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Influence of an extract from kudzu symbiosomes containing leghemoglobin on *in vitro* cutaneous procollagen production

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Synopsis

Cytoglobin is a hexacoordinate globin protein that was recently discovered in mammals. Interestingly, of the four human globin proteins that are now known, hemoglobin, myoglobin, neuroglobin and cytoglobin, the latter appears to have the closest resemblance to strikingly similar proteins expressed in plants. In legumes, these proteins accumulate in symbiosomes (root nodules) of various legumes and are called leghemoglobin.

The paper will discuss the ability of an aqueous extract from *Pueraria lobata* (kudzu) symbiosomes that contains leghemoglobin to stimulate procollagen production in human dermal fibroblasts. This effect may be partly due to the possibility that leghemoglobin may mimic the function of cytoglobin by shuttling oxygen to prolyl-4-hydroxylase, the enzyme responsible for oxidizing proline residues in procollagen bundles. This hypothesis is supported by DNA microarray sequencing data that demonstrate that treatment of normal human dermal fibroblasts (NHDF) with highly purified cytoglobin or leghemoglobin upregulates a number of key collagen-related genes including COL1A1 and COL1A2.

INTRODUCTION

The recent discovery of a fourth globin protein in humans, called cytoglobin, which appears to be expressed in human dermal fibroblasts and other cells that express collagen (such as osteoblasts), has raised the distinct possibility that these heme-based proteins may influence biochemical cascades in growing skin (1–3). Cytoglobin has been identified within many tissue types in the body although its expression in skin cell fibroblasts is likely, but still not firmly confirmed (4,5). The protein appears to be highly conserved in animal species (6,7). The possibility that expression of cytoglobin in skin cells that express collagen and other extracellular matrix fibrous proteins, namely fibroblasts, can be upregulated by hypoxia (oxygen-deficient growing conditions) has been discussed, indicating that cytoglobin either plays a role in oxygen perfusion or acts as a oxygen sensor or perhaps both (4,8–10). Cytoglobin also appears to have important antioxidant properties that may play a role in cellular defenses (3,11–13). Recent work has demonstrated that cytoglobin can enter the nuclear envelope and influence the genome, directly raising the possibility that these proteins can influence cellular functions at the genetic level (14).

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Because cytoglobin appears to influence oxygen's effects at the cellular levels, it has been suggested that cytoglobin may influence procollagen production by moving oxygen to key enzymes responsible for the early synthetic steps in collagen production (4,5,15). In particular, an enzyme called prolyl-4-hydroxylase, which is also a heme-based enzyme, requires oxygen to be attached at its active site in order to facilitate conversion of proline to hydroxyproline in early-stage procollagen production (Figure 1) (16).

This paper will explore the possibility that plant-based substitutes for human globin proteins, namely leghemoglobin, may function in a similar capacity to assist procollagen production. All green plants express heme-based globin proteins, which are thought to function as free-radical scavengers and oxygen-transport molecules, much as they do in humans (17). While all green plants express globin proteins (called phytoglobins), certain plants, namely legumes, which are known to fix nitrogen, accumulate this protein in special structures found on the plant roots called symbisomes (generally known as root nodules). *Pueraria lobata*, a plant more commonly known as kudzu, is a legume that grows in many parts of the world. It grows rapidly and can extensively cover many other plants, which causes problem in countries where the plant has no real predators. The roots of kudzu contain symbisomes, and these can be easily harvested and extracted to provide useful quantities of leghemoglobin. The ability of plant-based hemoglobins to scavenge reactive oxygen species has been established (18). The paper will examine whether a plant-based extract containing leghemoglobin can influence the genes responsible for upregulation of extracellular matrix proteins in normal human dermal fibroblasts comparably to human-derived cytoglobin.

MATERIALS AND METHODS

PUERARIA LOBATA (KUDZU) SYMBIOSOME EXTRACT AND PUERARIN

Using previously published methods, an aqueous extract taken from root nodules isolated from *Puerraria lobata* (kudzu) symbiosomes was standardized to 0.2–0.5 mg/ml leghemoglobin (3). Purified puerarin was obtained from Sigma Chemical Company (Milwaukee, WI).

