Published in final edited form as:

Mol Cell Endocrinol. 2009 October 15; 309(1-2): 76–81. doi:10.1016/j.mce.2009.03.004.

Sulfation of LH Does Not Affect Intracellular Trafficking

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Abstract

LH and FSH are produced by the same gonadotrope cells of the anterior pituitary but differ in their mode of secretion. LH secretion is primarily episodic, or regulated, while FSH secretion is primarily basal, or constitutive. The asparagine (N)-linked oligosaccharides of LH and FSH terminate with sulfate and sialic acid, respectively. TSH also contains sulfated N-linked oligosaccharides and is secreted through the regulated pathway. It has been hypothesized that sulfate plays a role in segregating LH to the regulated pathway. Using a mouse pituitary model, we tested this hypothesis by examining the secretory fate of LH from pituitaries treated with sodium chlorate, a known inhibitor of sulfation. Here we show that mouse LH is sulfated and secreted through the regulated pathway, while FSH is secreted constitutively. LH secretion from chlorate treated pituitaries, which showed complete inhibition of sulfation, was similar to untreated pituitaries. These data suggest that the metabolic role for sulfated N-linked oligosaccharides is not for intracellular trafficking but for the extracellular bioactivity of LH.

Keywords

LH; FSH; Pituitary; Mouse; Sulfation

Introduction

Luteinizing hormone (LH), follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG) are glycoprotein hormones consisting of a common α subunit and a hormone specific β subunit. LH and FSH are produced by the same gonadotrope cells of the anterior pituitary but differ in their mode of secretion. The differences in secretion are linked to normal gonadal function and gamete production in both males and females. LH secretion is primarily episodic, or regulated; it is stored in secretory granules and released upon stimulation by GnRH (Knobil, 1981, Wildt *et al.*, 1981, Filicori *et al.*, 1986, Conn *et al.*, 1987, Hall *et al.*, 1988, Muyan *et al.*, 1994, Hayes & Crowley, 1998, Crawford *et al.*, 2002, McNeilly *et al.*, 2003, Pawson & McNeilly, 2005). FSH secretion, in contrast, is primarily basal, or constitutive, linked to its rate of synthesis (McNeilly, 1988, Spratt *et al.*, 1988, Chappel, 1991, Muyan *et al.*, 1994, Padmanabhan *et al.*, 1997, McNeilly *et al.*, 2003, Crawford *et al.*, 2009). Immunolocalization studies in sheep and L β T2 cells have demonstrated that LH and FSH segregate to different granules populations (Thomas & Clarke, 1997, Nicol *et al.*,

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2004), supporting the notion that LH and FSH are secreted through independent pathways. However, there is a basal component of LH secretion and some release of FSH associated with GnRH pulses (Farnworth, 1995, Padmanabhan *et al.*, 1997, Crawford *et al.*, 2002, McNeilly *et al.*, 2003).

The structural signals encoded in the LH and FSH subunits that govern the intracellular sorting to different secretory granules are largely unknown. Given that LH and FSH are synthesized in the same cell and have an identical α subunit, the signals for sorting to their respective pathways must reside in the β subunits. Previous studies of glycoprotein hormone assembly and secretion relied on CHO cells (Kaetzel *et al.*, 1985, Corless *et al.*, 1987, Matzuk *et al.*, 1989, Muyan *et al.*, 1996) which do not possess a regulated pathway; therefore, sorting of the glycoprotein hormones could not be addressed. Using GH₃ cells, which are derived from rat pituitary somatotropes, and provide an *in vitro* model to study the intracellular trafficking of the gonadotropins, LH and FSH were shown to sort to the regulated and constitutive pathways, respectively (Muyan *et al.*, 1994). Our laboratory identified that the seven amino acid carboxy tail of LH β is a sorting signal for LH in GH₃ cells (Jablonka-Shariff *et al.*, 2008). When the heptapeptide was deleted from LH β , the truncated dimer was more constitutively secreted. Addition of the heptapeptide to FSH β resulted in a more regulated secretion of the FSH dimer.

