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Modulation of rat pituitary growth hormone by 670 nm light

W.C. Hymer [a,*], J. Welsch [a], E. Buchmann [b,1], M. Risius [a], H.T. Whelan [b,*,1]

[a] Centralized Biological Laboratory, Penn State University, University Park, PA 16802-4803, United States
[b] Dept. Neurology, Medical College of Wisconsin, Milwaukee, WI 53201, United States

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ABSTRACT

In rat pituitary somatotrophs, cytochrome oxidase is co-packaged with growth hormone (GH) in some storage granules. Because this enzyme is thought to be the molecular photoacceptor of red-near infrared light, and because exposure of diverse tissue systems to 670 nm visible light affects their biological responses (e.g., wound healing), we tested the idea that exposure of rat pituitary cells, rat hemi-pituitary glands and rat pituitary homogenates to 670 nm light *in vitro* might alter GH storage and/or release. In this report we offer evidence to show that light treatment (670 nm, 80 s, intensity 50 mW/cm^2, energy density 4 J/cm^2) up-regulates GH release, in part by breakdown of intracellular, oligomeric GH as determined by gel filtration chromatography.

1. Introduction

Activation of cellular metabolism by monochromatic visible light is a redox-regulated phenomenon [1]. Cytochrome c oxidase is the functional photoacceptor for light in the red to near infrared region (670 nm) of the visible spectrum; up-regulation of the enzyme by light can activate signaling cascades thereby leading to biological responses. For example, results from three recent studies demonstrate the beneficial effects of near infrared light in wound healing. Thus, 670 nm light (a) protects the retina of rats against the toxic actions of methanol-derived formic acid [2]; (b) increases the metabolism of primary rat visual cortex neurons functionally inactivated by KCN [3] and (c) mitigates oxidative stress and energy deficit resulting from dioxin-induced toxicity in the developing chick embryo [4].

Recent reports indicate that some growth hormone (GH) secretion granules in rat pituitary somatotrophs contain components of a redox system, viz. chrochrome c [5] and cytochrome oxidase subunits I and II [6]. These components, identified by immunogold electron microscopy, appear to be specific to the GH storage granule, i.e., they are not found in granules of other hormone-producing cell types.

Collectively, these reports provided the impetus for the current study. We sought to determine what effect in vitro exposure of rat pituitary cells or intact pituitary tissue to 670 nm light might have on GH release. The results show (a) that this treatment increases growth hormone release and further (b) that pituitary homogenates are also responsive to light treatment. We propose that the mechanism of light activation is via breakdown of oligomeric GH stores in the granule, which might, in turn, be mediated via intragranular cytochrome oxidase.

2. Materials and methods

2.1. Animals

Male rats (150–200 gm, Harlan Sprague Dawley) were used in protocols approved by the Animal Resource Center at the Medical College of Wisconsin. They were fed ad lib and maintained on a 12 h light/dark schedule under temperature and humidity controlled conditions. In every experiment anterior pituitary glands were harvested by 10 am to minimize possible effects of preparation time on phase of cultured pituitary cells [7].

2.2. 670 nm light treatment

GaAlAs light emitting diode (LED) arrays (25 × 10 cm), with a peak wavelength at 670 nm, were obtained from Quantum Devices Inc. (Barnaveld, WI). These devices have a band width of 25–30 nm at 50% power. The power intensity chosen was 50 mW/cm^2; the energy density was 4 J/cm^2 when applied for 1 min and 20 s. Light treatments were administered at room temperature.

2.3. Cell culture

In two experiments, fresh suspensions of rat anterior pituitary cells were prepared by trypsinization and subsequently cultured in modified Eagles medium (alpha modified MEM) containing

* Corresponding authors. Fax: +1 814 863 7024 (W.C. Hymer).
E-mail addresses: wch@psu.edu, (W.C. Hymer), ddye@mcw.edu, hwhelan@mcw.edu (H.T. Whelan).
[1] Tel.: +1 414 266 7544; fax: +1 414 456 6517.