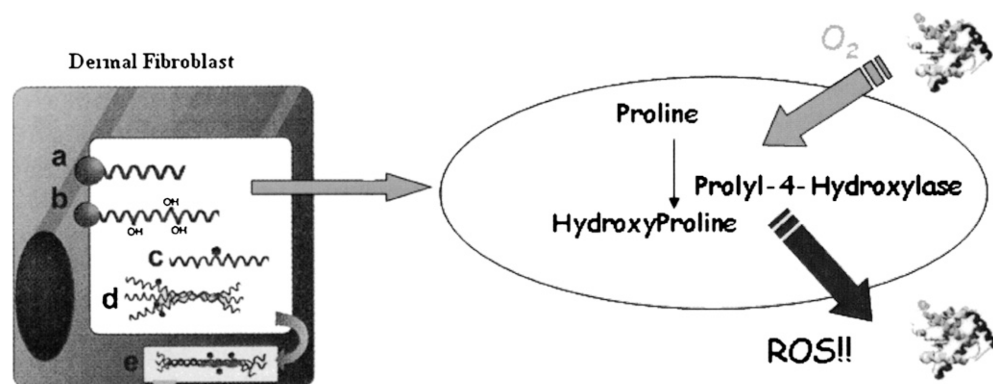

Figure 1. The proposed role of cytoglobin to act as an oxygen shuttle to deliver oxygen to prolyl-4-hydroxylase, which, in turn, oxidizes proline residues to hydroxyproline residues in early-stage procollagen (steps a–b). The collagen figure was adapted from reference 16.

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MICROARRAY ANALYSIS—NORMAL HUMAN DERMAL FIBROBLAST CELL CULTURES

Cultured normal human dermal fibroblasts (NHDF, Cascade Biologics) were grown in T-75 flasks until confluent using appropriate culture conditions. Upon reaching confluence, the cells were treated with culture media supplemented with test materials at the concentrations specified or with culture media alone, which acted as the untreated control.

After applying the test materials to the cells, they were incubated for 24 hours at 37 ± 2°C and 5 ± 1% CO_2. At the end of the incubation period, the culture media was removed by aspiration and the cells were washed once with cold phosphate-buffered saline using approximately 1 ml per well. After the washing step, a trypsin/EDTA solution was added to the wells to release the cells. After the cells were released, an appropriate volume of trypsin-neutralizing solution was added to the wells. The treated cells and the untreated cells were pooled into separate 15-ml centrifuge tubes and pelleted by centrifuging at 1000 rpm at 4 ± 2°C. After removing the supernatant, the pelleted cells were lysed by adding 300 μl of guanidinium thiocyanate lysis solution to each tube and then repeatedly drawing and releasing the solution into a pipet until the cell pellet dissolved. The cell lysate was then stored at −75°C until the RNA extraction process could be completed.

RNA ISOLATION (AMBION RNAqueous KIT)

To the thawed fibroblast lysate described above was added an equal volume of 64% ethanol, and the culture tubes were mixed via vortex mixing. Seven hundred microliters of the alcoholic mixture was transferred to a glass-fiber filter cartridge, the filter cartridge was loaded into a 1.5-ml collection tube, and the entire apparatus was centrifuged for 1 minute at 14,000 rpm. The filtration process was repeated until all of the mixture was filtered. The filter was then washed to remove any residual cellular debris from the RNA bound to the glass fibers by subsequently applying 700 μl of wash solution followed by an additional two washes with 500 μl of wash solution, each time removing the wash solution by centrifuging the tube at 14,000 rpm for one minute. The filtrate was spun to remove any residual wash solvent. RNA bound to the glass fibers within the cartridge was eluted by applying 30 μl of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, preheated to 70–80°C) to the cartridge and centrifuging the cartridge in a new collection tube at 14,000 rpm for one minute. After the RNA had eluted, it was analyzed as described below.

