Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;19:313-322

Accepted: November 13, 2006

Procollagen Synthesis is Increased in Hypothyroid Rat Ovary by a Parallel and Compensatory Pathway

Samir Kumar Saha[1], Pamela Ghosh[1], Samir Bhattacharya[2] and Sib Sankar Roy[1]

[1]Molecular Endocrinology Laboratory, Indian Institute of Chemical Biology, Kolkata, [2]School of Life Science, Visva Bharati University, Santiniketan

Key Words
Procollagen • HSP-47 • Prolyl Hydroxylase • Ovary• Hypothyroid

Abstract
Collagen biosynthesis is a multistep process that starts with the transcription and translation of the individual collagen gene. It is characterized by the presence of a large number of co- and posttranslational modifications. Hydroxylysine is found only in animal proteins and mostly in collagens. Procollagen lysyl hydroxylation is the first step in collagen biosynthetic pathway and lysyl hydroxylases (Plod isoforms) are responsible for this enzymatic process. Previously we showed the down regulation of Plod isoforms in hypothyroid ovary. As hypothyroidism is a stress for normal animals, we wanted to explore whether any compensatory pathway exists to balance the reduced lysyl hydroxylation of collagen in hypothyroid rat ovary. In this report we have shown that procollagen I and III are increased in hypothyroid condition and subsequently decreased upon T_3 add-back. Heat Shock Protein-47 is a collagen-specific molecular chaperone and its existence in ovary has been documented. The genes encoding HSP-47, prolyl-4-hydroxylase-α and -β (P4H- α and - β) are increased in hypothyroid condition. Down regulation of lysyl hydroxylase in hypothyroid condition results less collagen formation. At the same time over production of procollagens, HSP-47 and P4H is very significant as they may compensate the damage whatsoever caused due to hypothyroidism in ovarian tissue.

Introduction

Collagens are a large family of structural proteins that form a diverse range of highly organised supramolecular assemblies in the extracellular matrix (ECM). The role of ECM in the formation and maintenance of follicles and corpora lutea has been mentioned earlier [1-5]. The cells interact with matrix through cell surface adhesion receptors including the integrins. These focal adhesions can transduce multiple intracellular signals as well as provide the cells with anchorage. Although there are many heterodimeric combinations of the integrins, only a few of them have been localized to granulosa cells [6-8]. The role of thyroid

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1015-8987/07/0196-0313$23.50/0

Accessible online at:
www.karger.com/cpb

Sib Sankar Roy
Rm 154, Molecular Endocrinology Laboratory Indian Institute of Chemical Biology
4 Raja S. C. Mullick Road Kolkata-700032 (India)
Tel. +91-33-2473-3491, Ext. 258 Fax +91-33-2473-5197
E-Mail sibsankar@iicb.res.in

hormone in regulating the ECM protein expression has already been elucidated [9], in which ECM protein has been shown to alter due to hypothyroidism.

Biosynthesis, folding, and assembly of procollagens are complex and involve post-translational modification by at least nine ER-resident enzymes [10]. Some of these modifications, particularly hydroxylation of lysine and proline residues, are essential for efficient folding and secretion. Several studies have now demonstrated that newly synthesized procollagens are associated in the ER with the molecular chaperones HSP-47, immunoglobulin heavy-chain binding protein (BiP), glucose regulated protein 94 (GRP94) and protein disulphide isomerase (PDI) [11, 12] suggesting that they assist in the procollagen folding pathway. Hydroxylation of proline residues, and interaction with the molecular chaperone HSP-47 are unique collagen biosynthetic events.

Prolyl 4-hydroxylase (P4H) plays a critical role in collagen biosynthesis by catalysing the hydroxylation of proline residues in X-Pro-Gly triplets [13]. Almost complete hydroxylation of appropriate proline residues is necessary for stability of the fibrillar collagen triple helix at 37°C [14]. Upon reactivation of P4H, collagen folding and secretion is rapidly restored [15-17]. P4H is one candidate molecule that may monitor collagen conformation and regulate secretion. *In vitro* experiments have demonstrated that the substrate binding affinity of P4H is reduced by three orders of magnitude when collagen is fully hydroxylated, and is reduced even further when a triple helical conformation is achieved [18]. HSP-47 is a collagen-binding, heat-inducible protein, resident in the vertebrate endoplasmic reticulum. There are a number of potential phosphorylation sites in the HSP-47 sequence, and phosphorylated forms of HSP-47 can be detected in some, but not all cells [19-21], although the function and importance of HSP-47 phosphorylation is not known. Shortly after its initial discovery as a collagen-binding molecule, HSP-47 was found to be a heat-shock protein. HSP-47 mRNA is also up regulated at the transcriptional level by TGF-β [22]. The expression of HSP-47 closely correlates with that of collagen in a range of cell lines, during cell differentiation *in vitro*, and during development. HSP-47 is found in cells that synthesize collagens I and III, such as primary fibroblasts [23], 3T3 [24] and 3T6 cells [25], and in cultured chondrocytes synthesizing collagen II [26]. However, it is not expressed in cells that do not produce collagens. Expression of HSP-47 correlates both temporally and spatially with that of collagens I and II [27]. These data supports the idea that HSP-47 has an important function in collagen biosynthesis. Within the ER, procollagen is continuously bound to HSP-47, from synthesis through triple helix folding. HSP-47 continues to bind completely folded molecules and only dissociates when procollagen enters the Golgi. This ability to recognize both unfolded and mature folded proteins is unusual, and distinguishes HSP-47 from other molecular chaperones. HSP-47 may assist translocation of collagen chains into the ER during synthesis [27].

In this study we have shown that HSP-47 expression is increased in hypothyroid ovary at the mRNA as well as at the protein level. Procollagen I and -III expression was also increased in this condition. Expression levels of P4H isoforms were similarly up regulated in hypothyroid condition. As there is clear disintegration of ovarian ECM in hypothyroid condition due of down regulation of lysyl hydroxylases and activation of matrix metalloproteinases (MMPs), we hypothesize that up regulation of P4H and HSP-47 as well as that of procollagen I and -III genes could be a compensatory mechanism in the stressful condition due to hypothyroidism.

