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N-LINKED PROTEIN GLYCOSYLATION IN THE RAT PAROTID GLAND DURING AGING

ELENI E. KOUSVELARI*, DIPAK K. BANERJEE**, LALITA MURTY and BRUCE J. BAUM

Clinical Investigations and Patient Care Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892 (U.S.A.)

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SUMMARY

N-Linked protein glycosylation was examined *in vitro* in dispersed rat parotid acinar cells from young adult (3—6 months) and aged (22—24 months) rats. A small decrease in general protein production was observed with cells from aged animals (~20% lower incorporation of [^{14}C]leucine into 10% CCl_3 COOH insoluble protein during continuous pulse labeling). Incorporation of [^{3}H]mannose into N-linked glycoproteins by aged cells was further reduced (~35%). Similarly microsomal membranes from parotid glands of aged animals showed ~50% reduction in the synthesis of mannosylphosphoryl dolichol, a key intermediate in the dolichol pathway of protein N-glycosylation. Man-*P*-Dol synthase, the microsomal enzyme responsible for production of this saccharide-lipid, displayed no change in apparent K_m for GDP-mannose when preparations from aged animals were utilized, but did show ~50% reduction in V_{max}. Following β-adrenoreceptor activation, cells from both young adult and aged glands showed increased N-linked protein glycosylation almost to the same extent (~2-fold). The data suggested that in aged rat parotid cells there is a basal reduction of activity in the pathway responsible for asparagine-linked protein glycosylation, but that following exocytotic stimuli this pathway responds in a manner comparable to cells from young adult glands.

Key words: Asparagine-linked oligosaccharides; Man-*P*-Dol synthase; Dolichol pathway; Salivary gland; β-Adrenoreceptor

INTRODUCTION

Glycoproteins have multiple and varied roles in biology and often it appears to be the oligosaccharide moieties which are particularly important for glycoprotein

*To whom all correspondence should be addressed.
***Present address:* Department of Biochemistry and Nutrition, School of Medicine, University of Puerto Rico, San Juan, Puerto Rico 00936-5067, U.S.A.

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function [1]. To that end considerable attention in recent years has been focused on understanding events central to the assembly and transfer of asparagine-linked (N-linked) oligosaccharides [2,3]. It is now well established that these types of oligosaccharides are preassembled on a polyisoprenoid anchor (dolichol pyrophosphate) in the lumen of the rough endoplasmic reticulum (RER) and are co-translationally transferred to appropriate asparagine residues on nascent polypeptides [2,3]. These oligosaccharides are modified during subsequent transit through the RER and golgi apparatus en route to a final destination (e.g. lysosome, plasma membrane, extracellular locale). Perturbations at specific sites on this assembly or modification pathway (e.g. *via* genetic, pharmacologic or pathologic manipulations) can lead to altered glycoprotein production and consequently altered glycoprotein, as well as cellular, function [4—7].

Aging frequently has been associated with alterations in protein production and processing [e.g. 8—10]. In particular, several reports have described disturbances related to glycoproteins in senescent cells [e.g. 11—14]. Most recently, Blondal *et al.* have reported specific alterations in plasma membrane N-linked glycoproteins in senescent human diploid fibroblasts *in vitro* [15]. These changes were associated with significant reductions of mannose incorporation into mannosylphosphoryl dolichol, a key saccharide-lipid intermediate of the dolichol-cascade pathway of protein N-glycosylation [15]. Our laboratory, for several years, has been interested in studying regulatory mechanisms controlling N-linked glycosylation of secretory glycoproteins [e.g. 16—19]. We have used acinar cells from the exocrine rat parotid gland as a model cell for these studies. In aggregate, these studies showed that subsequent to β-adrenoreceptor activation there occurs a cyclic AMP-mediated, up-regulation of the dolichol linked pathway of protein glycosylation. Enhanced glycosylation (2—3-fold) is seen in four secretory glycoproteins and is accompanied by increased formation of saccharide-lipid intermediates (including mannosylphosphoryl dolichol) and increased activity of the dolichol-linked glycosyltransferases required for formation of these saccharide-lipids. Our laboratory also has had an interest in the status of exocrine secretory mechanisms during aging and we have characterized several aspects of stimulus-secretion coupling in the senescent rat parotid acinar cell [e.g. 20—23]. Accordingly, we were especially intrigued by the observations of Blondal *et al.* [15] and decided to determine if similar changes in N-linked protein glycosylation status during aging could be extended to a different cell type, one of epithelial origin. The present paper reports the results of this study.