RNA CONCENTRATION ASSAY (Molecular Probes Ribogreen Assay)

Ribogreen reagent was provided as a stock solution in DMSO. Prior to use, the reagent was diluted 2000-fold in TE buffer. The RNA assay requires 200 μl of diluted Ribogreen reagent per sample to be tested and 1 ml of the reagent for standards. Once prepared, the diluted reagent was stored in the dark.

A series of RNA standards was prepared by diluting purified ribosomal RNA derived from *E. coli* to the following concentrations: 2 μg/ml, 1 μg/ml, 200 ng/ml, 40 ng/ml, and 0 ng/ml (blank). Prior to assaying, the RNA samples, prepared as described above, were diluted 1000-fold in TE buffer. For the RNA assay, 100 μl of the diluted samples or standards

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was transferred to the wells of a black 96-well plate. The samples and standards were assayed in duplicate. After the samples/standards were added to the plate, 100 μl of the diluted Ribogreen assay reagent was added to the wells, and the plate was gently mixed and allowed to incubate for 5–10 minutes protected from light. After incubation, the plate was read by a fluorometer using an excitation wavelength of 500 nm and an emission wavelength of 525 nm.

mRNA AMPLIFICATION (Ambion MessageAmp aRNA Kit)

First-strand cDNA synthesis. Five micrograms of total RNA for each sample was placed into 600-μl PCR tubes, and the total volume of liquid in each tube was adjusted to 12 μl with DEPC·H_2O. To each tube, 1 μl of T7 Oligo(dT) primer was added, and the tube was incubated at 70 ± 2°C for 10 minutes to denature the RNA. The sample was then placed onto ice to allow the primer to anneal to the poly A ends of the mRNA. After cooling, 2 μl of 10X first-strand buffer, 1 μl of RNAse inhibitor, and 4 μl of dNTP mix was added to each tube and the tubes were placed at 42 ± 2°C. As soon as the tube was heated, 1 μl of reverse transcriptase was added and the tubes were returned to the 42 ± 22°C bath for two hours. After heating, the tubes were briefly centrifuged to gather the fluids at the bottom of the tube, and then they were cooled on ice.

Second-strand synthesis and cDNA amplification. For the synthesis of the second strand of cDNA, the following items were added to the tubes described above in the following order: 63 μl of DEPC·H_2O, 10 μl of 10X second-strand buffer, 4 μl of dNPT mix, 2 μl of DNA polymerase, and 1 μl of RNAse H. Each tube was mixed and then incubated at 16 ± 2°C for two hours. Toward the end of the two-hour incubation, a sufficient quantity of DEPC·H_2O was added, the tube was warmed to 50 ± 2°C, and a cDNA purification filter cartridge was equilibrated with 50 μl of cDNA binding buffer (one cartridge per sample) for at least five minutes. After the samples had finished incubating, 250 μl of cDNA binding buffer was added to each tube and the tubes were thoroughly mixed. The contents of the each PCR tube were transferred to the cDNA purification filter cartridge. The cartridge was then placed in a collection tube and centrifuged at 10,000 rpm for one minute. The flow-through was discarded and 650 μl of cDNA wash solution was added to the cartridge. The cartridge was centrifuged again and the flow-through was discarded. The contents were centrifuged one more time to ensure that the wash buffer was completely emptied from the filter. The cDNA was eluted by applying 10 μl of preheated DEPC·H_2O to the filter, and the contents were centrifuged in a new collection tube at 10,000 rpm for one minute. The elution was repeated one additional time to give a total volume of 16–18 μl of cDNA.

In vitro transcription to synthesize aRNA and aRNA purification. The *in vitro* transcription began by adding the following to the cDNA solution: 4 μl of 10X reaction buffer and 4 μl of T7 enzyme mix. The tube was mixed and then incubated at 37 ± 2°C for 6–14 hours. Towards the end of the incubation period, a sufficient volume of elution solution was warmed to 50°–60°C and an aRNA filter cartridge was equilibrated with 100 μl of aRNA binding buffer for at least five minutes. At the end of the incubation period, 350 μl of aRNA binding buffer was added to the sample tubes and thoroughly mixed. An additional 250 μl of absolute ethanol was added to each tube. The mixture was transferred to an aRNA filter cartridge, and the cartridge was then inserted into a collection tube and centrifuged at 10,000 rpm for one minute. The flow-through was discarded and 650 μl of aRNA wash buffer was added to the cartridge, followed by further centrifugation

