were obtained at the highest grade from Sigma-Aldrich Corporation (St. Louis, MO).

2.5 Data analysis

Each experiment was repeated at least three times, and data are reported as mean ± standard error of the mean (SEM). Significant differences between various experimental treatment groups were determined by analysis of variance (ANOVA) and Student's t-test. Densitometric analysis of the scanned images of Western blots was done using Scion Image Software (Scion Corporation, Frederick, MD). Use of * in figures designates $p \leq 0.05$.

3. Results

3.1 Lymphocyte GH expression in response to hypoxia

The cellular response during the inflammatory and immune reactions results in multiple changes in the local environment, including a decrease in the oxygen content [1]. Although a series of genes involved in glycolytic energy metabolism and cell survival are upregulated during hypoxia [22;23], nothing is known about what effect oxygen deprivation may exert on lymphocyte GH expression. Therefore, to examine the GH response by cells of the immune system exposed to hypoxia, we cultured EL4 cells stably harboring a specific GH promoter luciferase construct under normoxic and hypoxic conditions for 18 h. After culture, we prepared cell extracts and measured luciferase activity (Fig. 1). The results show a significant increase (6.8-fold, $p=0.0001$) in luciferase activity from cells containing the GH promoter luciferase construct compared to the basic luciferase control vector alone cultured under hypoxic conditions. Most importantly, the results also show a significant increase

(2.3-fold, p=0.00216) in luciferase activity from the GH promoter luciferase construct (GHP-Luc) in cells cultured under hypoxic conditions compared to the normoxic controls (black columns).

The biological response induced by hypoxia can be mimicked after treatment of cells with cobalt ions presumably via their influence on oxygen-sensing processes and induction of HIF-1 [30]. To investigate the effects of $CoCl_2$ on lymphocyte GH expression, we treated EL4 cells containing the basic luciferase control construct or a GH-promoter luciferase construct (−417 to +13 bp) with different doses of $CoCl_2$ (Fig. 2). Treatment with $CoCl_2$ (20 μM to 160 μM) stimulated significant luciferase activity in cells harboring the GH-promoter luciferase construct (GHP-luc-417 bp) compared to the basic luciferase construct (basic-Luc) (50-fold, p=0.0001). Similar results were obtained utilizing the transition metals $NiCl_2$ and $MnCl_2$ to mimic hypoxia (data not shown). These results further support the notion that the GH gene is activated by hypoxia and that it may contain a functional HIF-1 response element.

HIF-1α transactivates oxygen-sensitive promoters by binding to a consensus hypoxic response element (HRE) comprising the core sequence 5′-ACGTG-3′ [31]. Inspection of the GH promoter revealed one putative HRE sequence located at −176 bp to −172 bp 5′ to the transcription start site. To determine the potential contribution of this site to promoter induction by hypoxia, a double stranded region comprising only −183 bp to −162 bp (5′-AGATCAGGGACGTGACCGCAG-3′) was prepared and cloned into our basic luciferase vector (pGL2) and transiently transfected into EL4 cells. The culture of the cells under hypoxic conditions (2% 0_2) or with $CoCl_2$ (50 μM) showed a significant ($p<0.05$) increase in luciferase activity compared to the vector alone control (Fig. 3). The increase in the expression of luciferase was 1.7-fold in cells treated with $CoCl_2$ compared to the control (p=0.004) and 0.5-fold in cells cultured under reduced oxygen compared to the control (p=0.01). The results suggest that the putative HRE site in the GH promoter may play a role in hypoxic regulation of lymphocyte GH.

Since culture of cells under hypoxic conditions or treatment with $CoCl_2$ both increased GH gene expression, it was of interest to determine whether guanylate cyclase activity, was involved in the cell signaling pathway. Soluble guanylate cyclase (sGC) catalyzes the formation of cGMP, is the receptor for nitric oxide, and activates PKG, pathways essential for the control of a number of physiological processes [32]. To investigate a role for sGC in GH induction, we incubated control Basic-Luc EL4 cells and EL4 cells harboring the specific GH promoter luciferase construct with the selective sGC inhibitor Ly83583. Luciferase expression from the GH promoter construct showed that the sGC inhibitor (0.4-4.0 μM) significantly diminished $CoCl_2$-induced luciferase expression (Fig. 4).