Materials and Methods

Animals and treatment

Pregnant Sprague Dawley rats raised in our animal facilities were housed in a well ventilated and temperature controlled room with a 12h light and 12h darkness schedule. They were fed with standard balanced rat pellet and drinking water was made available ad libitum. Rats were divided into two groups, 1) euthyroid, were provided with normal drinking water and their pups were used as control. 2) hypothyroid, mother rats were administered 0.02% 6-N-propyl-2-thiouracil (PTU, Sigma, St. Louis, MO) dissolved in drinking water until the end of experiment and their pups were rendered hypothyroid as they take the drug through mother's milk [28]. Hormone treatment consisted of daily single ip injections of 15ng T_3 (Sigma) per gm body weight. At 28 days of age, the total ovaries were isolated and were pooled to isolate RNA. 10 pups from each group were pooled in each experiment. At the end of the treatment periods, animals were euthanised under ether anesthesia. The Institutional Animal Ethics Committee approved all animal protocols that were followed during the experiments.

Radio Immuno Assay (RIA)

For determination of plasma thyroid hormone levels, 100µl blood from rats was collected and quickly mixed with 100µl ice-cold 0.9% NaCl containing 0.24mg EDTA. Plasma was separated by cold centrifugation and the samples were stored at -80°C until the assays were performed. Plasma T_3 was determined by RIA using T_3 RIA kit (Riak-4, Board of Radiation and Isotope Technology, Bhaba Atomic Research Center, Mumbai, India). After incubation, the tubes were thoroughly decanted and the

Table 1. Serum T_3 and TSH levels in control and in experimental rats. Serum samples were collected from the control, hypothyroid, T_3-treated control and T_3-treated hypothyroid rats and T_3, TSH were assayed as mentioned in the methods section.

	T_3(ng/ml)	TSH (μIU/ml)
Con	2.6 ± 0.03	0.32 ± 0.032
Con+PTU-T_3	0.495 ± 0.025	3.75 ± 0.021
Con+PTU+T_3	2.2 ± 0.01	0.22 ± 0.015
Con-PTU+T_3	3.65 ± 0.008	0.25 ± 0.011

Gene Product	GenBank Acc.no.	Forward Primer	Reverse Primer	Size of the amplicon
GAPDH	BC059110	5GCCATCAACGACCC CTTC3`	5AGCCCCAGCCTTCTC CA3`	237 bp nt-937-1173
Procollagen I	Z78279	5TAAAGGGTCATCGT GGCTTC3`	5ACTCTCCGCTCTTCC AGTCA3`	501 bp nt-3287-3787
Procollagen III	XM_343563	5GATCAGATGGTCAG CCAGGT3`	5CATCTTTTCCAGGAG GTCCA3`	495bp nt-2894-3388
HSP-47	D12907	5AGGTCACCAAGGAT GTGGAG3`	5CCAGATGTTTCTGCA GGTCA3`	405bp nt-557-961
P4Hα	BC078703	5GGCAATCTTCCAGG AGTGAA3´	5GGCAGGTAGTCCAC AGCAAT3`	401bp nt-451-851
P4Hβ	NM-012998	5CTACGATGGCAAAT TGAGCA3`	5CTTCCACCTCATTGG CTGTT3`	500bp nt-804-1303

Table 2. The oligonucleotide primers and the respective amplicon sizes of the different genes used in the Q-PCR have been listed.

bound radioactivity was determined by a γ-counter (Electronics Corporation of India Limited, India). Standard curves were constructed by plotting the amount of total radioactivity bound against the hormone concentration. The sensitivity of T_3 was 0.24ng/ml of the sample based on 90% B/B_0 intercept.

ELISA

ELISA was performed for serum TSH using Pathozyme TSH kit (Omega Diagnostics Ltd., Alva, UK) following manufacturer's instructions. In short, the samples, standards and enzyme conjugate were dispensed into antibody coated well, incubated 1hr at RT (20-25°C) and washed. To this, substrate TMB was added and incubated in the dark for 20min for colour development. The absorbance was noted immediately in plate reader (Qualigens, Mumbai, India) using 450nm primary filter. The inter assay CV was 6% and intra assay CV was less than 5%. The minimum detectable concentration of TSH by Pathozyme TSH kit was estimated to be 0.2μIU/ml.

RNA isolation and cDNA preparation

Total RNA was isolated from the ovary using TRIReagent (Sigma) following the manufacturer's instruction and the method described earlier [29] and cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (MBI fermentas, Hanover, MD) following the manufacturer's instruction.

Reverse Transcription-Polymerase Chain Reaction

2μl of the cDNA, prepared by RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas) was used as template for RT-PCR with gene specific primers and relative expression was observed with GAPDH primer. A 50μl PCR volume was made by adding 2.5U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), to a PCR mixture containing 1X reaction buffer [50mM KCL, 10mM Tris-HCl (pH 8.3), 0.1% Triton-X-100 and 2.5mM $MgCl_2$], 200μM of each dNTPs (MBI Fermentas), 20pmol of each primers. The PCR was performed for 25 cycles, each cycle consisting of denaturation at 94°C for 30s (5min in the first cycle), annealing at specific temperature for each set of primers for 30s, extension at 72°C for 30s (10min in the last cycle; Perkin-Elmer 9700).

Real Time quantitative PCR

Relative Quantitative RT-PCR (Q-PCR) was performed on iCycler real time PCR machine (Bio-Rad Laboratories, Hercules, CA) using DyNAmo SYBR Green Q-PCR Kit (Finnzyme, Finland) following the instructions provided by the vendor to confirm the changes in gene expression observed during semi-quantitative RT-PCR. An internal control GAPDH gene was amplified in separate tubes in each experiment. The oligonucleotide primers used in the reactions have been listed in Table 2.