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at 10,000 rpm for one minute. After the flow-through was discarded, the cartridge was spun one final time to remove traces of the wash buffer. The cartridge was transferred to a new collection tube and 25 µl of prewarmed elution solution was added to the cartridge. The cartridge was incubated for two minutes at room temperature and then aRNA was eluted by centrifuging for one minute at 10,000 rpm. This elution was performed one additional time to give a total volume of 45–50 µl of aRNA solution. The final concentration of the aRNA was determined by the Ribogreen assay described above. In addition, the quality of the aRNA was checked via gel electrophoresis as described below.

Labeling of aRNA with fluorescent dyes (Perkin Elmer ASAP RNA Labeling Kit) and purification of labeled aRNA.

Labeling: Two tubes were prepared for the labeling process, one for Cy3 labeling (green) and one for Cy5 labeling (red). To the Cy3 tube was added 2 µg of aRNA prepared from the untreated control sample, and enough DEPC·H_2O was added to bring the total volume up to about 4 µl. To the Cy5 tube was added 2 µg of aRNA prepared from the sample treated with the test material, and enough DEPC·H_2O was added to bring the total volume up to 4 µl. To both tubes was added 5 µl of ASAP labeling buffer and 1 µl of the specific dye for each tube (Cy3 or Cy5). The tubes were incubated for 15 minutes at 85 ± 2°C. At the end of the 15-minute incubation period, the tubes were placed on ice to cool and then 2.5 µl of ASAP stop solution was added to each tube. The proportions provided enough sample to run one microarray chip.

Purification: To purify the labeled aRNA, a Microcon YM-30 filter column (Millipore) was inserted into a collection tube filled with 400 µl of TE buffer. The Cy3 and Cy5 probes were combined (12.5 µl of each) and then added to the Microcon filter; the mixture was thoroughly mixed with the TE buffer. The mixture was centrifuged at 12,000 rpm for eight minutes and the flow-through was discarded. The column was then washed twice with 400 µl of TE buffer and the flow-through was again discarded. After the final wash, the filter column was inverted, placed into a new collection tube, and centrifuged at 12,000 rpm for two minutes to collect the probe. The probe was further diluted to a volume of 2–30 µl with residual TE buffer.

Microarray hybridization and washing (Agilent Technologies Microarrays). For hybridization, 45 µl of 10X control target RNA (Agilent Technologies In Situ Hybridization Kit) was mixed with 160 µl of DEPC water and 9 µl of 25X Agilent fragmentation buffer. This mixture was incubated at 60°C for approximately 30 minutes in a hybridization oven. At the end of the incubation period, 225 µl of Agilent hybridization buffer was added, along with the fluorescent aRNA probes prepared above. During the incubation period, an Agilent SUREHYB hybridization chamber was prepared by inserting a glass gasket slide into the bottom half of the chamber. At the end of the incubation period, the hybridization mixture (approximately 450 µl) was applied to the glass gasket slide and an Agilent Human 1A Oligo microarray chip was placed face down on top of the gasket in such a way that the hybridization solution was sandwiched between the glass gasket slide and the microarray face of the chip. The top half of the chamber was then attached and the connecting thumbscrew tightened. After verifying that there was good bubble formation in the chamber, it was placed into the hybridization chamber for approximately 17 hours at 60°C and rotated at 4 rpm). At the end of the hybridization period, the microarray glass gasket assembly was removed from the SUREHYB chamber and placed in 50 ml of wash solution 1 (6X SSC, 0.005% Triton X-102) at room temperature. After the gasket

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had fallen away from the microarray chip, the array was transferred to 300 ml of fresh wash solution 1 on a magnetic stir plate. The array was washed while the solution was mixed at medium speed for ten minutes and then transferred to 300 ml of wash solution 2 (0.1X SSX, 0.005% Triton X-102) at 4°C for five minutes. After the final wash, the array was centrifuged at 500 rpm for five minutes for drying.