Sulfation of LH has been proposed to act as a sorting signal (Pierce & Parsons, 1981, Kohli & Muralidhar, 1990, Boime *et al.*, 1999, Bousfield George R. *et al.*, 2006). Analysis of the asparagine (N)-linked oligosaccharides of human, bovine and ovine gonadotropins revealed that these structures terminate with sulfate or sialic acid (Pierce & Parsons, 1981, Green & Baenziger, 1988a). However, sulfate and sialic acid are the primary moieties on LH and FSH, respectively (Parsons & Pierce, 1980, Green & Baenziger, 1988b). It is unknown if mouse LH and FSH show the same difference in sulfation/sialylation as human, bovine and ovine glycoproteins. Sulfation of the N-linked oligosaccharides occurs after subunit assembly, but prior to entry into the secretory vesicles (Hoshina & Boime, 1982, Green *et al.*, 1984, Blomquist & Baenziger, 1992), consistent with the hypothesis that sulfate is a sorting signal. TSH, which is secreted from the thyrotrope cells of the pituitary in a regulated manner, contains sulfated N-linked oligosaccharides (Gesundheit *et al.*, 1986, Green & Baenziger, 1988b). The β subunit of CG, which evolved from LH β contains sialylated N-linked oligosaccharides exclusively, and CG dimer is secreted constitutively from the placenta (Parsons & Pierce, 1980). Thus, the sulfated glycoprotein hormones (LH, TSH) are secreted through a regulated pathway while sialylated glycoproteins (FSH, CG) are secreted constitutively. Interestingly, equine pituitary LH and placental CG have identical amino acid sequences (Bousfield G. R. *et al.*, 1987). However, eLH is sulfated and secreted in a regulated manner, while eCG is sialylated and secreted constitutively (Matsui *et al.*, 1991, Smith *et al.*, 1993). These correlative data also support the hypothesis that sulfate is a sorting signal for LH.

Mice lacking GalNac-4-sulfotransferase, the enzyme responsible for the addition of sulfate to oligosaccharides, have recently been described (Mi *et al.*, 2008). These mice had an increased concentration of plasma LH which leads to increased precocious sexual maturation and increased fecundity in females. The authors report that the increased plasma concentration of LH was due to an increased half-life and decreased rate of clearance. Increased plasma concentrations of LH could also be explained by a less regulated and more constitutive secretion of LH from the pituitary, but this was not examined.

Previous studies of protein sulfation and regulated secretion indicate that sulfation is not involved with regulated secretion. Sulfation of tyrosine residues was found not to be involved with the sorting of proopiomelanocortin in *Xenopus* neurointermediate pituitary (van Kuppveld *et al.*, 1997), of chromogranin A in porcine parathyroid glands (Gorr & Cohn, 1990), and of prolactin and growth hormone from GH₃ cells (Hinkle *et al.*, 1992). Proteoglycan

sulfation did not contribute to sorting of salivary gland proline-rich-proteins (Castle & Castle, 1998). Carbohydrate sulfation did not contribute to the sorting of lysosomal enzymes in *Dictyostelium discoideum* (Cardelli et al., 1990). However, no studies on the role of carbohydrate linked sulfation and regulated secretion exist for the glycoprotein hormones. Here we tested the hypothesis that carbohydrate linked sulfate participates in the sorting of LH. Mouse pituitaries were treated with sodium chlorate, an inhibitor of protein and carbohydrate sulfation, to investigate the synthesis and secretion of sulfated and non-sulfated LH. The results demonstrate that sulfation of LH does not function as sorting signal.

Methods

Animals

Eight week old female, C57B/6J mice (Jackson Laboratories; Bar Harbor, ME) were euthanized with CO₂ and pituitaries were collected. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Division of Comparative Medicine at the Washington University School of Medicine.

Metabolic Labeling of Pituitaries

Individual pituitaries were labeled with 75 µCi/ml [³⁵S]cysteine (MP-Biomedicals; Solon, OH) in cysteine-free Ham's F-12 media for 24 hours at 37 °C in the absence or presence of 5 mM sodium chlorate (Sigma; St. Louis, MO). Labeling with inorganic sulfate was performed for 24 hours in Ham's F-12 sulfate-free medium with 1.4 mCi/ml carrier-free sulfate (Na₂[³⁵S]O₄; MP-Biomedicals). Labeling media was supplemented with 7.5% dialyzed fetal bovine serum, glutamine and antibiotics. Sodium chlorate has been demonstrated to be an effective inhibitor of sulfate addition to tyrosine residues, N-linked oligosaccharides and proteoglycans (Baeuerle & Huttner, 1986, Hortin *et al.*, 1988). Furthermore, use of sodium chlorate did not inhibit protein synthesis or show any other toxic effects on the cells in those studies. At the end of incubation, pituitary culture media were aspirated and added to an equal volume of media containing protease inhibitors (Complete Mini; Roche Diagnostics; Indianapolis, IN). Pituitaries were rinsed with PBS and homogenized in PBS containing protease inhibitors (Complete Mini; Roche Diagnostics) using a hand held pestle. Media and homogenate samples were centrifuged to remove debris and stored at -20 °C until immunoprecipitation.