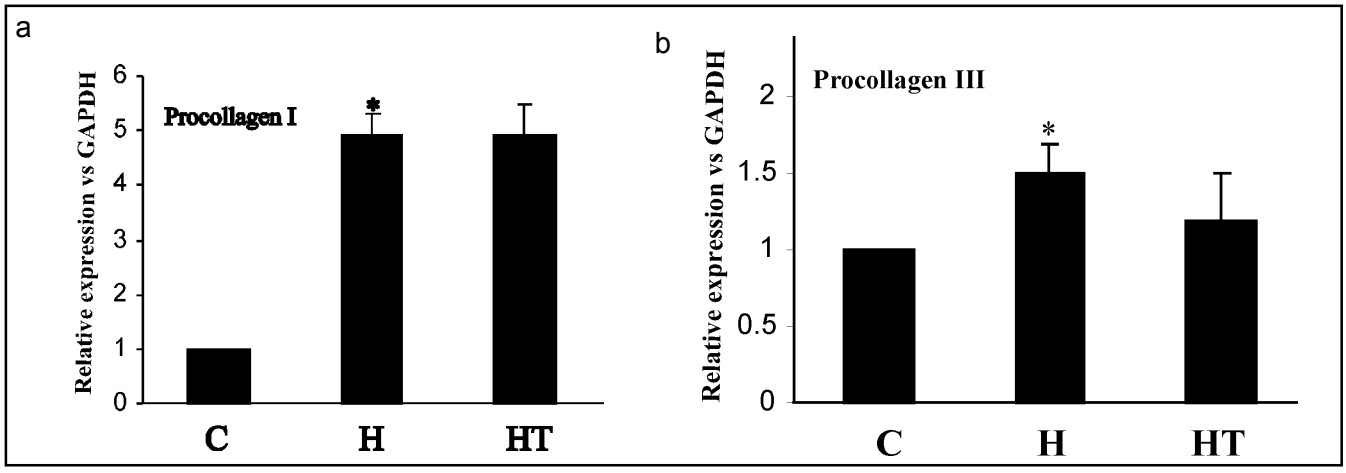

Fig. 1. Expression of procollagen I and -III in hypothyroid condition. The expression pattern of procollagen I (a) and -III (b) transcripts in control (C), hypothyroid (H) and T_3-treated hypothyroid (HT) rat ovaries as measured by Q-PCR. Data are represented as fold changes in experimental condition compared to control by analyzing the C_T values corrected by those of corresponding internal GAPDH controls. Data from four experiments (mean ± SD) were determined as fold changes compared to normal rat. *, $P<0.001$. RNA utilized for the RT-PCR was isolated from control, hypothyroid and T_3-injected hypothyroid ovaries.

Western blot analysis

Total ovaries from 10 individual rats were isolated for each group, i.e. for control, hypothyroid and T_3-treated hypothyroid groups, for each experiment. The ovaries, thus pooled, were homogenized in homogenizing buffer (150mM NaCl, 500mM Tris and 10mM EDTA) supplemented with protease inhibitors (1µg/ml aprotinin, 1µg/ml pepstatin, 1µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, and 1µg/ml trypsin inhibitor) and 1% Triton X-100 (all from Sigma chemical co, USA). The homogenate was then centrifuged at 8000g for 10min at 4°C. The supernatant was collected and (an aliquot of it was used for protein concentration estimation) resolved on a 10% SDS-PAGE gel and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). The membrane was incubated with 5% blocking solution (TBS containing 0.1% Tween 20, and 5% non fat dried milk) for 1h, washed twice with TBS containing 0.1% Tween 20 and then incubated for 16h with goat anti-procollagen I, anti-procollagen III, rabbit anti-HSP-47 antibodies respectively (1:1000 dilution in 5% blocking solution) and mouse anti-actin antibody was used for loading control (1:2000 dilution in 5% blocking solution, all from Santa Cruz Biotechnology, USA). Immunoreactive bands were visualized by reaction of horseradish peroxidase (HRP)-labeled secondary donkey anti-goat, goat anti-rabbit or rabbit anti-mouse antisera at 1:2000 dilutions with HRP substrate.

C_T value calculation

In Q-PCR, relative quantification was performed by comparative C_T value calculation. In this method arithmetic formulae are used to calculate relative expression levels, compared with a calibrator, which can for instance be a control (non-treated) sample. The amount of target normalized to an endogenous housekeeping gene and relative to the calibrator, is then given by $2^{-\Delta\Delta CT}$, where $\Delta C_T = \Delta C_T$ (sample) $-\Delta C_T$ (calibrator), and ΔC_T is the C_T of target gene subtracted from the C_T of the housekeeping gene [30].

Statistical analysis

All data are expressed as the mean±SD, and statistical analysis were performed using *Student's t-test*. $P<0.05$ was considered to be significant. Experiments were repeated at least three times in duplicate unless otherwise stated. To make the variance independent of the mean, statistical analysis of the real-time PCR data was performed after logarithmic transformation.

Results

Serum T_3 and TSH level in the experimental animals

To see the thyroid hormone status of the control, hypothyroid, and other experimental rats, we collected the serum from each set of rats and performed RIA and ELISA for T_3 and TSH assay respectively. Serum T_3 and TSH levels were decreased and increased respectively in PTU-treated hypothyroid rats compared to control rats (Table 1). The serum T_3 level was increased and serum TSH level was decreased when hypothyroid rats were injected with T_3.

Up regulation of procollagen I and - III gene in hypothyroid rat ovary

To elucidate the expression level of procollagen I and -III in hypothyroid ovarian tissue, Q-PCR was