3.2 Effect of $CoCl_2$ on the expression of GH protein in primary rat spleen cells

To confirm the expression of GH in cells of the immune system after treatment with $CoCl_2$, we studied the presence of the GH protein in primary spleen cells by Western blot analysis. The results from whole cell extracts of control and $CoCl_2$-treated rat spleen cells are shown in Figure 5. The data show significant increases ($p<0.001$) of GH in $CoCl_2$-treated cells (25 and 50 μM) compared to control. In another study, Western blot analysis showed that treatment with the sGC inhibitor Ly83583 as expected blocked approximately 75% of the expression of GH induced after treatment of cells with $CoCl_2$ (Fig. 6). In a previous study [21], we reported the identification of bona fide GH in mouse spleen cells by nano LC-tandem mass spectrometry. This same analysis was successfully applied to the 48 kDa GH isoform from rat spleen cells confirming the presence of GH protein in the Western analysis of rat tissues (data not shown).

3.3 Effect of intracellular alkalinization on the expression of GH in primary rat spleen cells

Acidic extracellular pH generated at sites of inflammation and in the tumor microenvironment has been shown to result in cytoplasmic alkalinization [27]. The role of intracellular alkalinization in cell systems has been routinely studied in vitro utilizing weak bases, such as trimethylamine (TMA), that induce cytoplasmic alkalinization [33]. Therefore, to determine whether cellular alkalinization alone can lead to altered GH production, primary rat spleen cells were treated overnight with a weak base, trimethylamine (TMA), to increase intracellular pH. As seen in the Western blot for the GH protein shown in Figure 7, TMA induced a dose-dependent increase in the intracellular levels of GH. Incubating lymphocytes for 18 h with TMA (5 mM) and TMA (10 mM) increased the intracellular pH by 0.11 ± 0.02 pH units and 0.16 ± 0.04 pH units, respectively, and the levels of GH approximately 3-fold ($p<0.05$). An increase in rat spleen lymphocyte GH was observed after 8 h of incubation with TMA and was dependent on protein synthesis since cycloheximide blocked the effect (data not shown).

3.4 Lymphocyte GH expression in enriched T-cell and B-cell populations in response to $CoCl_2$ and TMA

In previous studies, we have shown that basal levels of lymphocyte GH mRNA and protein in primary B cells appeared to be significantly higher than the levels seen in primary T cells [28]. This observation was seen both from an earlier work in rats [28] and more recently in mice [21]. In order to determine if both T and B cell subpopulations of cells respond to hypoxia and alkalinization of the cytoplasm with elevated GH production, nylon column enriched T and B cells were incubated for 18h with $CoCl_2$ and TMA and the level of GH protein was measured by Western blot analysis (Fig. 8). The data show higher levels of basal lymphocyte GH in B-cell enriched cell populations compared to T-cell enriched cell populations as expected. In addition, the T-cell enriched cell population significantly increased GH production after treatment with $CoCl_2$ (3-fold) or TMA (2-fold) compared to enriched B cells.

4. Discussion

The present investigation has shown that GH expression by cells of the immune system can be upregulated by hypoxia and alkalinization of the cytoplasm. The conclusions are based on the results of expression studies of GH-promoter luciferase constructs and Western blot analysis showing increased GH expression in cells exposed to conditions of hypoxia and alkalinization of the cytoplasm.

The transcription factor, hypoxia-inducible factor-1 (HIF-1) is a heterodimer composed of HIF-1α and HIF-1β subunits that functions as a global regulator of O_2 homeostasis [34]. The HIF-1 complex has been shown to bind to the HIF-1 binding site in the hypoxia response elements of erythropoieitin [35], transferrin [36], vascular endothelial growth factor, and glucose transporter-3 [37] genes. In the present study, we identified an HRE core sequence, 5′-ACGTG-3′, located between −183 bp and −162 bp, upstream of the GH gene. We confirmed this region may function as an HRE by reporter analysis (Fig. 3). Although a single copy of the HRE motif has been shown to be sufficient for hypoxic induction [38], two copies of the 5′-ACGTG-3′ sequence separated by 4 bp [39] as well as the presence of a HIF-1 ancillary sequence (HAS) motif (5′-CAGGT-3′) with a spacing of 8 bp may be essential or at least important for a strong response to hypoxic stimuli [40]. The GH-promoter appears to contain only a single HRE motif and seems to lack a HAS motif. Further studies analyzing deletion/mutation of the putative HRE site, HIF-1 overexpression studies and gel shift analysis are needed to confirm and establish the exact nature of the HRE in the promoter for GH.