Buserelin Stimulation

Individual pituitaries were labeled for 24 hours with [³⁵S]O₄ or [³⁵S]cysteine in the absence or presence of 5 mM sodium chlorate. Media was collected at the end of 24 hours to serve as controls for differences in the extent of synthesis by individual pituitaries. Pituitaries were washed and then incubated in chase medium (+/- chlorate) for four hours with or without 5 ng/ml Buserelin. At the end of the four hour incubation, media and homogenates were collected for immunoprecipitation.

Immunoprecipitation

Media and pituitary homogenates were pre-cleared with 7.5 µl/ml of normal rabbit serum (Sigma) and Pansorbin (EMD BioSciences Inc., La Jolla, CA). The supernates were immunoprecipitated for three hours at room temperature with anti-human CGβ (Muyan *et al.*, 1996) or anti-rat FSH polyclonal serum (National Hormone Peptide Program). The antisera cross react with the mouse LHβ and FSHβ subunit, respectively. Immune complexes were precipitated with Pansorbin and subjected to SDS-PAGE electrophoresis on 12.5% acrylamide gels. The gels were soaked in 1 M sodium salicylate for 15 min, dried and exposed to Iso-Max film (Sci-Mart; St. Louis, MO).

Statistical Analyses

Band intensity was quantitated by densitometry using Quantity One software (Bio-Rad Laboratories; Hercules, CA). Homogenate/medium ratios (H/M) correspond to the ratio of band intensity observed in the homogenate divided by the band intensity observed in the medium. Fold stimulation of LH corresponds to the ratio of the band intensity obtained with and without Buserelin. To account for differences in unstimulated secretion, the fold difference of secreted protein after 24 hours of labeling was used to adjust the post-Buserelin secretion values. Data was analyzed by paired t-tests with $p < 0.05$ considered significant (MiniTab; State College, PA) and are expressed as the mean $\pm$ SEM.

Results

This study was based on two premises: LH and FSH are secreted through the regulated and constitutive secretory pathways, respectively, and that differences exist in oligosaccharide processing between LH and FSH. To address if the difference in LH and FSH secretion patterns can be recapitulated in the mouse, individual pituitaries were labeled with [^{35}S] cysteine for 24 hours; the media and tissue homogenates were immunoprecipitated with antisera against the CG β or FSH β subunit (Fig. 1). The α and β subunits of LH were observed in both the media and homogenate with more than 2 fold (2.28 ± 0.18) greater amounts of subunit present in the homogenate (Fig. 1, lanes 1-4). The presence of LH in the homogenate indicates that it accumulated in the pituitary, which is consistent with LH secretion through the regulated pathway. In contrast, α and β subunits of FSH were observed exclusively in the media and not in the homogenates (Fig. 1, lanes 5-8). This is consistent with a protein that is constitutively secreted. Also, heterogeneity of the FSH β subunit was observed, consistent with previous observations of FSH synthesis in other species (Ulloa-Aguirre et al., 1995).

Mouse pituitaries were next labeled with [^{35}S]O $_4$ for 24 hours to assess the extent of sulfation for LH and FSH. Both the α and β subunits of LH were sulfated (Fig. 2A, lanes 1-4; see also Fig. 4, lanes 1-4). While there was variability between individual pituitaries, the amount of accumulated LH was 3 fold (2.92 ± 0.38) higher than secreted LH. This indicates that the majority of sulfated LH accumulated in the pituitary. FSH was also sulfated in the mouse, although to a lesser extent than LH (Fig. 2A, lanes 5-8). As seen for cysteine labeled FSH, there was heterogeneity of the labeled β subunit. In contrast to LH, no accumulation of FSH was observed in the homogenate, even after prolonged exposure (Fig. 2B). Thus, despite synthesis of a significant fraction of sulfated FSH, none of it was detected intracellularly, suggesting that sulfate does not function as a sorting signal for the regulated pathway.

