An autoradiographic study using [³H]glucosamine of gonadotrophin regulation of proteoglycan and glycoprotein synthesis in developing mouse follicles

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Summary. Autoradiography of serial sections of ovaries of immature mice was used, after an injection of [³H]glucosamine, to follow the migration of newly synthesized macromolecules into preantral follicles during the period of treatment with PMSG and hCG. [³H]Glucosamine was injected at the same time as the PMSG or 2-h before the time of autopsy. PMSG stimulated a modest uptake of [³H]glucosamine into the zona pellucida of preantral follicles. However, the in-vivo synthesis of labelled macromolecules increased substantially during the period of hCG stimulation, especially in those mice in which the label was injected at the same time as the PMSG. After both short and longer term exposure to [³H]glucosamine, the maximum uptake of label in preantral follicles occurred 4–8 h after the injection of hCG, indicating that hCG rather than PMSG probably exerts the greatest control over the uptake and incorporation of [³H]glucosamine into the zona pellucida and oocyte of preantral follicles. It is suggested that [³H]glucosamine is largely incorporated into non-sulphated glycosaminoglycans.

Introduction

Carbohydrate–protein complexes in the form of proteoglycans or glycoproteins are an important component of the developing follicles for they are found in substantial quantities in the follicular fluid of Graafian follicles (Yanagishita *et al.*, 1979; Ax & Ryan, 1979a), in the secretions of granulosa cells (Ax & Ryan, 1979b, Yanagishita & Hascall, 1979; Yanagishita *et al.*, 1981) and the secretions of the oocyte–cumulus complex (Eppig, 1979, 1981). They also form an integral part of the structure of the zona pellucida which surrounds the developing oocyte (Da Silva Sasso, 1959; Kang, 1974). Autoradiographic studies using carbohydrates such as [³H]fucose and acetyl [³H]glucosamine have demonstrated that the label is incorporated into zona macromolecules concentrated next to the plasma membrane of the oocyte 2–3 days after the injection and that, 7–9 days later, the labelled carbohydrates are located further from the oocyte surface (Oakberg & Tyrell, 1975; Haddad & Nagai, 1977). Quantitative and qualitative patterns of proteoglycan synthesis are almost certainly hormonally dependent since the biosynthesis of proteoglycans by rat granulosa cells cultured *in vitro* is modified by gonadotrophins and steroid hormones (Yanagishita *et al.*, 1981). In the present study, we have investigated the effect of gonadotrophins on the in-vivo synthesis of labelled macromolecules within the ovary following an injection of [³H]glucosamine into immature mice at various times after treatment with PMSG and human chorionic gonadotrophin (hCG). Autoradiography of serial sections enabled us to follow closely the synthesis and migrations of newly synthesized macromolecules into preantral follicles during the time-course of the study. The dynamic activity of the in-vivo system enabled us to investigate labelling changes in different components of the ovary induced by hormone treatments which stimulate follicular development and oocyte maturation.

Materials and Methods

The 24 MF1 albino mice, 3 weeks of age, were treated with PMSG followed after 40 h by an injection of hCG (Intervet Laboratories Ltd, Cambridge): 12 of these mice received an injection of 100 μ Ci D-[6- 3 H]glucosamine hydrochloride (Amersham International, Amersham, Bucks, U.K.: sp. act. 22.6 Ci/mmol) which was injected intraperitoneally in 0.1 ml 0.9% (w/v) NaCl. At 2, 20, 40, 42, 48 and 51 h after the injection of PMSG, 2 mice were killed. The remaining 12 mice were injected with [3 H]glucosamine 2 h before the time of autopsy and were killed at 2, 40, 44, 46, 48 and 50 h after the injection of PMSG (2 mice at each time).

Five untreated immature 3-week-old MF1 mice were also injected with 100 μ Ci [3 H]glucosamine and one was killed at 2, 8, 40, 44 and 50 h after the injection. Radiolabelled glucosamine is incorporated into carbohydrate-protein complexes which include both proteoglycans and glycoproteins. At autopsy, both ovaries and oviducts were washed in isotonic saline and then fixed in buffered formaldehyde-glutaraldehyde as described by McDowell & Trump (1976) containing 1.0% cetylpyridinium chloride.