In order to shed light on the signaling pathway leading to increased GH synthesis during $CoCl_2$ treatment and hypoxia, the role of guanylate cyclase was examined. One of the major targets of hypoxia is soluble guanylate cyclase which is activated causing an increase in cGMP levels which activates PKG [32]. We show here that $CoCl_2$-mediated luciferase expression from a GH-promoter construct was inhibited by Ly83583 (a guanylate cyclase inhibitor). The results imply that activation of guanylate cyclase may be important in the mechanism of hypoxic induction of GH. Although the primary action of cGMP is considered to be an increase in the intracellular concentration of PKG, cGMP may also exert effects independent of PKG on cyclic nucleotide-gated ion channels, phosphodiesterases and possibly cyclic nucleotide regulated exchange factors that activate GTPases [32]. In the present study, the sGC inhibitor Ly83583 inhibited GH expression (Figure 4), whereas the PKG inhibitor KT-5823 only partially inhibited GH expression (data not shown) suggesting that PKG-dependent and independent pathways may both be involved in lymphocyte GH expression.

In the studies presented here, cytoplasmic alkalinization via the action of TMA stimulated the production of GH by cells of the immune system. Although our result is an observation, a characteristic feature of the inflammatory locus is local acidosis [41]. The interstitial fluid of tumors is also acidic [42] which may inhibit immune cell function [43]. Acute and chronic acidosis causes an increase in the mRNA and activity of the NHE-1 exchanger in lymphocytes [44] whose primary physiological role is to maintain intracellular pH homeostasis by extruding metabolically generated H^+ ions [45]. Increases in RNA and protein synthesis for NHE-1 and an abrupt rise in intracellular pH is found after activation of mature lymphocytes [46] and other cellular processes including autophagy, migration, adhesion and chemotaxis [47]. Although the exact mechanism underlying the regulatory induction of GH during alkalinization is unknown, it may be attributable to the activation of HIF-1 [48]. Thus, decreasing the electrochemical H^+ gradient in the mitochondria via alkalinization would decrease ATP synthesis and activate phosphofructokinase (PFK) and glycolysis [49] which then may activate HIF-1 [50]. Further, intracellular alkalinization is known to enhance superoxide generation which is known to increase HIF-1 [51]. It has also been reported that the ligands of tyrosine kinase receptors (i.e., EGF, IGF-1, PDGF) induce intracellular alkalinization and increase HIF-1 [52]. There was no synergism or additive effects with a combination of TMA and $CoCl_2$ on GH expression in rat spleen cells (data not shown). In fact, in studies where TMA and $CoCl_2$ were combined, there was actually a small decrease in lymphocyte GH expression accompanied by a decrease in cell viability suggesting that in our studies combined treatment may be toxic (data not shown). It should also be noted that intracellular alkalinization in endothelial and neural cells has been shown to activate nitric oxide synthase and increase the production of nitric oxide [53;54]. In our studies, the inhibitor of sGC, Ly83583, also significantly attenuated lymphocyte GH production after treatment with TMA (data not shown), suggesting such a NO-mediated regulatory mechanism may be important in lymphocyte GH production in cells of the immune system.

In this study, we have confirmed previous findings that greater basal GH expression occurs in B-cell enriched cell populations compared to T-cells. The relative degree of up-regulation of GH expression via hypoxia or alkalinization, however, appears different. The greater level of GH expression in T cells above basal levels after treatment with $CoCl_2$ (3-fold) or TMA (2-fold) relative to B cells suggests the existence of distinct regulatory mechanism(s) of GH expression between T and B-cell types. It is known that T cell interactions with B cells in secondary lymphoid tissues, such as the spleen, provides help to B cells that influence the cytokine milieu and facilitate B cell survival, differentiation, and antibody production [55]. In this context, the substantial increase in GH production by T cells alone during hypoxia may increase T cell survival and/or enhance the ability of T cells to provide

optimal help to B cells during a normal immune response. Thus, T cell overproduction or lack of GH production may account for certain autoimmune diseases or immunodeficiencies, respectively.