MATERIALS AND METHODS

Animals

Male, Wistar-derived rats were used in these studies. All rats were obtained from the Gerontology Research Center (NIA) colony. These rats have a mean life span of

~23 months. In the present study animals 3—6 months in age are termed young adult and animals 22—24 months in age are termed aged. Animals were maintained on NIH-Purina laboratory chow and water *ad libitum*.

Cell preparation and incubation procedures

Animals were sacrificed between 0930 and 1100 h. after ether anesthesia and cardiac puncture. Parotid glands were removed, trimmed of fat and connective tissue and enzymatically dispersed cells prepared essentially as described earlier [16,17,20]. After preparation, cells were incubated with Ham's F-12 medium (NIH Media Unit), either containing low glucose (0.55 mM) or leucine-free, when [2-^{3}H]mannose (2—3 Ci/mmol, 25 μCi/ml) or [U-^{14}C]leucine (302 mCi/mmol, 2 μCi/ml) were used, respectively. Radiolabeled compounds were obtained from Amersham, Arlington Heights, IL. Cells were incubated in the absence or presence of the β-adenergic agonist isoproterenol (10 μM/ Sigma, St. Louis, MO) for 1 h [17]. Thereafter, cells were separated from medium by centrifugation (15 s at 40 *g*), media saved on ice and cells washed three times with phosphate-buffered saline (pH 7.4, PBS). Cells were resuspended in PBS and homogenized with a Brinkman Polytron (setting 5 for 10 sec). The incorporation of radiolabeled mannose or leucine into media and cellular protein was determined as 10% (w/v) CCl_3COOH insoluble material collected on Millipore filters (HA, pore size 0.45 μm). Filters were dissolved in 10 ml of Ready-Solv (Beckman, Palo Alto, CA) and radioactivity assayed by liquid scintillation spectrometry. Data are reported as cpm/μg DNA. DNA was measured according to Richards [24].

Preparation of parotid gland microsomal membranes and measurement of mannosylphosphoryl dolichol formation

Parotid glands from six rats of each age group studied were homogenized in 0.1 M Tris—HCl (pH 7.0), containing 0.25 M sucrose, 1 mM EDTA and microsomal membranes isolated by differential centrifugation following previously described procedures [18,25]. Membranes were frozen in multiple aliquots until used to measure mannosylphosphoryl dolichol formation. Enzymatic formation of mannosylphosphoryl dolichol by Man-*P*-Dol synthase was assayed by incubating microsomal membranes in 2 mM Tris—HCl (pH 7.0), containing 5 mM sucrose, 20 μM EDTA, 5 μM $MnCl_2$, 26—27.6 μg membrane protein (assayed as described by Bradford [26]) and 2.5 μM GDP-[U-^{14}C]mannose (Amersham, 203 mCi/mmol) for 5 min at 37°C [18]. Each assay was initiated by addition of GDP-mannose and data are expressed as pmol mannosylphosphoryl dolichol formed/mg protein · 5 min. In some experiments exogenous dolichol phosphate (50 μg; Sigma, St. Louis, MO) was added to incubation tubes. For kinetic experiments the concentration of GDP-[U-^{14}C]mannose was varied over a range of 0.025—0.5 μM. Following incubation, newly formed mannosylphosphoryl dolichol was extracted into chloroform/methanol (2:1, v/v) and identified by previously described procedures [18].

RESULTS

Parotid cells from aged rats show a small decrease in general protein production compared to cells obtained from young adult animals. The incorporation of [^{14}C]leucine into CCl_3 COOH insoluble material was ~20% lower (Table I). These data are similar to results reported previously by Kim and colleagues [27,28]. The incorporation of [^{3}H]mannose into parotid gland protein, which over a 1-h time period represents assembly of N-linked oligosaccharides [16,17], was also reduced in cells from aged animals but to a greater extent than seen with leucine incorporation (~35%). The ratio of [^{3}H]mannose/[^{14}C]leucine incorporation, a useful index of parotid N-linked glycosylation status [16], was ~25% lower in cells from aged rats (Table I). These data suggest the possible reduction of basal N-linked protein glycosylation in the aged rat.