The ovaries were processed as described previously (Fowler & Grainge, 1985) and serial sections cut at 5 μ m. Tissue sections were deparaffinized, hydrated and coated with Kodak AR10 stripping film. After exposure for 4 weeks the sections were developed in Kodak D19, fixed in Johnsons Fixol, washed and then air-dried. Sections were stained with Ehrlich's haematoxylin, air-dried and mounted in Dammar xylene. Free [3 H]glucosamine not incorporated into macromolecules and that incorporated into glycolipids is extracted during the course of fixation and other treatments in preparation for autoradiography. Preantral follicles were classified according to the number of layers of granulosa cells surrounding the oocyte. In general, the location of the label was clearly defined and well above background.

Results

Incorporation of [3 H]glucosamine into the ovaries of untreated immature mice

After 2 h there was little label in the autoradiographs of ovarian sections except in the follicular fluid of the larger antral follicles and in some atretic follicles. The labelling density within the ovary had increased rapidly by 8 h especially within the zona pellucida and follicular fluid of large antral follicles (Fig. 1). The density of labelling in most preantral follicles remained relatively low (Fig. 1) when compared with the labelling in large antral follicles. The intensity of labelling within the zona pellucida of preantral follicles after 40 h was essentially similar to that found after 8 h exposure to [3 H]glucosamine (Fig. 2).

Indeed, the intensity of label specifically located within the zona pellucida of preantral follicles was maintained during the time-course of the experiment (Fig. 9).

Injection of [3 H]glucosamine at the time of the injection of PMSG

After 2 h, there was a significant uptake of radio-labelled glucosamine into the zona pellucida and follicular fluid of antral follicles, although the density of silver grains within the zonae of preantral follicles was lower. Significant amounts of label remained incorporated within the ovary after stimulation with PMSG for 20–40 h. The intensity of labelling within the small preantral follicles remained relatively low (Figs 3 & 4). The highest silver grain density during this period was found in the follicular fluid and the zona pellucida of the larger antral follicles which develop in response to PMSG (Figs 10 & 11b). However, the intensity of label within the largest antral follicles appeared to have decreased by 40 h (Fig. 11).

After stimulation with hCG, there was an overall increase in silver grain density within both the thecal and interstitial layers of the ovary in addition to the high density of labelling observed in preantral (Figs 5–8) as well as preovulatory follicles (Fig. 12). Indeed, the overall increased density of labelling within the ovary reached a peak about 8 h after the injection of hCG, i.e. 48 h after the injection of labelled glucosamine. Counts made of the number of silver grains within the zona pellucida of sectioned 2–3-layer preantral follicles during the period of PMSG and hCG stimulation also reached a maximum 8 h after hCG, the density of silver grains being at least 4 times higher than that found in untreated immature mice during the same period of labelling (see Fig. 9). In many preantral follicles, the highest density of label was located on the inner edge of the zona

Fig. 1. An autoradiograph of a section through the ovary of an untreated 21-day-old mouse autopsied 8 h after the injection of [^3H]glucosamine. The follicular fluid (FF) is heavily labelled. There is specific labelling on the zona pellucida (ZP) of the oocyte from the largest antral follicles (AF) but silver grain density is much lower on the zona pellucida of the smaller preantral follicles (PF). Dark field, $\times 175$.

Fig. 2. An autoradiograph (a) and a photomicrograph (b) of two 2-layer preantral follicles 40 h after the injection of label into an untreated 21-day-old mouse. Note the presence of a germinal vesicle (GV) in one of the follicles. (a) Dark field, $\times 350$; (b) haematoxylin, $\times 350$.

Fig. 3. An autoradiograph (a) and a photomicrograph (b) of a 2-layer preantral follicle from a mouse autopsied 20 h after stimulation with PMSG and injection of [^3H]glucosamine. The specific labelling of the zona is relatively low compared with the high density of labelling in the zona of oocytes in large antral follicles in the same ovary (see Fig. 10). A germinal vesicle is present. (a) Dark field, $\times 350$; (b) haematoxylin, $\times 350$; 20 h label.

Fig. 4. An autoradiograph (a) and a photomicrograph (b) of two 2–3-layer preantral follicles 40 h after stimulation with PMSG and injection of [^3H]glucosamine. (a) Dark field, $\times 350$; (b) haematoxylin, $\times 350$; 40 h label.

Fig. 5. A large preantral follicle with a germinal vesicle 8 h after the injection of hCG (a) and an autoradiograph of the same follicle (b) illustrating the high density of label in the zona pellucida, particularly in the region of the egg plasma membrane (48 h label). The labelling of the oocyte is now well above background. (a) Haematoxylin, $\times 700$; (b) dark field, $\times 700$.