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10% FCS for 4 days in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C [8]. After media removal, cultures (2.5×10^5 cells/well) were exposed to 670 nm light by placing the multiwell plate directly on the surface of the LED unit using energies of 4 J/cm^2 daily as described previously [3]. As controls, other cells from the same pool, but contained in a different multiwell plate not exposed to 670 nm light, were kept under otherwise identical environmental conditions (i.e., same room temperature and time). After incubation for 24 h, media from each plate was removed, flash frozen, and kept at −80 °C until GH assay. The experimental group was re-exposed to 670 nm light after addition of fresh medium and the culture continued for another 24 h. This cycle was repeated for three additional days.

2.4. Hemi-pituitary gland culture

In each of two experiments intact glands from four rats were carefully removed and the posterior pituitary discarded. Four anterior pituitary halves were placed into one well of a multiwell plate containing 2 ml of serum free MEM and protease inhibitor cocktail (Complete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche Diagnostics. According to the manufacturer, this cocktail inhibits several proteases such as serine-, cysteine- and metalloproteases). The four contralateral halves were placed in a well of another multiwell plate containing the same medium formulation. Thus each of the two wells contained a total of two right lobes and two left lobes. In the first experiment (Fig. 1C), pituitary halves in the experimental well were exposed to 670 nm light (4 J/cm^2) within 3 min after removal from the animal followed by a 15 min incubation period at 37 °C. Aliquots of media were then removed, contents in the experimental well re-exposed to 670 nm light and incubation continued for 15 min. This cycle was repeated throughout the total incubation period (1.25 h). In the second experiment (Fig. 1D), an initial pre-incubation of 30 min was used before a single light treatment was applied 45 min into the 1.5 h incubation period. In these designs, the experimental groups were therefore exposed to light either 1 or 5 times.

2.5. Homogenates

Pituitary homogenates (four glands/homogenate; five separate homogenates for each of two experiments) were prepared in a ground glass homogenizer containing 0.25 M sucrose + 0.2 mM $ZnCl_2$ (40 strokes, 4 °C, 1 pituitary/ml). As judged by phase contrast microscopy, cell breakage was >99%. One half of the homogenate, contained in a multiwell plate, was exposed to 670 nm light (4 J/cm^2) while the other half (control) was kept under identical environmental conditions, but in a separate multiwell plate. In one of these experiments, an aliquot of the homogenate was first centrifuged to obtain a GH granule/mitochondrial fraction (40,000×*g*, 1 h pellet) and a (40,000×*g*, 1 h) microsomal supernatant fraction prior to light treatment. In all cases, GH was extracted from particle-containing fractions with 0.01 N NaOH at 4 °C for 18 h.

2.6. Incubation studies

In two experiments, each requiring pituitaries from 12 animals, four individual homogenates (three glands/homogenate; 1 gland/ml) were prepared as above. Two of these homogenates were prepared in 0.25 M sucrose containing 0.2 mM $ZnCl_2$ while the other two were prepared in 0.25 M sucrose without $ZnCl_2$. Each homogenate was then divided into two equal aliquots, treated with or without 670 nm light, and 100 l samples then incubated in media containing 0.25 M sucrose, 0.04 M Tris–HCl buffer, pH 8.0 for 1 h at 30 °C (total incubation volume 500 μl). Those homogenates prepared in the presence of zinc were also incubated in media containing $ZnCl_2$, but at 10× concentration (i.e., 2 mM). These incubation conditions were based on a previous study [9]. Combinations of these media also contained a protease inhibitor cocktail. These media combinations tested effects of 670 nm light, zinc, and protease inhibitor on GH concentrations in the homogenates after incubation.