Earlier studies in our laboratory have shown that β-adrenergic receptor stimulation of rat parotid cells can considerably enhance N-linked glycosylation of parotid glycoproteins [16,17]. We also have reported that the β-adrenergic receptor signal transduction coupling system is functionally intact in parotid cells from aged animals [20]. Accordingly, we next examined the ability of β-adrenergic receptor stimulation to modulate N-linked glycosylation of parotid glycoproteins in aged cells. As can be seen in Table I, treatment of cells from both young adult and aged animals with the β-adrenergic agonist isoproterenol resulted in a 2-fold enhancement of protein N-glycosylation (Table I, ratio of mannose/leucine incorporation). β-Adrenergic receptor stimulation elicits a modest increase in protein production in these cells but a marked increase in N-linked glycosylation [16]. Although absolute levels of tracer incorporation are lower in aged cells, the proportional effects in the two groups are essentially identical. Thus the β-adrenoreceptor stimulated response remains stable with age, much like β-adrenoreceptor coupled protein exocytosis [20]. Only basal glycosylation was altered in aged rats.

We next decided to more specifically evaluate basal N-linked protein glycosylation in parotid cells from aged rats. We chose to study the ability of microsomal membranes from these cells to synthesize mannosylphosphoryl dolichol, a key saccharide-lipid intermediate in the N-glycosylation pathway. Formation of this saccharide-lipid is catalyzed by the enzyme Man-*P*-Dol synthase and Blondal *et al.* [15] have reported reduced activity of this enzyme in aged fibroblasts. As can be seen in Table II, microsomal membranes from young adult parotid glands synthesize ~2-fold more mannosylphosphoryl dolichol than comparable membranes from glands of aged rats. When incubations were performed in the presence of exogenous dolichol phosphate, a condition which will promote more saccharide-lipid formation [18], membranes from both age groups showed increased synthesis of mannosylphosphoryl dolichol (~2-fold). Importantly, the proportional difference between young adult and aged samples remained the same (~2-fold) as seen with incubations without exogenously added dolichol phosphate. This suggests that any differences in saccharide-lipid formation

TABLE I

INCORPORATION OF [^{3}H]MANNOSE AND [^{14}C]LEUCINE INTO NEWLY SYNTHESIZED PROTEINS AFTER *IN VITRO* INCUBATION OF RAT PAROTID ACINAR CELLS[a]

	Young adult			*Aged*		
	[^{3}H]mannose	*[^{14}C]Leucine*	*[^{3}H]/[^{14}C]Ratio*	*[^{3}H]Mannose*	*[^{14}C]Leucine*	*[^{3}H]/[^{14}C]Ratio*
Basal	7032 ± 1439	31 462 ± 3837	0.238 ± 0.052	4453 ± 1024	25 096 ± 3712	0.182 ± 0.035*
+ Isoproterenol (10 μM)	17 643 ± 3881	35 394 ± 3829	0.488 ± 0.095	12 368 ± 2957	30 113 ± 4657	0.397 ± 0.069**

[a]All experiments were performed with cells from young adult and aged rat parotid glands incubated in parallel. Data are the mean ± S.E.M. of seven experiments (each assayed in triplicate). Data are expressed as cpm incorporated per μg DNA and were obtained as described in Materials and Methods. Data for incorporation of radiolabeled compounds into media (secreted) and cellular proteins are combined for presentation here.

*Different statistically from young adult results by paired "*t*" test, $t = 1.71$, $P = 0.069$.

**Different statistically from young adult results, $t = 1.99$, $P = 0.047$.

TABLE II

INCORPORATION OF GDP-[^{14}C]MANNOSE INTO MANNOSYLPHOSPHORYL DOLICHOL IN RAT PAROTID MICROSOMAL MEMBRANES[a]

	Young adult	*Aged*
Basal	49.5 ± 5.1 (10)	27.2 ± 1.2 (10)*
+ Dolichol phosphate (50 μg)	108.9 ± 15.0 (8)	50.7 ± 2.2 (10)**

[a] All experiments were performed with microsomal membranes from young adult and aged rat parotid glands. Data are the mean ± S.E.M. from the number of experiments indicated in parentheses. Data are expressed as pmole/mg protein · 5 min and were obtained as decribed in Material and Methods.
*Different statistically from young adult results by unpaired "*t*" test, $t = 4.19$, $P = 0.001$.
**Different statistically from young adult results, $t = 3.82$, $P = 0.003$.

seen with membranes from aged rats are not due to levels of endogenous dolichol phosphate, but are more likely related to the status of the enzyme Man-*P*-Dol synthase. Therefore, we directly examined kinetic characteristics of the enzyme in membranes from both age groups of animals. These results are shown in Table III. Enzyme activity in all membrane preparations followed Michaelis-Menten kinetics. No differences were detected in the K_m for GDP-mannose of the enzyme between young adult and aged preparations. Conversely, a marked difference in the V_{max} of the enzyme was seen. Membranes from young adult rats showed ~2-fold higher V_{max} for Man-*P*-Dol synthase compared to that seen with membranes from aged rats.