A recent and accumulating literature has implicated GH-mediated signal transduction in the development and progression of a number of malignancies most notably in breast cancer [56]. Autocrine GH promotes cell growth, survival, migration, invasion and oncogenic transformation via modulation of gene expression [57;58]. Interestingly, exogenous or endocrine GH has less oncogenic potential than autocrine GH [59] yet both presumably act through the GH receptor [60]. Microarray analysis identified 305 genes regulated by autocrine GH and 167 genes regulated by both autocrine and exogenous GH [61]. This same group of investigators in studies comparing the cellular response between exogenous versus endogenously produced GH, have shown that endogenously produced GH was only partially inhibited by an exogenously applied GH antagonist and that the effects of combined exogenous GH administration and endogenous GH production were primarily additive instead of synergistic [62]. It seems clear that autocrine GH appears to have a role in oncogenesis while exogenous GH does not, whereas the role of endogenous GH alone in tumorigenesis, if any, is yet to be realized.

A majority of our previous studies taken together suggests the presence of complex autocrine/intracrine modes of synthesis and action of GH-axis hormones within cells of the immune system [63-68]. On the one hand, some lymphocyte GH signaling pathways promote proliferation, transformation, and protection from apoptosis. On the other hand, this growth does not appear to be uncontrolled but may be modulated by additional signaling pathways. Most recently, we have identified high molecular weight isoforms of GH that can be induced by oxidative stress [21]. It is our bias that lymphocyte GH acts at site(s) within cells (i.e., nucleus [4]/mitochondria [5;69;70]) during occasions of stress (i.e., oxidative, hypoxic or altered pH) to facilitate homeostatic processes that promote immunological activity and survival. In most cases, this can be viewed as a beneficial response designed to resolve/heal at sites of inflammation, prevention of autoimmunity or attack tumor cells. It seems clear also that dysregulation of this pathway in a particular cell, such as a tumor cell, may introduce survival advantages during tumor growth and metastasis. Although nuclear and mitochondrial actions for lymphocyte GH have been proposed [13], the actual site(s) of action of endogenously produced GH (i.e., intracellularly and/or extracellularly) and the differences between GH receptor-mediated events have not been definitely established. Our own previous studies [21] along with those reported here suggest the production of lymphocyte GH during states of oxidative stress, hypoxia and/or altered pH may serve an important survival role and a potential therapeutic target.

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HIGHLIGHTS

- We report that culture of lymphocytes under hypoxic conditions induced growth hormone.
- We identified a Hif-response element in the growth hormone promoter.
- Cytoplasmic alkalinization of lymphocytes induced growth hormone.
- T-cell populations produce greater levels of growth hormone than B-cells during hypoxia.

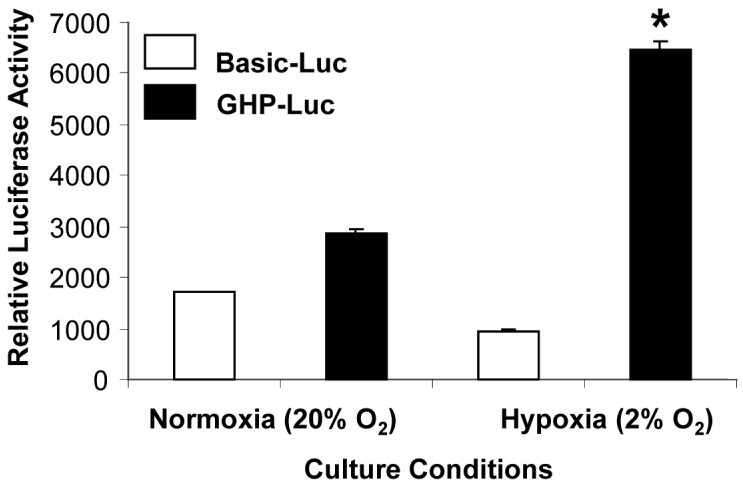

Fig. 1.

GH promoter activity from a GH promoter luciferase construct and the basic luciferase vector alone control in EL4 cells. Cells were cultured as described in the Materials and Methods under normal and hypoxic conditions. Eighteen hours later, cells were harvested and the expression of luciferase measured in cell extracts and normalized by protein concentration as described in the methods. The results are the mean of three experiments with the standard error of the mean represented by error bars. *p=0.0001 compared with the GHP-Luc normoxia control.