2.7. Sephadex chromatography

Gel filtration chromatography was used to study distribution profiles of GH isoforms contained in either media or alkaline pituitary extracts. Samples (500 μl) were pumped into a pre-packed Superdex 75 10/300 GL column at a flow rate of 0.5 ml/min (<260 psi). This column is best suited for separation of globular proteins of Mr 3000–70,000 (Amersham Biosciences). The running buffer was 0.1 M K_2HPO_4 + 0.05 M NaCl (pH 7.0). Before collection (75 fractions/run, 250 μl/fraction), the column eluate passed through a 10 μl flow-through cuvette within a diode array detector, thereby permitting ~4000 continuous samplings of absorbance (230 or 280 nm) during the entire run. The column was calibrated using a Sigma molecular weight standard kit (Sigma MW-GF-70) that consisted of aprotinin, cytochrome c, carbonic anhydrase, bovine albumin and blue dextran. A graph of log molecular weight vs. Ve/Vo (R^2 = 0.991) of these protein standards enabled estimation of apparent molecular weight of GH forms in the sample. In this system rhGH ran with apparent molecular weight of 21,300 kD.

2.8. Enzyme linked immunoassay

After chromatography, the concentration of rat GH in each of the 75 fractions was determined by enzyme immunoassay (EIA). This assay, previously validated and developed in our laboratory for rat GH [10], uses a polyclonal antiserum to rat GH (kindly provided by Dr. R. Grindeland, NASA Ames Research Center) at a final dilution of 1:80,000. At this dilution the cross-reactivity to rat prolactin is <0.3%. Each sample was analyzed in duplicate at two or three dilutions. Results are expressed relative to a rat GH standard preparation (NIDDK-rGH-B-13; AFP-87401; 1.8 I.U./mg) provided by the National Hormone and Peptide Program, NIDDK, and Dr. A. Parlow.

2.9. Statistics

In all experiments, the proc mixed procedure (SAS Institute, 2003. SAS system for Windows. SAS Institute Inc., Cary, NC) was used to analyze data and the least square means procedure was used to compare control vs. experimental treatments. Statistical significance was assumed for $p < 0.05$.

3. Results

3.1. Effects of 670 nm light on GH release from primary rat pituitary cell cultures

In two experiments, daily exposure of cell cultures 670 nm light, stimulated daily release of GH in the 4-day trials (Fig. 1A and B). When the results of these two experiments were combined, statistical analysis indicated that the GH content of media from the experimental cultures was significantly higher after the first and second culture days ($p < 0.05$), and tended to be greater after the third or fourth day, but not significantly so ($p > 0.05$).

3.2. Effects of 670 nm light on GH release from rat hemi-pituitary glands

The difference in the total amount of GH released from hemi-pituitary glands exposed to 670 nm light five times at 15 min