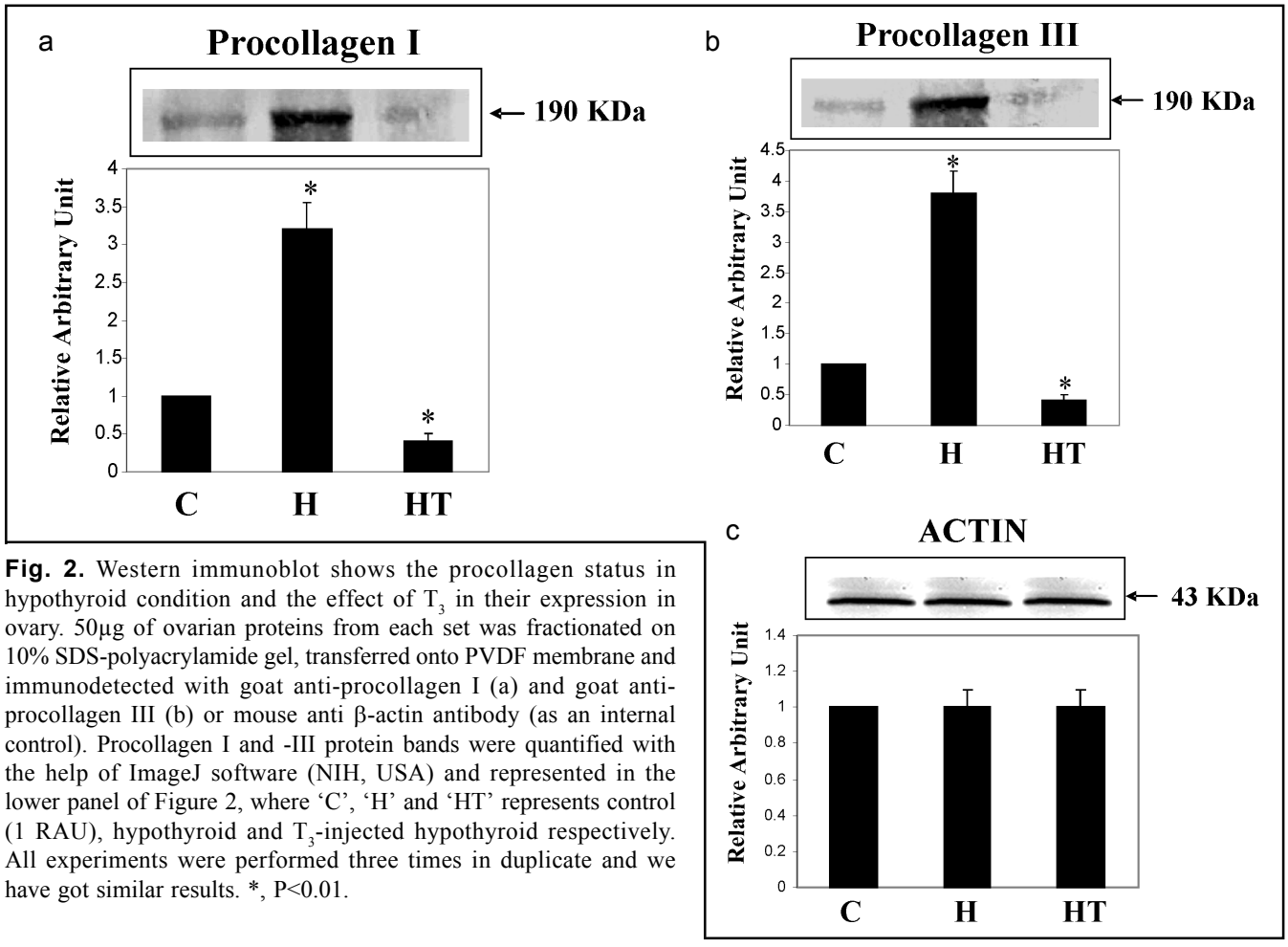

Fig. 2. Western immunoblot shows the procollagen status in hypothyroid condition and the effect of T_3 in their expression in ovary. 50µg of ovarian proteins from each set was fractionated on 10% SDS-polyacrylamide gel, transferred onto PVDF membrane and immunodetected with goat anti-procollagen I (a) and goat anti-procollagen III (b) or mouse anti β-actin antibody (as an internal control). Procollagen I and -III protein bands were quantified with the help of ImageJ software (NIH, USA) and represented in the lower panel of Figure 2, where 'C', 'H' and 'HT' represents control (1 RAU), hypothyroid and T_3-injected hypothyroid respectively. All experiments were performed three times in duplicate and we have got similar results. *, $P<0.01$.

performed with the ovarian RNAs isolated from control, hypothyroid and T_3-treated hypothyroid animals. In this experiment, gene specific primers of procollagen I and -III were used. The data suggests that the expression of procollagen I and -III were significantly increased in hypothyroid ovary and their expression was not significantly altered after T_3 re-addition (Fig. 1 a-b).

Procollagen I and -III protein expression is up regulated in hypothyroid condition

Total ovarian protein was quantified by the Lowry method and then resolved on a SDS-PAGE (4% stacking gel laid over 10% resolving gel) and transferred onto Immobilon-P membranes. The membrane was incubated with 5% blocking solution (TBS containing 0.1% Tween 20, and 5% non-fat dried milk) for 1h, washed twice with TBS-T and then immunodetected with the antibodies against procollagen type-I and type-III. Immunoblotting in parallel sets were performed with antimouse β-actin antibody as loading controls. The protein levels of procollagen type-I and -III (approximately 190KDa bands) were significantly increased by more than 3 fold in the ovary of hypothyroid rats compared to control (Fig. 2a-b). The level of these proteins, however, significantly decreased in the ovarian tissue of hypothyroid rats that injected with T_3. Actin antibody was used as a loading control in this experiment (Fig. 2c).

Increased expression of HSP-47, P4H-α and -β in hypothyroid ovarian tissue

To elucidate the expression level of HSP-47, P4H-α and -β in ovarian tissue of hypothyroid rats, Q-PCR was performed with ovarian RNAs isolated from control, hypothyroid and T_3-treated hypothyroid animals, using gene specific primers. The Q-PCR data suggests that the expression of HSP-47, P4H-α and -β were significantly increased in hypothyroid ovary (Fig. 3a-c), compared to control, which was not reduced to normal