Fig. 6. A 2-3-layer preantral follicle with a germinal vesicle 8 h after the injection of hCG (a) and an autoradiograph of the same follicle (b) shows the high density of labelled macromolecules within the zona pellucida and the increased labelling within the oocyte itself (compare Fig. 4a). (a) Haematoxylin, $\times 700$; (b) dark field, $\times 700$: 48 h label.

next to the plasma membrane of the egg (Fig. 5) but this was by no means always so (Fig. 6). Between 4 and 8 h after the injection of hCG many of the oocytes in preantral follicles also showed significant labelling (Figs 5b & 6b). The oocytes in many preovulatory follicles were also heavily labelled during this period as illustrated in Fig. 13(b). However, the labelling of preovulatory oocytes appeared to be transitory because, by the time of the extrusion of the first polar body, the labelling of oocytes was reduced to background levels.

Fig. 7. An autoradiograph of a group of small preantral follicles 8 h after the injection of hCG. Note high density labelling of the zona pellucida as compared with Fig. 4(a). Dark field, $\times 175$: 48 h label.

Fig. 8. An autoradiograph of a selection of follicles within the ovary 13 h after the injection of hCG (53 h label). The zonae are still heavily labelled although the density of label is not as high as that found 4 h before ovulation (compare Fig. 7). Dark field, $\times 175$: 53 h label.

Fig. 9. The effect of PMSG and hCG on silver grain density within the zona pellucida of 2–3-layered preantral follicles. Grain counts were made from autoradiographs of sectioned ovaries. Only those sections in which the germinal vesicle was present were used to obtain mean estimates ($\pm$ s.e.m.) of the silver grain density within the zona pellucida. Ovaries were labelled *in vivo* by injecting the [3 H]glucosamine at the time of the injection of PMSG (see 'Methods') or by injecting the label 2 h before death. Untreated immature mice injected with [3 H]glucosamine, and autopsied at similar times to those treated with gonadotrophins, served as controls. The first estimation of silver grain density was after 2 h in both groups treated with PMSG and after 8 h in untreated controls (see text).

Fig. 10. An autoradiograph of a large antral follicle 20 h after stimulation with PMSG. Note the high density of label in the follicular fluid (FF) and the specific labelling of the zona pellucida of the oocyte. Dark field, $\times 175$: 20 h label.

Fig. 11. A photomicrograph (a) and an autoradiograph (b) of a large preovulatory follicle containing an oocyte with a germinal vesicle 40 h after stimulation with PMSG. The intensity of label over the zona pellucida and follicular fluid appears to have declined (compare Fig. 10). (a) Haematoxylin, $\times 350$; (b) dark field, $\times 350$: 40 h label.

An accumulation of labelled macromolecules within the zona pellucida of preantral follicles persisted after ovulation, i.e. 13 h after hCG (Fig. 8) although the developing corpora lutea remained largely unlabelled (Fig. 14).

Incorporation of [^3H]glucosamine for 2-h periods during the stimulation of immature mice by PMSG and hCG

PMSG stimulated a rapid uptake of labelled macromolecules into the follicular fluid and zona pellucida of the largest antral follicles within the 2-h exposure period although the labelling of

Fig. 12. A photomicrograph (a) and an autoradiograph (b) of a large preovulatory follicle 8 h after the injection of hCG (i.e. 48 h label). The oocyte contains metaphase I chromosomes. There is a large increase in silver grain density within the follicular fluid (compare Fig. 11b) and over the cumulus cells immediately surrounding the preovulatory oocyte. (a) Haematoxylin, $\times 175$; (b) dark field, $\times 175$.

Fig. 13. A photomicrograph of a large preovulatory follicle with an oocyte containing metaphase I chromosomes 8 h after the injection of hCG, and an autoradiograph of the oocyte (b). Note the heavy labelling of the oocyte (O) in this follicle and the almost complete lack of labelling within the zona pellucida itself. (a) Haematoxylin, $\times 175$; (b) dark field, $\times 700$: 48 h label.

Fig. 14. An autoradiograph of a section through a corpus luteum (CL) 13 h after the injection of hCG (i.e. ~ 1 h after ovulation). Note the high density of label in the follicle fluid of antral follicles and the zona pellucida of a preantral follicle while the developing corpus luteum remains largely unlabelled. Dark field, $\times 175$: 53 h label.

preantral follicles remained low (Figs 15 & 16). The labelling of the zona pellucida of the preantral follicles during the period of PMSG stimulation increased steadily (Fig. 9) but remained below that of untreated controls. However, there was a rapid increase in the density of label within the zonae of ovarian follicles during the period of hCG stimulation (Figs 17 & 18) which reached a maximum 8 h after hCG (Fig. 9). This is similar to that observed when the [^3H]glucosamine was injected at the

Fig. 15. An autoradiograph (a) and a photomicrograph (b) of a single-layer follicle 40 h after stimulation with PMSG showing little specific labelling of the zona pellucida. (a) Dark field, $\times 700$; (b) haematoxylin, $\times 700$; 2 h label.