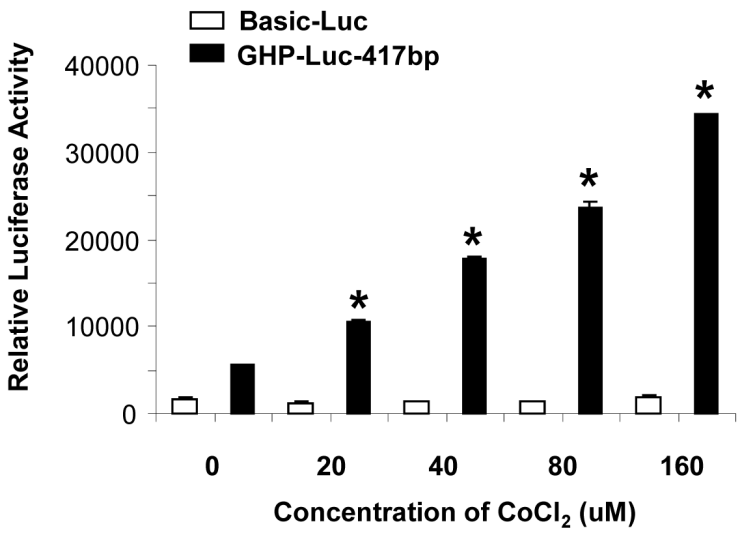

Fig. 2.

GH promoter activity in a GH promoter luciferase construct (-417bp) and the basic luciferase vector alone control in EL4 cells treated with $CoCl_2$ and cultured as described in the methods under normoxic conditions. Eighteen hours later, cells were harvested and the expression of luciferase measured in cell extracts and normalized by protein concentration as described in the methods. The results are the mean of three experiments with the standard error of the mean represented by error bars. *p=0.0001 compared with the GHP-Luc-417bp untreated control.

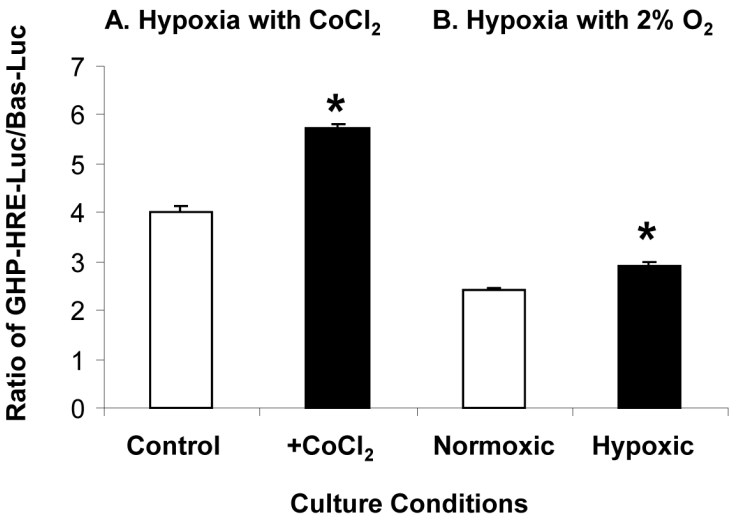

Fig. 3.

Luciferase activity of the putative HRE region identified in the GH promoter. Cells were transiently transfected by electroporation as described in the methods with either a control basic Luc vector or a GH promoter luciferase construct containing the putative HRE region (−183 bp to −162 bp) along with β-Gal reporter constructs. Cells were then either cultured under normoxic and hypoxic conditions and with and without $CoCl_2$ treatment (50 μM) for 18 h. After cell harvest, cell extracts were prepared and luciferase and β-Gal assays performed as described in the Methods. The results shown are the ratio of the luciferase activity of the Basic-Luc control and GHP-HRE-Luc transfected cultures normalized by β-Gal and are the mean represented by error bars. *p=0.004 compared between the untreated and $CoCl_2$ treatrd cultures and *p=0.01 compared between the normoxia and hypoxia-treated cultures. .