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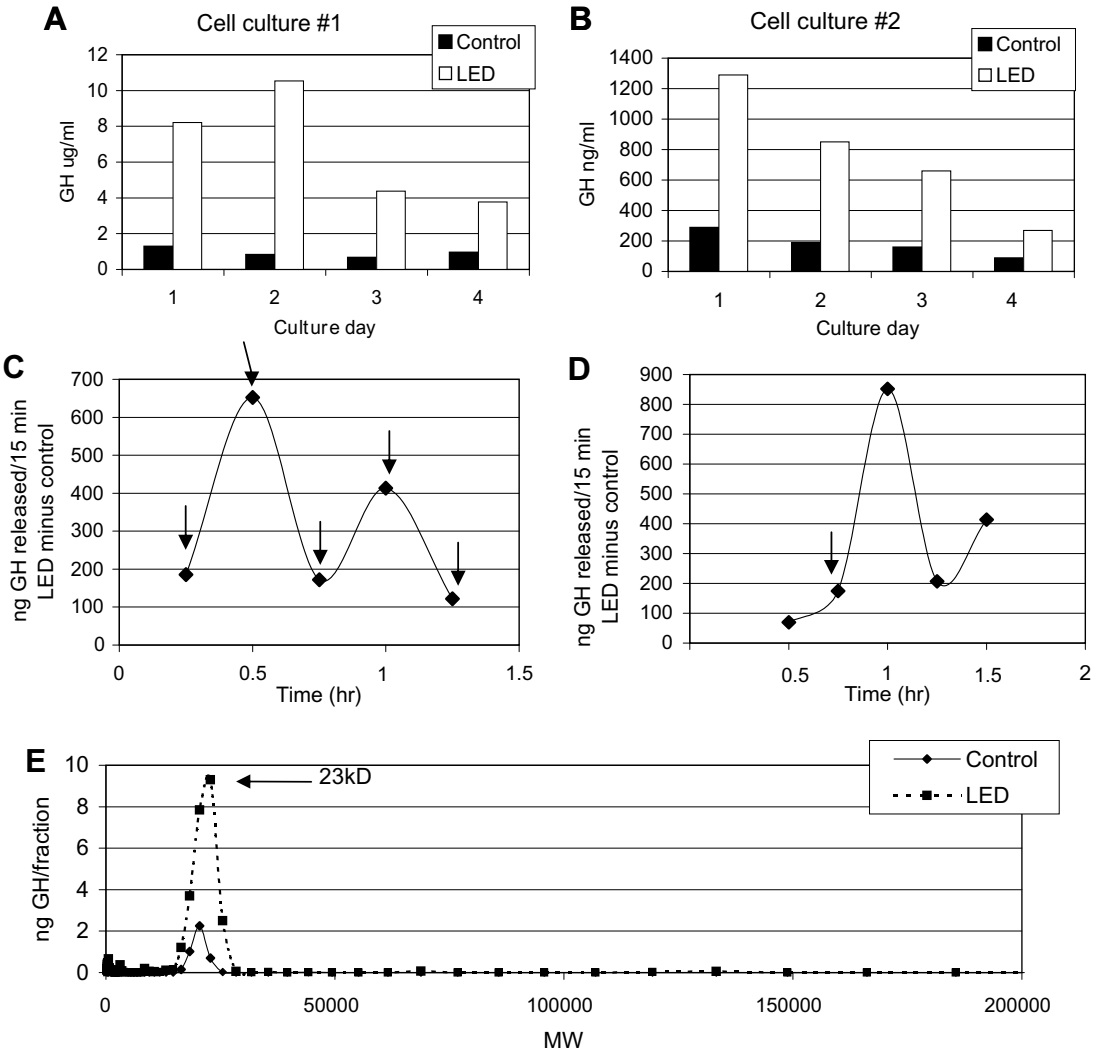

Fig. 1. Panels A and B: Effect of daily light treatment of primary rat pituitary cell cultures on GH contents of culture media, relative to non-exposed control cultures. Duplicate experiments (see Section 2). Considered together, the GH concentration of the experimental groups in both experiments 1 and 2 was significantly greater than controls ($p < 0.05$) for days 1 and 2, but not thereafter. Panel C: Effect of light treatment (arrows), administered at 15 min intervals, on GH released from four hemi-pituitary glands during a 1.25 h incubation. Light treatment started immediately after removal from the rats (see Section 2). Data are expressed as ng GH released from the experimental group *minus* the GH released from the four contra-lateral hemi-pituitary glands of the control group. The difference in total GH concentration in media between the LED vs. control group was significant ($p < 0.02$). Panel D: Effect of a single light treatment (arrow) on GH release from four hemi-pituitary glands *minus* GH released from the four contra-lateral hemi-pituitary glands of the control group. In this case there was a 30 min pre-incubation period prior to light exposure. The difference in total GH concentration in media between the LED vs. control group was significant ($p < 0.035$). Panel E: Gel filtration profile of GH obtained from pooled aliquots (125 μl each) of four media samples (45, 60, 75 and 90 min) from the experimental wells (panel D) relative to that using the identical pooling and fractionation scheme from the control samples is shown.

intervals, relative to the control group (expressed as LED minus control), was significantly greater in the light-treated group (Fig 1C, $p < 0.02$). In a similar experiment, the difference in the total amount of GH released from the experimental hemi-pituitaries following a single exposure to 670 nm light was again significantly greater (Fig. 1D, $p < 0.035$). In this latter experiment (Fig. 1D), GH profiles following gel filtration showed that hormone released from both the experimental and control samples had an apparent mass of 23 kD (Fig. 1E).