Fig. 16. A photomicrograph (a) and an autoradiograph (b) of a sectioned 2-layer preantral follicle with a germinal vesicle 40 h after stimulation with PMSG. Note the density of silver grains specifically associated with the zona pellucida. (a) Haematoxylin, $\times 700$; (b) dark field, $\times 700$.

Fig. 17. An autoradiograph showing a selection of preantral follicles 8 h after the injection of hCG. Note the increased density of silver grains in the zonae pellucidae of most of the follicles. Dark field, $\times 175$; 2 h label.

Fig. 18. An autoradiograph (a) and a photomicrograph (b) of a preantral follicle 8 h after the injection of hCG illustrating the high specificity of labelling associated with the zona pellucida. There was no evidence for an accumulation of label next to the egg plasma membrane as observed in Fig. 5(b). (a) Dark field, $\times 700$; (b) haematoxylin, $\times 700$; 2 h label.

Fig. 19. A photomicrograph (a) and an autoradiograph (b) of a 2–3-layer follicle with a germinal vesicle 10 h after the injection of hCG. Note that the specific labelling of the zona pellucida is very much reduced (see Fig. 18a). (a) Haematoxylin, $\times 700$; (b) dark field, $\times 700$; 2 h label.

time of PMSG, but the peak was lower, presumably due to the short incubation time. However, the zonae were uniformly labelled (Fig. 18) and a concentration of labelling next to the egg plasma membrane was not observed (compared with Fig. 5b).

Between 8 and 10 h after hCG the uptake of label within the preantral follicles was markedly reduced (Fig. 19), as illustrated in Fig. 9. Maximum labelling of preovulatory follicles was also

observed between 4 and 8 h after hCG with a progressive increase in label over the cumulus cells before ovulation. During 2-h incubations with [^3H]glucosamine, the labelling of oocytes was generally not above background.

Discussion

The presence of protein-carbohydrate complexes in the form of proteoglycans or glycoproteins in such a wide variety of ovarian components such as the zona pellucida, follicular fluid, the secretions of granulosa cells and the oocyte-cumulus complex presupposes a variety of functions although it is probable that their physiological role cannot be deduced from an analysis of glycosaminoglycans alone.

The present study clearly demonstrates that the synthesis and migration of carbohydrate-protein complexes into the preantral as well as the larger antral follicles is controlled by gonadotrophins. Although PMSG stimulated a modest uptake of [^3H]glucosamine into the zona pellucida of preantral follicles, hCG and therefore probably endogenous luteinizing hormone would appear to exert the greatest control over the uptake and incorporation of [^3H]glucosamine into macromolecules of the zona pellucida and oocyte of a preantral follicle. The maximum uptake of label into preantral follicles occurred between 4 and 8 h after the injection of hCG, after both short and longer term exposure to [^3H]glucosamine.

The distribution of label within the preantral follicle during this period appeared to depend on the duration of exposure to [^3H]glucosamine. After short-term labelling *in vivo* (2 h) 6–8 h after the injection of hCG, the zona pellucida was uniformly labelled and the density of labelling within the oocyte remained low, whereas after long-term exposure to [^3H]glucosamine (46–48 h) the oocyte labelling was well above background and the labelling intensity of the zona pellucida was frequently highest on the inner surface of the zona pellucida next to the egg plasma membrane. The rapid increase in silver grain density during short-term labelling of the ovary and more especially within the follicular fluid of antral follicles and the zona pellucida of preantral follicles after the injection of hCG probably reflects the incorporation of [^3H]glucosamine into glycoprotein molecules because the specific activity of the serum glycoprotein reaches a peak between 4 and 8 h after the injection of label (Haddad & Nagai, 1977). Indeed, studies using [^3H]fucose as a precursor indicate that the renewal and synthesis of glycoproteins is very rapid indeed (Coffey *et al.*, 1964; Bekesi & Winzler, 1967). In addition, the turnover of glycoprotein in mouse follicular fluid is relatively fast compared to the renewal of complex sulphated carbohydrates in rabbit follicles (Zachariae, 1957).