DISCUSSION

The results of this study support the conclusion that parotid cells from aged rats displayed an alteration in the basal performance of the N-linked protein glycosylation pathway. Experiments both with intact cells measuring precursor incorporation into proteins and with microsomal membranes measuring mannosylphosphoryl dolichol synthesis are consistent with this conclusion. The

TABLE III

KINETIC CHARACTERISTICS OF MAN-P-DOL SYNTHASE IN MICROSOMAL MEMBRANES PREPARED FROM YOUNG ADULT AND AGED RAT PAROTID GLANDS[a]

	K_m (μM)	V_{max} (pmol/mg protein · min)
Young adult	0.11	42.7
Aged	0.11	23.6

[a] All experiments were performed with microsomal membranes from young adult and aged rat parotid glands incubated in parallel. Data are the mean of three determnations. Data were obtained following incubation of microsomal membranes, as described in Materials and Methods, with varying concentrations of GDP-[^{14}C]mannose (0.025—0.5 μM) and analysis by the Lineweaver-Burk method.

ability of aged parotid cells to respond to a stimulus capable of upregulating basal N-linked protein glycosylation in young adult cells was, however, unchanged. β-Adrenoreceptor activation elicited the same proportional increase in response in both groups of cells. This finding is not surprising since we have preivously shown that β-adrenoreceptor signal transduction coupling is unchanged with age, with respect to exocrine protein secretion, in the rat parotid gland [20].

Several previous investigations have suggested perturbed glycosylation is associated with increased age [e.g. 11—15]. Interestingly, our results are especially consonant with findings recently reported by Blondal *et al.* [15]. These workers showed that senescent human diploid fibroblasts had significant differences related to protein N-glycosylation compared to younger cells. Several characteristics of plasma glycoproteins were altered (concanavalin A binding; mannose, fucose and glucosamine radiolabeling) and these changes were accompanied by reduced mannosylphosphoryl dolichol formation in older cells. Thus in two quite different cell types (mesenchymal and epithelial), from two diverse mammalian species (human and rat), aging has been associated with general changes in N-linked protein glycosylation and, most importantly, with a specific diminution in activity of a key enzyme in the dolichol-cascade pathway, Man-*P*-Dol synthase. This enzyme is responsible for the formation of mannosylphosphoryl dolichol, the saccharide-lipid intermediate by which are added the four mannose residues to the growing oligosaccharide chain [2,3]. Full mannosylation is necessary to permit addition of three glucose residues to the oligosaccharide core, forming the complete oligosaccharide-lipid suitable for transfer to an appropriate asparagine residue on a nascent protein [2,3]. Without commplete saccharide addition to the core, oligosaccharide transfer is greatly impaired (~10—100-fold) and altered protein glycosylation results [29,30].

Glycoproteins have many important functions in biology and often it is the oligosaccharide moieties which are of particular import to biologic activity [1]. Alterations in the extent or nature of the N-linked glycosylation process could lead to decremental changes in glycoprotein processing and routing, cell surface recognition phenomena, physical characteristics such as solubility and tertiary or quaternary structure, signal transduction events, etc. [1]; i.e. an alteration in the glycosylation pathway would be considerably amplified biologically. Blondal *et al.* [15] suggested that the changes they observed in the dolichol-linked pathway of protein N-glycosylation reflect a fundamental modification in cell biology during senescence. Our observations indeed support the possibility that alterations in protein glycosylation may underlie a number of the changes in protein function which have been associated with aging [13]. Although unequivocal proof for the existence of fundamental, senescence-associated perturbations in N-linked protein glycosylation remains to be established, the available data and the permissable speculation are intriguing. Clearly, more effort is needed to assess both the ubiquity of such changes in a broad spectrum of biological systems as well as to determine the mechanistic explanation(s) for the already observed phenomena.

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