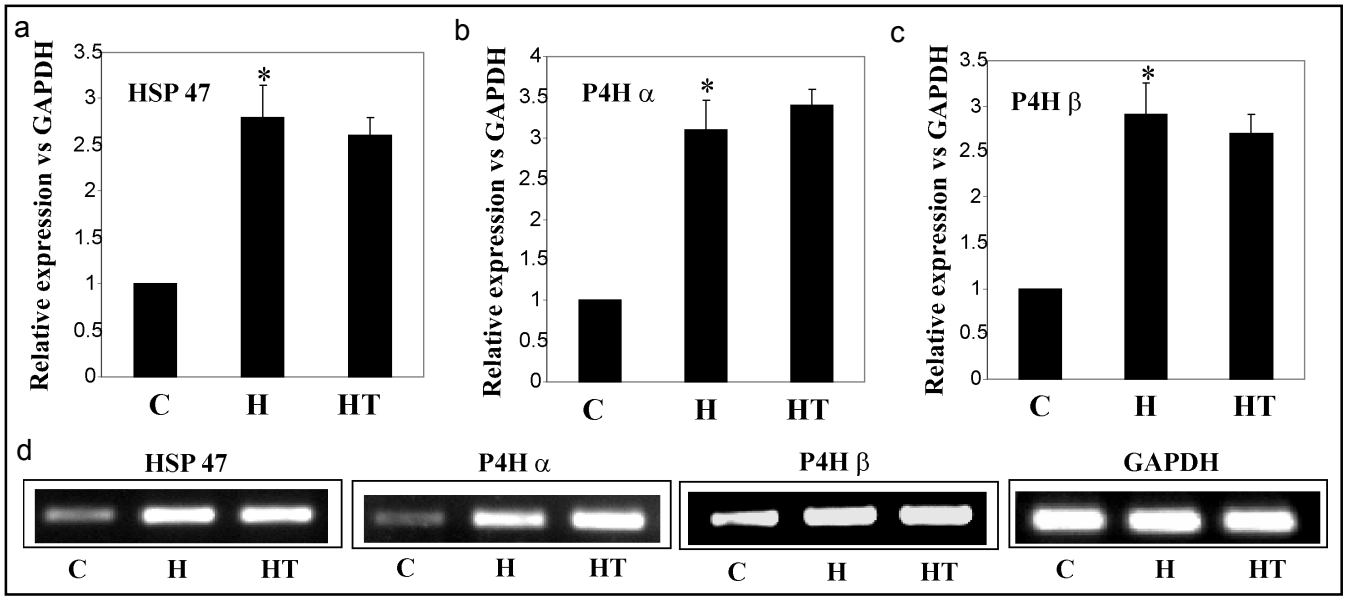

Fig. 3. Expression of HSP-47, P4H-α and -β in hypothyroid condition. The expression pattern of HSP-47 (a), P4Hα (b) and P4Hβ (c) transcripts in RNAs isolated from control (C), hypothyroid (H) and T_3-treated hypothyroid rat ovaries (HT) as measured by Q-PCR. Data are represented as fold changes in experimental condition compared to control by analyzing the C_T values corrected by those of corresponding internal GAPDH controls. Data from four experiments (mean±SD) are determined as fold changes compared to normal rat. *, P<0.001. The Q-PCR-amplified products were then electrophoresed on agarose gel and ehidium bromide stained bands are shown (d).

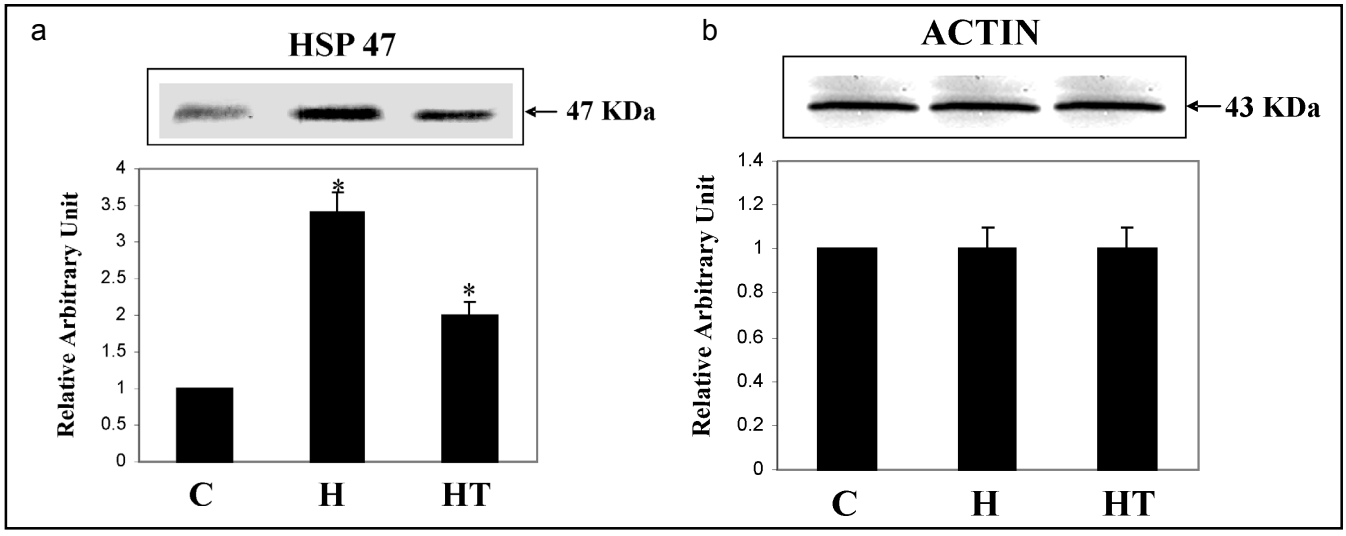

Fig. 4. Western immunoblot shows the HSP-47 status in hypothyroid condition and the effect of T_3 in their expression in ovary. 50μg of ovarian proteins from each set were fractionated on 10% SDS-polyacrylamide gel, and subjected to immunodetection with either rabbit anti-HSP-47 (a) or mouse anti β-actin antibody as an internal control (b). HSP-47 protein bands were quantified with the help of ImageJ software (NIH, USA) and represented in the lower panel of figure 4, where 'C', 'H' and 'HT' represents control (1 RAU), hypothyroid, and T_3-injected hypothyroid respectively. All experiments were performed three times in duplicate and we have got similar results. *, P<0.001.

level even after 15 days of T_3 treatment. GAPDH gene specific primers were used in these reactions as internal control. The Q-PCR-amplified products were then electrophoresed on agarose gel and the band intensity supports the Q-PCR data as mentioned above (Fig. 3d).