To confirm that LH was in the regulated pathway, pituitaries were treated with the GnRH analog, Buserelin (Fig. 3). Pairs of pituitaries were incubated with [^{35}S]O $_4$ for 24 hours at which point the media was collected. Subsequently, one pituitary was incubated for four hours with Buserelin, and the other incubated without Buserelin. To account for variations in synthesis between the two pituitaries, differences in the amount of secreted protein after 24 hours of labeling (Fig. 3, lanes 1 and 2), were used to normalize the post-Buserelin secretion values. Buserelin stimulated secretion of sulfated LH 2.30 ± 0.33 fold (Fig. 3, lanes 3, 4) with a decrease in the homogenates of 1.17 ± 0.13 fold (Fig. 3, lanes 5, 6). The secretory response of LH to Buserelin demonstrates that LH was stored in the regulated pathway. The change in intracellular content did not correspond to the enhanced secretion likely due to a fraction of LH present in a non-releasable pool.

To test whether sulfate acts as a sorting signal for LH, pituitaries were metabolically labeled in the presence of sodium chlorate, an inhibitor of sulfation. To test the effectiveness of sulfate inhibition, pituitaries were first incubated with [^{35}S]O $_4$. After immunoprecipitation and electrophoresis, labeled LH α and β subunits were observed in untreated pituitaries (Fig. 4,

lanes 1-4). No labeled proteins were detected in the media or homogenates of pituitaries treated with chlorate (Fig. 4, lanes 5-8). This indicates that chlorate effectively inhibited incorporation of sulfate into LH. The dose of 5 mM was chosen because it was the lowest dose that completely inhibited sulfate labeling of LH. To exclude the possibility that chlorate disrupted protein synthesis, pituitaries were incubated with ^{35}S]cysteine and treated with chlorate (Fig. 5). It is evident that LH synthesis was unaffected by chlorate. To determine if the amount of storage and secretion was altered by lack of sulfation, pituitary homogenate to media (H/M) ratios were calculated for ^{35}S]cysteine labeled LH. The H/M ratio was 2.28 ± 0.18 for LH secreted from untreated pituitaries and 2.02 ± 0.18 for LH secreted from chlorate treated pituitaries. Similar H/M ratios suggest that the accumulation and secretion of LH was not significantly affected ($p = 0.3$) by the lack of sulfation.

The LH responsiveness of chlorate-treated pituitaries to Buserelin was examined (Fig. 6). To account for differences in unstimulated secretion, the amount of secreted protein after 24 hours of labeling (Fig. 6A; lanes 1 and 2, 3 and 4) was used to adjust the post-Buserelin values (Fig. 6B; lanes 5 and 6, 9 and 10). Buserelin stimulated LH secretion 2.39 ± 0.28 fold from untreated pituitaries. The fold stimulation observed varied from 1.44 to 3.29 with one value at 4.76. Buserelin stimulated LH secretion 1.82 ± 0.12 fold with a range of 1.31 to 2.63 from chlorate treated pituitaries. The fold decrease of LH observed in the homogenate was 1.16 ± 0.13 for untreated pituitaries and 1.30 ± 0.08 for chlorate-treated pituitaries. The LH response to Buserelin was not significantly different (media, $p = 0.09$; homogenates, $p = 0.37$) suggesting that the efficiency of sorting to secretory granules was unaltered by the lack of sulfate. These results, together with the data showing no change in the ratio of stored and secreted LH, demonstrate that sulfation of LH is not required for sorting to secretory granules of the regulated pathway.

Discussion

Protein exocytosis from cells occurs through either the constitutive or regulated secretory pathway. Secretion of protein through the constitutive pathway occurs without an external stimulus and vesicles travel from the Golgi to the plasma membrane within minutes (Kelly, 1985, Burgess & Kelly, 1987); therefore, secretion is coupled to the rate of protein synthesis. Proteins secreted through the regulated pathway are concentrated in granules, can be stored within the cell for extended periods, and require an external stimulus to trigger exocytosis of the granules (Kelly, 1985, Burgess & Kelly, 1987). In contrast to constitutively secreted proteins, synthesis and secretion of regulated secretory proteins occurs as two distinct steps allowing for large amounts of protein to be secreted at a specific time.