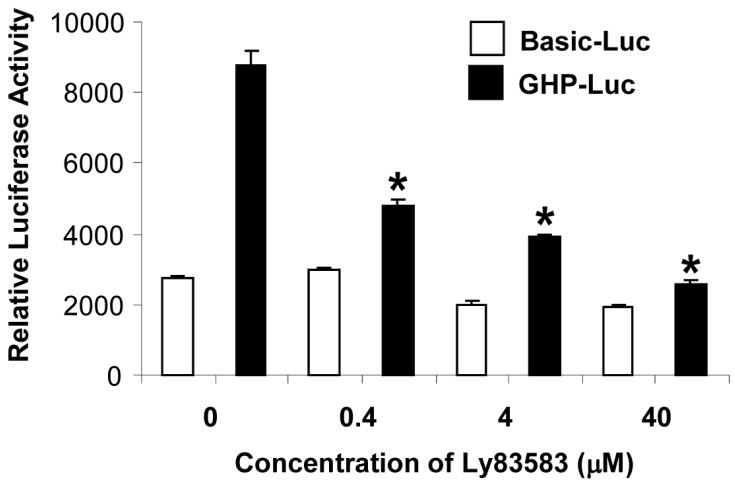

Fig. 4.

Luciferase promoter activity from a control and GH promoter (-417 bp) luciferase construct in EL4 cells treated with the sGC inhibitor Ly83583. Cells were treated at the indicated concentrations with Ly83583 for two hours, then treated with $CoCl_2$ and cultured overnight under normoxic conditions. At the time of cell harvest, the cell viability was 98% in untreated cell cultures. The viability in treated control Basic-Luc cell cultures was 98%, 93%, and 90% and in GHP-Luc-417 bp treated cell cultures was 95%, 90%, and 87% after culture with Ly83583 at 0.4, 4 and 40 μM, respectively. Cells were harvested and the expression of luciferase was measured in cell extracts and normalized by protein concentration as described in the methods. The results are the mean of three experiments with the standard error of the mean represented by error bars. *p=0.0001 compared with the GHP-HRE-Luc normoxia control.

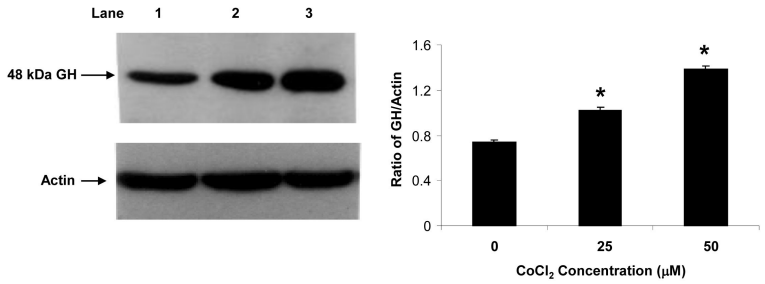

Fig. 5.

GH protein expression in rat spleen cells treated with $CoCl_2$. Cells were treated for 18 h with different doses of $CoCl_2$ after which whole cell extracts were prepared as described in the Methods. After SDS-PAGE (8%) and transfer to PVDF membranes, Western blot analysis was performed using commercial Ab to GH (Santa Cruz) and bands visualized using a chemiluminescence substrate for HRP. Blots were stripped and reprobed with specific Abs to actin. Asterisks (*) denote a significant difference ($p<0.001$) from control. The approximate molecular weight for GH is shown with an arrow on the left. The results shown are typical of an experiment repeated three times. Key: lane 1 (nontreated control); lane 2 ($CoCl_2$, 25 μM); lane 3 ($CoCl_2$, 50 μM).

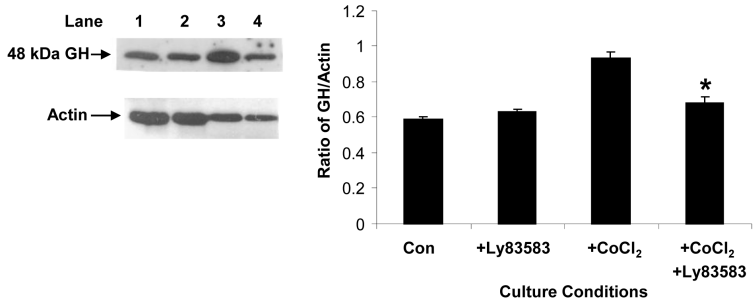

Fig. 6.