HSP-47 protein is up regulated in hypothyroid condition

Total ovarian protein was resolved on a SDS-PAGE (4% stacking gel laid over 10% resolving gel) and transferred onto PVDF membrane. The membrane was incubated with 5% blocking solution (TBS-T, and 5% non-fat dried milk) for 1h, washed twice with TBS-T and then incubated for 16 hrs with the HSP-47 antibody. Immunoblotting in parallel sets were performed with anti-mouse β-actin antibody as loading controls. HSP-47 protein level was increased by more than 3 fold in the hypothyroid rat ovary compared to control as demonstrated by Western immunoblot (Fig. 4a). The expression level of these proteins, however, decreased in hypothyroid rat ovary when they were injected with T_3. Actin antibody was used as loading control in this experiment (Fig. 4b).

Discussion

Collagens, a large family of glycoproteins, are the structural building blocks of tissues and are the major component of ECM. The ovary is a very dynamic organ where follicles and corpora lutea continually grow and regress. Cell movement, migration, division, specialization, differentiation and death are the events occurring continuously in this organ and the ECM participates in all these processes. Ovarian follicular development depends on ECM. It helps follicular fluid formation, filters soluble materials and provides rigid or elastic mechanical support for tissues. Nutrients, hormones and other extra cellular signaling molecules are often required to traverse the matrix to reach the target cells [31].

Collagen biosynthesis requires a number of post-translational modifications, one of the important steps being hydroxylation of lysine residues, which provide attachment sites for glycosylated hydroxylysine. 'Plod' encodes the enzyme procollagen lysyl hydroxylase, which regulates the hydroxylation of lysine residues in collagen biosynthesis. Among other secretory proteins, collagen possesses unique folding, assembly and processing characteristics [32]. Immediately after the α-chains of procollagen emerge in the ER lumen, protein disulfide isomerase (PDI) binds to the nascent α-chains and prevents the improper folding of those chains until all three α-chains assemble at the C-terminus [33]. At the same time, P4Hα and -β also bind to single α-chains and hydroxylate the proline residues at the Y-position of Gly-X-Y triplets [34]. During the progression of triple-helix formation from the C-terminus to N-terminus, PDI and P4H dissociate from the procollagen and HSP-47 associates in turn with the triple-helical part of the procollagen [35, 36]. After the formation of triple helix is complete, fully hydroxylated procollagen is transported from the ER to the Golgi apparatus via vesicular transport.

We earlier showed the down regulation of Plod isoforms, activation of collagenolytic MMPs and down regulation of specific TIMP in hypothyroid ovary. Altogether, these phenomena results in concomitant decrease in mature collagen formation [37]. It has also been reported that the ovarian ECM is severely disrupted under hypothyroid condition and consequently structural disintegration causes ovarian dysfunction which is a very common manifestation in hypothyroidism. In the present study, we have demonstrated that procollagen I and -III are up regulated at the protein as well as mRNA level in hypothyroid ovary and decreased upon T_3 add-back at the protein level, whereas no significant change was observed at their RNA level. Moreover, HSP-47 (collagen specific ER chaperone), P4H-α and -β gene expression and HSP-47 protein expression was increased in the ovarian tissue of hypothyroid rat. When T_3 was injected to the hypothyroid rats, HSP-47 protein level was decreased but there was no recovery at the RNA levels of any of these three genes. In contrast with reduced formation of the mature collagen in hypothyroid ovary, the significant up regulation of procollagen I and -III could provide a parallel pathway to compensate the loss of collagen. This result supports our hypothesis that procollagen formation increases whenever mature collagen is reduced. HSP-47, which is a well characterized chaperon molecule in collagen biosynthetic pathway, possibly up regulates in hypothyroidism due to the compensatory measure. We have schematically represented this hypothetic model in Fig. 5, where we propose that this compensatory pathway may enhance the collagen maturation, when there will be low abundance of mature collagen due to hypothyroidism induced defect in lysyl hydroxylase (Plod) pathway. The possible reason of this discrepancy at the RNA and protein levels is that probably the T_3-mediated regulation of expression of these genes in ovary lies at the level of translation, not at the transcriptional level (when T_3 was injected to the hypothyroid animals to show the direct effect of it on collagen gene and protein level). However, the up regulation of these genes due to severe hypothyroidism may happen due to some other factors which are active only in the hypothyroid condition. Further research in this direction is necessary to know the regulatory mechanism