LH and FSH are produced in the same gonadotrope cell but differ in their mode of secretion. LH and FSH can be localized in different secretory vesicles (Thomas & Clarke, 1997, McNeilly *et al.*, 2003, Nicol *et al.*, 2004) consistent with different secretory pathways. Portal blood sampling in sheep demonstrated that while there is some basal LH secretion, and episodic FSH secretion, LH is primarily secreted via the regulated pathway and FSH secretion is primarily constitutive (Midgley *et al.*, 1997, Padmanabhan *et al.*, 1997, McNeilly *et al.*, 2003). Similar results were observed in cell culture studies using GH₃ cells transfected with human LH and FSH (Muyan *et al.*, 1994). In mice, plasma concentrations of FSH are much higher than LH, consistent with constitutive and regulated secretion, respectively. However, the pituitary concentrations of the two hormones are similar suggesting similar amounts of hormone storage (Parkening *et al.*, 1980, Collins *et al.*, 1981, Parkening *et al.*, 1982b, a). Our results demonstrate that, as seen in other species, mouse LH accumulates in the pituitary and is secreted through the regulated pathway, whereas FSH does not accumulate and is constitutively secreted. Increasing the amount of FSH in mouse pituitaries by altering its mRNA expression so that it mimicked the expression profile of LH, did not affect the storage of FSH (Brown *et al.*,

2001); suggesting that intracellular hormone concentration does not affect sorting and storage within the pituitary.

The (N)-linked oligosaccharides of human, bovine and ovine gonadotropins terminate with sulfate or sialic acid (Pierce & Parsons, 1981, Green & Baenziger, 1988a). In these species, sulfate was present in 49-73% of the total oligosaccharides for LH, while sialic acid was present in 38-88% of the total oligosaccharides for FSH (Green & Baenziger, 1988b). Within each species, the oligosaccharides of LH were more sulfated and less sialylated than the oligosaccharides of FSH. Similar data for the mouse has not been reported. The results reported here are consistent with other species and demonstrate that mouse LH is highly sulfated, and while a significant portion of mouse FSH is sulfated, the degree of sulfation is much less than LH.

Two models have been proposed to account for how proteins enter the regulated pathway: sorting for entry or sorting by retention (Tooze, 1998). Both models require a sorting signal or determinant within the protein to either enter the secretory granule from the trans-Golgi or be retained within the secretory granule as other proteins are removed. Constitutive secretion appears to be the default pathway as proteins without a sorting signal are secreted through this pathway.

Sulfation of LH has been proposed to act as a sorting signal (Pierce & Parsons, 1981, Kohli & Muralidhar, 1990, Boime *et al.*, 1999, Bousfield George R. *et al.*, 2006). Kohli and Muralidhar (1990) compared the LH-RH response of LH by indirect double labeling experiments. From these data they suggested that sulfate could be a sorting signal for LH. Our experiments provide the first direct test of sulfation and its role in LH sorting. The results demonstrate that sulfation of LH does not function as a sorting signal to the regulated secretory pathway. Similar studies performed by us with GH₃ cells expressing human LH, also demonstrated that sulfate was not involved with sorting of LH to the regulated pathway (data not shown). These results are consistent with previous findings showing that carbohydrate sulfation does not contribute to the sorting of lysosomal enzymes in *Dictyostelium discoideum* (Cardelli *et al.*, 1990), nor does tyrosine sulfation contribute to the sorting of proopiomelanocortin in *Xenopus* neurointermediate pituitary (van Kuppeveld *et al.*, 1997).

The full complement of sorting signals for LH to the regulated pathway is currently unclear. The only signal that has been identified for the glycoprotein hormones is the carboxy-terminal heptapeptide of the LH β subunit (Jablonka-Shariff *et al.*, 2008). Cell culture studies using GH₃ cells showed that removal of the heptapeptide caused a less regulated secretion of LH whereas, addition of the heptapeptide to the FSH β subunit, induced a more regulated secretion of FSH. The mechanism by which the heptapeptide causes sorting is currently under investigation.

Based on the results reported here, sulfate is not involved in the intracellular sorting of the LH and FSH. However, the degree of sulfation/sialylation results in different charge isoforms of these hormones (Ulloa-Aguirre *et al.*, 2001) which exhibit varying degrees of receptor activation (Stanton *et al.*, 1996). Higher sulfate content also increases the rate of clearance from the circulation (Baenziger *et al.*, 1992, Wide *et al.*, 2008). Thus, the amount of sulfate present on LH and FSH is critically important for in vivo potency by 1) controlling the rate of clearance which contributes to hormone concentration and pulsatility and 2) influencing receptor activation and therefore target tissue response to the gonadotropin hormones.