3.3. Effect of 670 nm light on GH previously released into culture media

To test the possibility that 670 nm light does not act on the cell/tissue sample directly, but instead on GH molecules once released into the incubation medium, aliquots of media from control cell cultures (Fig. 1A) were exposed to 670 nm light (4 J/cm^2). Upon re-assay, the light treatment was without effect (data not shown).

3.4. Effect of 670 nm light on GH concentrations in rat pituitary homogenates

Direct exposure of cell-free homogenates to 670 nm light resulted in significant losses of GH (Fig. 2A; $p < 0.001$ and 0.01, experiments 1 and 2, respectively).

3.5. Gel filtration GH profiles extracted from sub-cellular fractions

As a part of the experiment shown in Fig. 2A (experiment 1), individual aliquots of the five homogenates taken *before* light treatment were each subjected to centrifugation to obtain a 40,000×*g* pellet and supernatant fraction. Aliquots of each fraction were

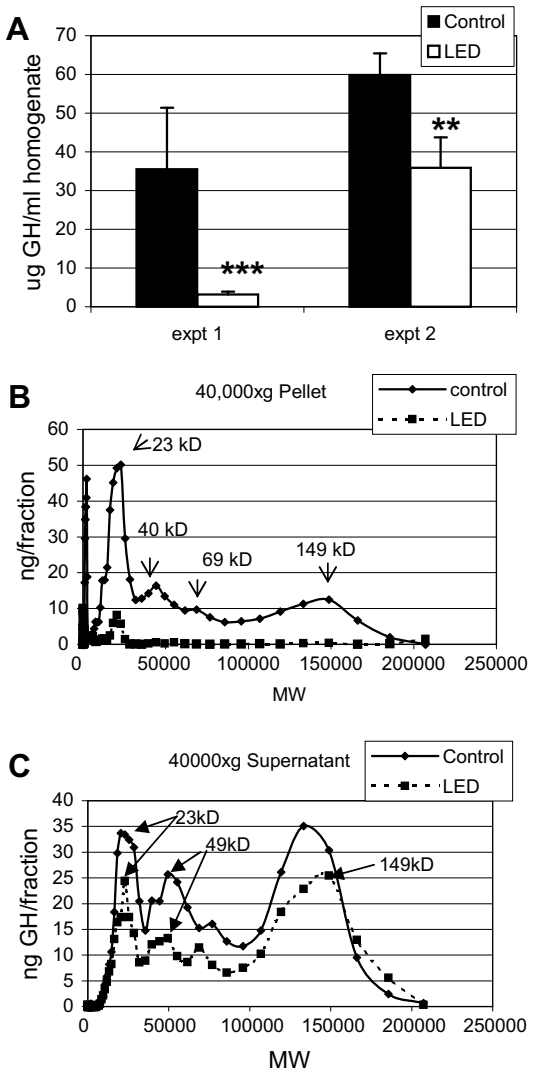

Fig. 2. Panel A: GH concentrations in alkaline extracts of rat pituitary gland homogenates exposed to 670 nm light. In 5 (experiment 1) and 4 (experiment 2) separate trials, each homogenate required 2 glands/trial. One half of each sample was exposed to light while the other half served as the control sample. In both experiments 1 and 2, the differences in GH contents between the experimental and control samples were significant ($p < 0.001$ and 0.01 for experiments 1 and 2, respectively). Panels B and C: Aliquots of the five homogenates from the *control* groups (panel A) were each centrifuged at 40,000×*g* for 1 hr to obtain a pellet (panel B) and supernatant fraction (panel C). After re-suspension of the pellet, both pellet and supernatant fractions were divided and half treated with light. Alkaline extracts of material in each of the treated and control groups were then pooled prior to gel filtration and subsequent determination of GH concentrations in each of the 75 fractions.

exposed to 670 nm light and alkaline extracts subsequently fractionated by gel filtration.