GH protein expression in rat spleen cells treated with an inhibitor to sGC. Cells were treated with $CoCl_2$ (50 μM) and/or Ly83583 (4 μM) for 18h after which cell extracts were prepared as described in the methods. After SDS-PAGE (8%) and transfer to PVDF membranes, Western blot analysis was performed using commercial Ab to GH (Santa Cruz) and bands visualized using a chemiluminence substrate for HRP. Blots were stripped and reprobed with specific Abs to actin. The approximate molecular weight for GH is shown with an arrow on the left. The results shown are typical of an experiment repeated three times. Key: lane 1 (nontreated control); lane 2 (Ly83583); lane 3 ($CoCl_2$); lane 4 ($CoCl_2$ and Ly83583). Asterisks (*) denote a significant difference ($p<0.05$) from the $CoCl_2$ treated control.

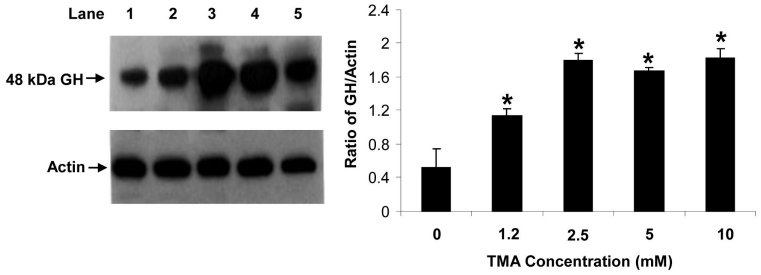

Fig. 7.

GH protein expression in rat spleen cells treated with trimethylamine-HCl (TMA). Cells were treated with TMA (1.2 to 10 mM) for 18 h after which whole cell extracts were prepared as described in Section 2. After SDS-PAGE (8%) and transfer to PVDF membranes, Western blot analysis was performed using commercial Ab to GH (Santa Cruz) and bands visualized using a chemiluminescence substrate for HRP. Blots were stripped and reprobed with specific Abs to actin. The approximate molecular weight for GH is shown with an arrow on the left. The results shown are typical of an experiment repeated three times. Key: lane 1 (nontreated control); lane 2 (TMA, 1.2 mM); lane 3 (TMA 2.5 mM); lane 4 (TMA, 5.0 mM); lane 5 (TMA, 10 mM). Asterisks (*) denote a significant difference ($p<0.05$) from the nontreated control.

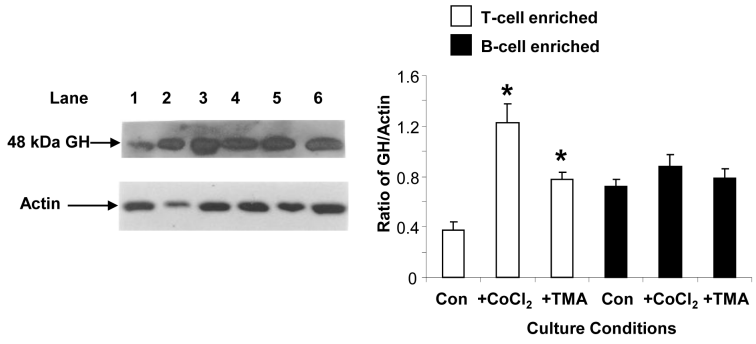

Fig. 8.

GH protein expression in rat spleen T-cell and B-cell enriched cell populations treated with trimethylamine-HCl (TMA) or $CoCl_2$. Nylon column purified T- and B-cells were treated with TMA (5 mM) or $CoCl_2$ (50 μM) as indicated for 18 h after which whole cell extracts were prepared as described in Section 2. After SDS-PAGE (8%) and transfer to PVDF membranes, Western blot analysis was performed using commercial Ab to GH (Santa Cruz) and bands visualized using a chemiluminescence substrate for HRP. Blots were stripped and reprobed with specific Abs to actin. The approximate molecular weight for GH is shown with an arrow on the left. The results shown are typical of an experiment repeated three times. Key: lanes 1,4 (nontreated controls); lanes 2,5 ($CoCl_2$); lanes 3, 6 (TMA). Asterisks (*) denote a significant difference ($p<0.05$) from the nontreated T cell control.