Acknowledgements

This work was supported in part by National Institutes of Health Grant DR065155. CAP was supported by Training Grant T32HD049305-03 from the National Institutes of Health/NICHD. We thank Drs. Alan McNeilly and Albina Jablonka-Shariff for their comments regarding the manuscript.

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Figure 1.

Storage and secretion of cysteine labeled LH (lanes 1-4) and FSH (lanes 5-8) from mouse pituitaries. Fourteen pituitaries were incubated for 24 hours with 75 μ Ci/ml [35 S]cysteine and immunoprecipitated with antisera against CG β (n = 7 pituitaries) or FSH β (n = 7 pituitaries). Two representative pituitaries are shown for both LH and FSH. More LH accumulated intracellularly than was secreted, while all of the FSH was secreted. The migration of the α and β subunits is indicated. H = pituitary homogenate, M = medium.

Figure 2.

Storage and secretion of sulfate labeled LH (lanes 1-4) and FSH (lanes 5-8) by mouse pituitaries. Ten pituitaries were incubated for 24 hours with 1.4 mCi/ml [^{35}S]O $_4$ and immunoprecipitated with antisera against CG β (n = 5 pituitaries) or FSH β (n = 5 pituitaries). Two representative pituitaries are shown for both LH and FSH. Three day exposure of LH and FSH (Panel A); eight day exposure of FSH (Panel B; lanes 5'-8'). Note that even after prolonged exposure, no sulfated FSH was detected in pituitary homogenates. The migration of the α and β subunits is indicated. H = pituitary homogenate, M = medium.

Figure 3.

Secretagogue stimulation of sulfate labeled LH. The experiment was repeated five times using two individual pituitaries in each replicate (ten total pituitaries); one representative replicate is shown. Lanes 1 and 2 show the secretion of LH from one pair of untreated pituitaries after 24 hours of labeling with 1.4 mCi/ml [^{35}S]O $_4$. Subsequently, pituitary *b* (lane 2) received 5 ng/ml Buserelin (lanes 4 and 6) for 4 hours, while pituitary *a* (lane 1) was untreated (lanes 3 and 5). All media and homogenates were immunoprecipitated with antiserum against CG β . Buserelin stimulated an approximately 2-fold release of LH from the pituitary (compare lanes 3 and 4). H = pituitary homogenate, M = medium.

Figure 4.

Sulfation of LH in pituitaries treated with chlorate. Sixteen pituitaries were incubated for 24 hours with 1.4 mCi/ml [35 S]O $_4$ in the absence (untreated, n = 8 pituitaries) or presence (treated, n = 8 pituitaries) of 5 mM sodium chlorate and then immunoprecipitated with antiserum against CG β . Two representative pituitaries are shown for each group. The absence of labeled subunits in the presence of chlorate indicates that sulfation of LH was inhibited. H = pituitary homogenate, M = medium.

Figure 5.

Synthesis of LH from pituitaries treated with chlorate. Sixteen pituitaries were incubated for 24 hours with 75 $\mu\text{Ci/ml}$ [^{35}S]cysteine in the absence (untreated, $n = 8$ pituitaries) or presence (treated, $n = 8$ pituitaries) of 5 mM sodium chlorate and then immunoprecipitated with antiserum against CG β . Two representative pituitaries are shown for each group. Protein synthesis was unaffected by incubation with chlorate and the amount of accumulated LH appears unaltered by the lack of sulfate. H = pituitary homogenate, M = medium.

A. Pre-Buserelin Media

B. Buserelin Treatment

Figure 6.

Secretagogue stimulation of LH in the absence (sulfated) or presence (non-sulfated) of 5 mM sodium chlorate. The experiment was repeated 12 times using four individual pituitaries in each replicate (48 total pituitaries); one representative replicate is shown. Four individual pituitaries (designated *a-d*) were incubated with 75 μ Ci/ml [35 S]cysteine for 24 hours in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of chlorate. Subsequently, pituitaries *b* and *d* were incubated for 4 hours with 5 ng/ml Buserelin (lanes 6, 8, 10, 12) while pituitaries *a* and *c* were untreated (lanes 5, 7, 9, 11). Each medium/homogenate set in panel B (e.g. pituitary *a*, lanes 5 and 7) corresponds to the same lettered pituitary in panel A (e.g. pituitary *a*, lane 1). All media and homogenates were immunoprecipitated with antiserum against CG β . The secretion of LH in response to Buserelin was similar for untreated and chlorate treated pituitaries. H = pituitary homogenate, M = medium.