GH profiles in both control samples revealed peaks with apparent mass ranges of ~20–23 kD; ~40–49 kD; ~70–80 kD and a broad peak of GH immunoreactivity at ~150 kD (Fig. 2B and C). In addition, a sharp peak of immunopositive material (~2 kD) was extracted from the control 40,000×*g* pellet (Fig. 2B) but not from the corresponding supernatant material (Fig. 2C).

After light treatment GH profiles of extracts prepared from the 40,000×*g* pellet material indicated an almost total loss of GH (Fig. 2B). However, profiles from the corresponding 40,000×*g* supernatant fraction were similar between the control and experimental extracts, but GH concentrations were reduced ~30% throughout the chromatogram of the experimental sample (Fig. 2C).

3.6. GH concentrations in incubated pituitary homogenates: effects of Zn^{+2}, protease inhibitor and 670 nm light

In an extensive experimental series, Lorenson and Jacobs described an assay to monitor protein, GH and PRL release from granules isolated from the bovine anterior pituitary [9,11]. Collectively their results revealed a number of experimental conditions which significantly changed the content and molecular form(s) of the hormones packaged within these particles. In two experiments, we used their identical procedures to determine if pre-exposure of fresh rat pituitary homogenates to 670 nm light would modify subsequent GH detectability after incubation at 30 °C for 1 h. The results revealed significant ($p < 0.05$) *increases* in GH in the light-exposed group when the incubation medium contained Zn ion and protease inhibitor (Fig. 3A). However, no other combination of medium formulation of zinc, protease inhibitor and 670 nm light treatment (i.e., +Zn,−PI; −Zn, +PI; −Zn,−PI) revealed significant differences in GH concentrations after incubation ($p > 0.05$, data not shown).

To determine which GH form(s) might account for the increased GH concentration in these experiments (Fig. 3A), extracts from the samples in Fig. 3A, experiment 2 were analyzed by gel filtration. The GH profiles from the control sample revealed two GH peaks; one at ~14 kD and the other at ~25 kD (Fig. 3B). However, the GH profiles from the experimental sample revealed not only more total GH in the chromatogram, but also showed three major GH peaks with apparent masses of ~25, 44 and 130 kD. In addition, GH was spread over a wide range between 44 kD and column void volume (Fig. 3B).

3.7. Other experiments

We also studied possible effects of 670 nm light exposure under conditions of (a) added thiols or (b) increased light dosage (8 and 30 J/cm^2). Neither addition of GSH nor longer light exposures affected GH concentrations that were different from those found using the 4 J/cm^2 dose.

4. Discussion

The results of this study show that both rat pituitary cells and intact hemi-pituitary glands respond to brief exposures of 670 nm visible light by releasing more GH into the culture medium relative to that of control cultures. Native cell structure is not required to obtain this response since pituitary homogenates are also affected by light treatment. We emphasize that (a) the different biological samples (cells, glands and homogenates) that served as controls in this study were always taken from the *same* sample pool that was used for light exposure testing and that (b) except for the light treatment, the conditions in the laboratory between the two groups were otherwise identical.

We favor the hypothesis that the target for 670 nm light is the pituitary GH storage granule. The GH gel filtration profiles support this idea. Thus, higher molecular weight GH isoforms, contained in extracts prepared from the 40,000×*g* pellet of the experimental, but not the control group, were totally absent after light treatment. The 40,000×*g* pellet is enriched in GH storage granules as well as GH oligomers [12]. Because several types of proteases and thiol dependant oxido-reductases are also present in the GH storage granule [9], we further suggest that that the light treatment may have activated proteolysis in these unprotected samples. The net result was essentially total loss of GH signal (Fig. 2B). This did not happen in the control sample (Fig. 2C). In contrast, the GH isoform patterns in the 40,000×*g* supernatant fraction were less affected by light treatment (cf Fig. 2B vs. C). In