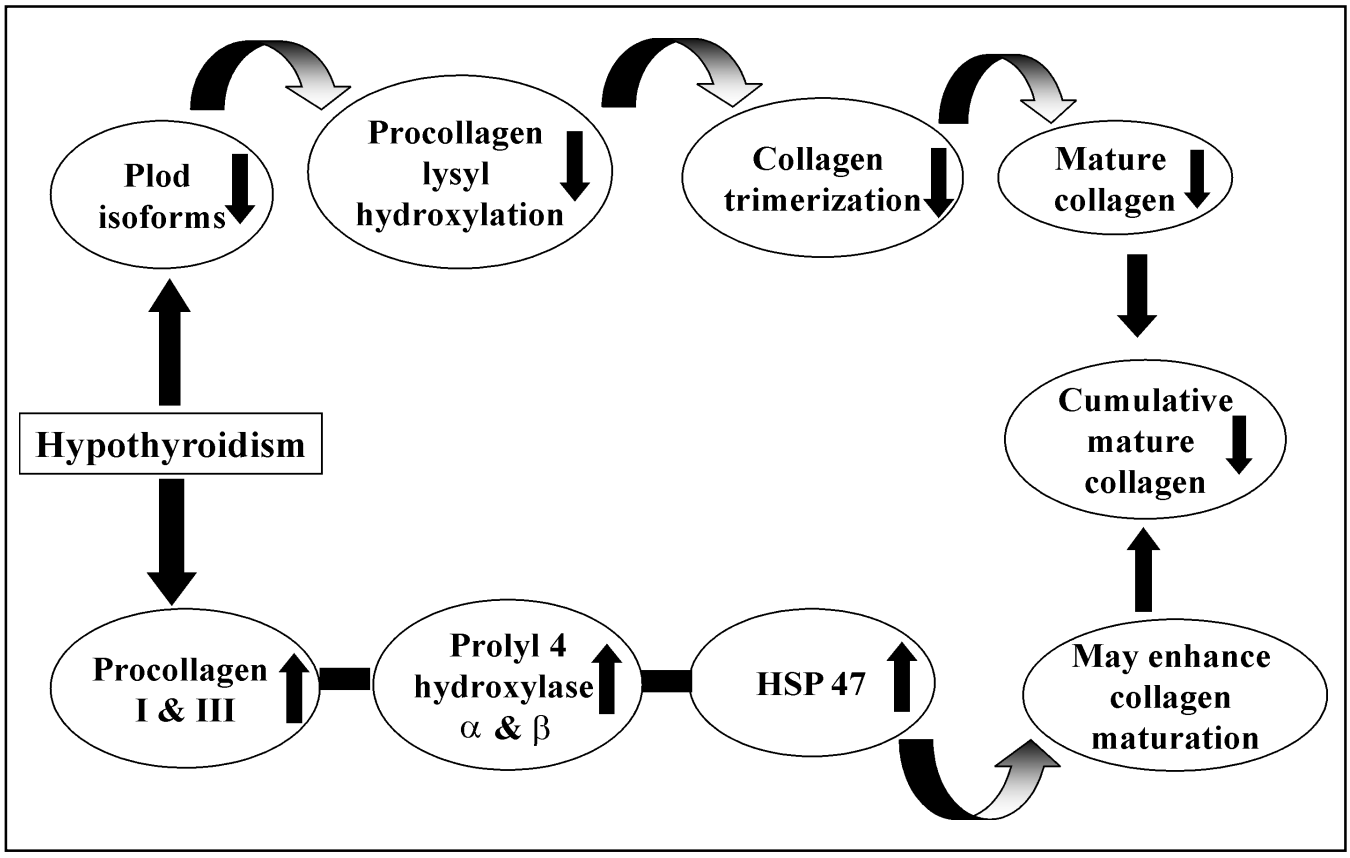

Fig. 5. The hypothetical model showing two different and parallel pathways of collagen synthesis and maturation, which exist in hypothyroid rat ovary. The cumulative effect is less collagen formation in hypothyroid ovarian tissue. Straight arrows inside the ellipse indicate up regulation (arrow directed upwardly) or down regulation (arrow directed downwardly).

of collagen gene and protein expression in hypothyroid ovary. Constellations of ovarian phenotypes are manifested in hypothyroidism among which abrogation of ovarian ECM significantly contributes in reproductive malfunction. Hence, some molecules engaged in collagen biosynthesis, provide adaptive response to compensate the reproductive damage in hypothyroidism, as it is HSP-47 molecule here. Lysyl hydroxylation is a very important step in collagen biosynthesis, since hydroxylysine derived aldehydes provide better attachment sites and hence provide foundation of ECM. Under hypothyroid condition lysyl hydroxylation is severely abrogated due to significant down regulation of Plod isoforms. We postulate that increased prolyl hydroxylation could be a compensatory mechanism for the loss of lysyl hydroxylation.

Despite the compensatory mechanisms provided by procollagens, HSP-47, P4Hα and -β, the structural disintegration of ovarian ECM is prevalent in hypothyroidism. This could be due to the fact that the increased levels of these molecules are not sufficient to compensate the extent of decreased collagen level caused due to the down regulation of Plod, TIMP and the up regulation of MMPs in hypothyroid ovary. Further study is needed to unravel the mechanisms interplaying under hypothyroidism as ovarian dysfunction is often caused in hypothyroid patients.

Abbreviations

ECM (Extracellular matrix); ER (Endoplasmic reticulum); GAPDH (Glyceraldehyde-3-phosphate dehydrogenase); PBS (Phosphate buffered saline); TBS (Tris-buffered saline); SDS (Sodium dodecyl sulfate); HRP (Horseradish peroxidase); Q-PCR (Quantitative Real Time PCR); TIMP (Tissue inhibitor of matrix metalloproteinase).

Acknowledgements

We gratefully acknowledge Council of Scientific and Industrial Research (CSIR), India for the financial support to SSR and fellowship and contingency grant provided to SKS (CSIR Award no.31/2/(439)/2001-EMR-I) and PG (CSIR Award no.31/2/(477)/2002-EMR-I) The skillful technical assistance of Mr. Prabir Kumar Dey and Mr. Swapan Mandal of Indian Institute of Chemical Biology is gratefully acknowledged. We also acknowledge Dr. Arun Bandyopadhyay and Dr. Aditya Konar of Indian Institute of Chemical Biology, Kolkata for stimulating discussion.

References

1 Vogel W, Gish GD, Alves F, Pawson T: The discoidin domain receptor tyrosine kinases are activated by collagen. Mol Cell 1997;1:13-23.

2 Luck MR: The gonadal extracellular matrix. Oxford Rev Reprod Biol 1994;16:33-85.

3 Rodgers HF, Irvine CM, Van Wezel IL, Lavranos TC, Luck ML, Sado Y, Ninomiya Y, Rodgers RJ: Distribution of the α1 to α6 chains of type IV collagen in bovine follicles. Biol Reprod 1998;59:1334-1341.

4 Rodgers RJ, van Wezel IL, Rodgers HF, Lavranos TC, Irvine CM, Krupa M: Roles of extracellular matrix in follicular development. J Reprod Fertil Suppl 1999;54:343-352.

5 Belkin AM, Stepp MA: Integrins as receptors for laminins. Microsc Res Tech 2000;51:280-301.

6 Fujiwara H, Honda T, Ueda M, Nakamura K, Yamada S, Maeda M, Mori T: Laminins suppresses progesterone production by human luteinizing granulosa cell via interaction with integrin $\alpha 6\beta 1$. J Clin Endocrinol Metab 1997;82:2122-2128.