Longer term exposure to radio-labelled glucosamine probably involves incorporation into glycosaminoglycans associated with large proteoglycan aggregates. Very little is known about the role of large protein-carbohydrate complexes within the zona pellucida of developing follicles. Certainly proteoglycans with large molecular weights are generally molecules of extracellular space. It is possible that the presence of larger proteoglycan molecules within the zona pellucida may act as a molecular sieve as in the glomerula basement membrane (Kanwar *et al.*, 1980) by preventing the passage of molecules of a particular size or configuration from entering the oocyte and stimulating oocyte maturation.

Quantitative and qualitative patterns of proteoglycan synthesis are clearly hormone-dependent. A variety of reproductive hormones such as FSH, LH, hCG, prostaglandins, oestradiol-17 β , oestrone and testosterone have all been found to stimulate proteoglycan synthesis by cultured granulosa cells (Yanagishita *et al.*, 1979) although all these substances also stimulate the production of progesterone (Channing & Tsafiriri, 1977). The rapid downturn in the rate of incorporation of [^3H]glucosamine into protein-carbohydrate complexes within the zona pellucida of preantral follicles would not be expected if proteoglycan synthesis was controlled by progesterone concentrations, because progesterone values rise during the period of hCG stimulation. Early studies

demonstrated that the incorporation of $^{35}\text{SO}_4$ into mucopolysaccharides varied during the oestrous cycle (Zachariae, 1959). This control was mediated by FSH, which stimulates the in-vivo uptake of $^{35}\text{SO}_4$ into ovarian follicular mucopolysaccharides in hypophysectomized immature female rats (Mueller *et al.*, 1978). The response was found to be linear with respect to log-dose of FSH in adult rats autopsied 18 h after the last injection of $^{35}\text{SO}_4$ (Ax *et al.*, 1978). In-vitro studies with rat ovarian slices or pig granulosa cells also show an increased incorporation of $^{35}\text{SO}_4$ or $[^3\text{H}]\text{glucosamine}$ into ovarian macromolecules after stimulation by FSH (Mueller *et al.*, 1978; Ax & Ryan, 1979b), although a decrease in $^{35}\text{SO}_4$ incorporation was evoked in rat ovarian slices by LH or progesterone (Gebauer *et al.*, 1978), suggesting antagonistic functions of FSH and LH in sulphated glycosaminoglycan accumulation. Our own histochemical observations (Fowler & Grainge, 1985; Fowler *et al.*, 1986) support this view since we found no evidence for a stimulation in the synthesis of sulphated glycosaminoglycan following the injection of hCG. After coitus in rabbits, there is also no further release of sulphated mucopolysaccharides from granulosa cells (Zachariae, 1957).

Our results suggest that hCG (or LH) stimulates the rapid incorporation of $[^3\text{H}]\text{glucosamine}$ into macromolecules which pass into the zona pellucida of preantral as well as the larger antral follicles. The role of hCG in initiating changes in the preovulatory follicle is well documented (Edwards, 1980). The rapid increase in silver grain density over the granulosa and cumulus cells of the preovulatory follicles which we observed after hCG stimulation is probably largely associated with the mucification process and the synthesis of hyaluronic acid, a non-sulphated glycosaminoglycan. Mucification and hyaluronic acid synthesis have been induced *in vitro* in rat and mouse cumulus-oocyte complexes by both FSH and LH (Dekel & Kraicer, 1978; Eppig, 1979) although our own in-vivo studies indicate that mucification is stimulated by hCG or LH (Fowler, 1985) rather than FSH. This study demonstrates that hCG appears to be important in regulating the incorporation of $[^3\text{H}]\text{glucosamine}$ into glycoproteins or proteoglycans of the preantral follicle. Since hCG or LH has generally been shown to inhibit production of sulphated glycosaminoglycans (see above), the incorporation of $[^3\text{H}]\text{glucosamine}$ into proteoglycans would largely be into non-sulphated forms.

The passage of labelled macromolecule into the oocyte of preantral and antral follicles only appeared to occur between 4 and 8 h after the injection of hCG. Glycoproteins of the zona pellucida are thought to be secreted by the oocyte itself (Haddad & Nagai, 1977; Bleil & Wassarman, 1980). However, it is possible that some components of the zona pellucida are not synthesized by the oocyte; indeed, different zona components may be synthesized at different stages of the oestrous cycle. Studies on the incorporation of radiolabelled compounds into oocyte and zona pellucida indicate that the control of $[^3\text{H}]\text{glucosamine}$ incorporation into labelled macromolecules is different from that of $[^3\text{H}]\text{fucose}$ into glycoproteins (R. E. Fowler, unpublished). The synthesis of the various components within the follicle during follicular growth and maturation are clearly complex and merit further study.

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