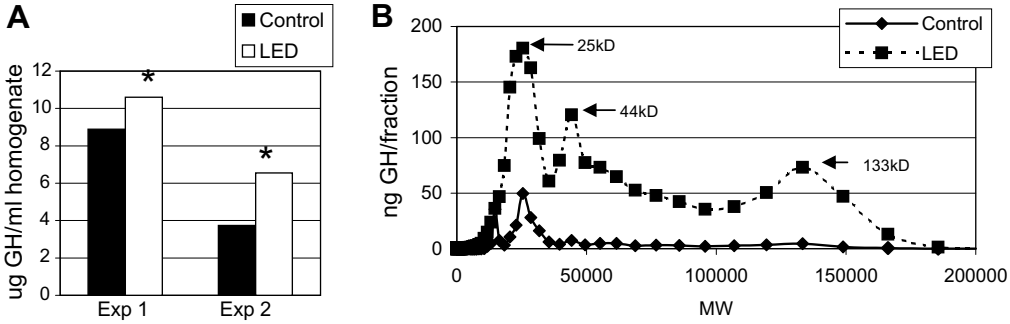

Fig. 3. Panel A: Effect of light treatment on GH contents of rat pituitary homogenates subsequently incubated at 30 °C for 1 h in media containing sucrose, Zn and protease inhibitor, pH 8.0. In each of two experiments, pituitaries from 12 animals were used to prepare four different homogenates (see Section 2). In both experiments the GH concentrations were significantly greater ($p < 0.05$) after light treatment. Panel B: Gel filtration profiles of GH in alkaline extracts prepared from the control and light-treated samples of experiment 2 (panel A).

this sample, the GH molecules are likely to be less concentrated (see below).

The literature offers insights as to how 670 nm light might promote disaggregation of GH oligomeric isoforms in the storage granule. The concentration of GH in a granule has been estimated to be 4 mM with two Zn^{+2} ions cooperatively associated per GH dimer [13]. Furthermore, under certain conditions GH molecules within the core of the granule will reversibly associate from monomers into large oligomers [14]. An *in vitro* assay, developed several years ago by Lorenson and colleagues, was used to study release of hormone molecules from isolated bovine GH granules. Zinc was found to be a potent inhibitor of GH release while EDTA, thiols (GSH) and other agents stimulated hormone release, eventually leading to storage granule disruption [9,11]. And in a previous study our laboratory studies showed that the antigenicity of GH aggregates increased 3–5-fold after addition of reducing agent [12].

If 670 nm light promotes disaggregation of oligomeric GH, we reasoned that use of a protease inhibitor cocktail in the "granule breakdown assay" [15], should also show an increase in apparent GH content of the sample. This prediction proved true (Fig. 3A). However, the result was not found when other combinations of zinc, protease inhibitor and 670 nm light treatment (i.e., +Zn,−PI; −Zn, +PI; −Zn,−PI) were used. Apparently the environmental conditions used in these trials (+Zn, +PI) was sufficient to capture at least some of the light-induced disaggregation process. The GH gel filtration profile (Fig. 3B) may reflect light-induced liberation of GH from a poorly immunoreactive pool of densely packed, oligomeric GH of very high molecular weight thought to be packaged in the granule.

The mechanism(s) by which 670 nm light might induce breakdown of intracellular stores of GH aggregates could be (1) direct, (2) indirect, or (3) both. Evidence, albeit circumstantial, for both can be gleaned from the literature. The case for a direct action is supported, for example, by studies of Thamann [16] and Miller et al. [17]. These reports show that photolysis of recombinant bovine GH in the solid state by near UV light targets tryptophan mediated photooxidation of disulfide bonds as well as a corresponding modification of the C–S stretching mode in the hormone aggregate. Other reports such as those of Prokhorenko et al. [18] and Banghart et al. [19] also support the direction action hypothesis by demonstrating light-induced conformational changes of proteins in systems containing bacteriorhodopsin and potassium channels.