7 Le Bellego F, Piselet C, Huet C, Monget P, Monniaux D: Laminin-$\alpha 6\beta 1$ integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells. J Endocrinol 2002;172:45-59.

8 Rapp G, Freudenstein J, Klaudiny J, Mucha J, Wempe F, Zimmer M, Scheit KH: Characterisation of three abundant mRNAs from human ovarian granulosa cells. DNA Cell Biol 1990;7:479-845.

9 Calloni WC, Alvarez-Silva M, Vituri C, Trentin AG: Thyroid hormone deficiency alters extracellular matrix protein expression in rat brain. Dev Brain Res 2001;126:121-124.

10 Bateman JF, Lamande SR, Ramshaw JAM: Collagen superfamily, (Comper, W.D., eds), 1996 pp. 22-67. Harwood Academic Press, Melbourne.

11 Ferreira LR, Norris K, Smith T, Hebert C, Sauk JJ: HSP47 and other ER-resident molcular chaperones form heterocomplexes with each other and with collagen type IV chains. Connect Tissue Res 1996;33:265-273.

12 Satoh M, Hirayoshi K, Yokota S, Hosokawa N, Nagata K: Intracellular interaction of collagen-specific stress protein HSP47 with newly synthesized procollagen. J Cell Biol 1996;133:469-483.

13 Kivirikko KI, Myllyharju J: Prolyl 4-hydroxylases and their protein disulfide isomerase subunit. Matrix Biol 1998;16:357-368.

14 Berg RA, Prockop DJ: The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. Biochem Biophys Res Commun 1973;52:115-120.

15 Bachinger HP, Fessler LI, Timpl R, Fessler JH: Chain assembly intermediates in the biosynthesis of type III procollagen in chick embryo blood vessels. J Biol Chem 1981;256:13193-13199.

16 Fessler LI, Fessler JH: Characterization of type III procollagen from chick embryo blood vessels. J Biol Chem 1979;254:233-239.

17 Fessler LI, Timpl R, Fessler JH: Assembly and processing of procollagen type III in chick embryo blood vessels. J Biol Chem 1981;256:2531-2537.

18 Bassuk JA, Berg RA: Protein disulphide isomerase a multifunctional endoplasmic reticulum protein. Matrix 1989;9:244-258.

19 Nagata K, Yamada KM: Phosphorylation and transformation sensitivity of a major collagen-binding protein of fibroblasts. J Biol Chem 1986;261:7531-7536.

20 Cates GA: Analysis of the phosphorylation state of a collagen-binding heat-shock glycoprotein from L6 myoblasts by isoelectric focusing. Biochim Biophys Acta 1991;1073:521-527.

21 Nakai A, Satoh M, Hirayoshi K, Nagata K: Involvement of the stress protein HSP47 in procollagen processing in the endoplasmic reticulum. J Cell Biol 1992;117:903-914.

22 Yamamura I, Hirata H, Hosokawa N, Nagata K: Transcriptional activation of the mouse HSP47 gene in mouse osteoblast MC3T3-E1 cells by TGF-b 1. Biochem Biophys Res Commun 1998;244:68-74.
23 Hirayoshi K, Kudo H, Takechi H, Nakai A, Iwamatsu A, Yamada KM, Nagata K: HSP47: a tissue-specific, transformation-sensitive, collagen-binding heat shock protein of chicken embryo fibroblasts. Mol Cell Biol 1991;11:4036-4044.
24 Nakai A, Hirayoshi K, Nagata K: Transformation of BALBr3T$_3$ cells by simian virus 40 causes a decreased synthesis of collagen-binding heat-shock protein (hsp47). J Biol Chem 1990;265:992-999.
25 Sauk JJ, Smith T, Norris K, Ferreira L: Hsp47 and the translation-translocation machinery cooperate in the production of α1-I. chains of type I procollagen. J Biol Chem 1994;269:3941-3946.
26 Kambe K, Yamamoto A, Yoshimori T, Hirayoshi K, Ogawa R, Tashiro Y: Preferential localization of heat shock protein 47 in dilated endoplasmic reticulum of chicken chondrocytes. J Histochem Cytochem 1994;42:833-841.
27 Masuda H, Hosokawa N, Nagata K: Expression and localization of collagen-binding stress protein Hsp47 in mouse embryo development: comparison with types I and II collagen. Cell Stress Chaperones 1998;3:256-264.
28 Braski SN: Effect of propylthiouracil-induced hypothyroidism on serum levels of luteinizing hormone and follicle-stimulating hormone in the rat. J Endocrinol 1973;59:655-656.
29 Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
30 Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C: An overview of real-time quantitative PCR applications to quantify cytokine gene expression. Methods 2001;25:386-401.
31 Rodgers RJ, Irving-Rodgers HF, Russell DL: Extracellular matrix of the developing ovarian follicle. Reproduction 2003;126:415-424.
32 Lamande SR, Bateman JF: Procollagen folding and assembly the role of endoplasmic reticulum enzymes and molecular chaperones. Semin Cell Dev Biol 1999;10:455-464.
33 Wilson R, Lees JF, Bulleid NJ: Protein disulfide isomerase acts as a molecular chaperone during the assembly of procollagen. J Biol Chem 1998;273:9637-9643.
34 Myllyharju J: Prolyl 4-hydroxylases, the key enzymes of collagen biosynthesis. Matrix Biol 2003;22:15-24.
35 Koide T, Asada S, Nagata K: Substrate recognition of collagenspecific molecular chaperone HSP47 Structural requirements and binding regulation. J Biol Chem 1999;274:34523-34526.
36 Tasab M, Batten MR, Bulleid NJ: Hsp47: a molecular chaperone that interacts with and stabilizes correctly-folded procollagen. EMBO J 2000;19:2204-2211.
37 Saha SK, Ghosh P, Konar A, Bhattacharya S, Roy SS: Differential expression of procollagen lysine 2-oxoglutarate 5-deoxygenase and matrix metalloproteinase isoforms in hypothyroid rat ovary and disintegration of extracellular matrix. Endocrinology 2005;146:2963-2975.