On the other hand, the recent report by Eells et al. [2] shows that 670 nm light up-regulates cytochrome oxidase. We suggest that our results could also be explained via up-regulation of cytochrome oxidase and cytochrome c since both of these complexes have been shown to be present within the pituitary GH granule itself [5,6]. Thus, the action of the light on the rat pituitary preparations *in vitro* would be indirect. The reason why elements of a redox system are present in only some GH granules, and not in granules containing other pituitary hormones, remains an open one. It is remarkable, and perhaps not coincidental, that similar elements of a redox system are also present in secretory granules of the acinar, but not beta, cells of the pancreas [6].

To our knowledge, the results in this report are entirely novel. Whether *in vivo* application of 670 nm light might result in similar GH responses remains an open question. However, our data invite further studies in which light is used as a tool to examine GH aggregation states in relationship to intragranular cytochrome oxidase in the rat pituitary gland.

How might LED exposure *in vivo* modify pituitary GH? Our preliminary result (pg 10) invites speculation as to how 670 nm light might interact either directly at the pituitary level or indirectly via cooperation with neurotransmitters.

A direct action of light at the pituitary level seems feasible in the rat because it has been shown that penetratibility is ~2 cm, a sufficient distance to activate the gland. In the human brain this has also been documented at 2 cm.

Since the minimum mean free photon path to the pituitary gland in the sella turcica (ST) from an external source is through the orbital fossa or nasal pharynx, these minimally invasive approaches will be evaluated. This approach will utilize a high power diode laser as identified above coupled into a bundle of four or more fibers. Each fiber will be inserted into a specially molded fiber holder suitable for insertion into the nasal or orbital cavity providing a direct "line of sight" approach to the ST. Due to the absence of highly scattering dermal tissue and minimal bone thickness at the cribriform plate, it is anticipated that this direct nasal approach may be the most efficient launch scheme for minimally invasive irradiation of the ST with IR light.

The literature also supports the idea of an indirect action of light *in vivo*. At least three mechanisms are noteworthy.

In the first case, it has been known for some time that photic cues, transmitted via the retinohypothalamic tract to superchiasmatic nuclei (SCN), entrain circadian rhythms to the light/dark cycle. More recently, nocturnal photic stimulation (light intensity 200–300 lux) has been shown to suppress spontaneous GH secretion in rats [20]. This suppression is attributed to induction of gene expression in both the SCN but also in periventricular, somatostatin neurons. In this experimental situation light dampened GRF neuronal activity. A different response to 670 nm light, acting via the retinohypothalamic tract, might be invoked for GRF containing neurons in the arcuate nucleus.

In the second case, modulation of GH secretion by hypothalamic peptides other than GHRH and SRIF is thought to be accomplished by certain neurotransmitters acting either directly at the pituitary

or indirectly at the hypothalamic level. For example, the role of dopaminergic systems in the control of GH, although still controversial, is reported to have either stimulatory or inhibitory effects on GH secretion [21]. Use of dopamine antagonists (D2 receptor) alters GH and IGF-1 secretion in mice [22].

The recent report by Smith et al. [23] offers a third, and exciting possibility as to how 670 nm light could act via ghrelin receptor agonists and dopamine receptor subtype (D1R). In this case, up-regulation of ghrelin and dopamine either at the hypothalamic or pituitary level, would amplify GHRH signal transduction thereby regulating the GH/IGF-1 pathway. Smith speculates that dopamine induced coupling of D1R to G protein subunits activates adenylate cyclase *in somatotrophs* as a consequence of ghrelin induced coupling. Smith's pivotal work clearly establishes the role of ghrelin, ghrelin receptor agonists and GH as potential interventive agents during human aging. The results of our study invite further investigations into 670 nm light effects on the pituitary GH system.

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