Microarray scanning and analysis. The microarrays were scanned with an Axon GenePix 4100A scanner with the scanning resolution set at 10 μm and analyzed with GenePix Pro software. During the initial scan, the PMT gains for the scanner were adjusted such that the Cy5/Cy3 image count ratios were between 0.88 and 1.12.

CALCULATIONS

RNA Ribogreen assay. To derive the standard curve for the Ribogreen assay, the relative fluorescent units versus the known RNA concentrations in μg/ml for the standards were plotted and subjected to regression analysis to establish the line that best fits the data points. Mean RFU values for the test materials and untreated samples were then used to estimate the amount of RNA present in each sample.

Microarray calculations. The level of gene expression is related to the fluorescent intensity of the probed gene marker on the microarray. Since it was possible to have differences in labeling efficiency when making the Cy3 and Cy5 probes, it was essential to normalize the fluorescence measurements between the two respective dyes before looking at changes in gene expression. Fluorescence intensities for the microarrays were subjected to global normalization. The total fluorescent signal for both dyes was normalized with a correction factor that makes the ratio of total intensities for both dyes equal to one. After normalization of the fluorescence measurements, changes in gene expression were then possible to examine. The criteria for evaluating gene expression values are summarized below:

1. The ratio of Cy3/Cy5 (untreated/treated) fluorescence intensity was greater than 1.3 or less than 0.7. This relates to a change in gene expression of at least ±30%.
2. The fluorescent intensity of the gene marker was greater than the background intensity.

HUMAN DERMAL FIBROBLAST PROCOLLAGEN (TYPE 1 C-PEPTIDE) ASSAY

Preparation of normal human dermal fibroblasts. Fibroblasts were seeded into individual wells of a 12-well plate in 1.0 ml of fibroblast growth media (FGM) and incubated overnight at 37 ± 2°C and 5 ± 1% CO_2. On the following day, the media was removed via aspiration to eliminate any non-adherent cells and replaced with 1.0 ml of fresh FGM. The cells were grown until confluent, with a media change every 48 to 72 hours. Upon reaching confluence, the cells were treated for 24 hours with DMEM supplemented with 1.5% FBS to wash out any effects from the growth factors included in the normal culture media. After this 24-hour washout period, the cells were treated with the test materials at the specified concentrations dissolved in DMEM with 1.5% FBS. Untreated cells (negative controls) only received DMEM with 1.5% FBS, while sodium ascorbate (100 μg/ml) was the positive control. The cells were incubated for 48 hours, and at the end of the incubation period, the culture media was collected and either stored frozen (−75°C) or assayed immediately. Samples were tested in triplicate.

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Procollagen assay (Takara ELISA Kit). A series of Type 1 C-peptide standards was prepared, ranging from 40 ng/ml to 640 ng/ml. An ELISA microplate was prepared by removing any unneeded strips from the plate frame. In each well to be used 100 µl of peroxidase-labeled anti-procollagen Type 1 C-peptide was added, followed by 20 µl of either sample (one part collected tissue culture media diluted with four parts fresh culture media) or standard. The microplate was then covered and allowed to incubate for 3 ± 0.25 hours at 37°C. After the incubation period, each well was aspirated and washed three times with 400 µl of wash buffer. After the last wash was removed, 100 µl of peroxidase substrate solution (hydrogen peroxide with tetramethylbenzidine as a chromagen) was added to each well and the plate was incubated for 15 ± 5 minutes at room temperature. After the incubation, 100 µl of stop solution (1 N sulfuric acid) was added to each well and the plate was read using a microplate reader at 450 nm.

Procollagen concentration calculations. A standard curve was generated using known concentrations of Type 1 C-peptide. A regression analysis was then performed to establish the line that best fit the data points. Mean absorbance values for the test materials and untreated samples were then used to estimate the amount of Type 1 C-peptide present in each sample.

RESULTS AND DISCUSSION

HUMAN DERMAL FIBROBLAST MICROARRAY RESULTS

Our interest in whether or not leghemoglobin, derived from symbiosomes of legumes, in particular symbiosomes of *Pueraria lobata* (kudzu), might behave like its human-derived counterpart, cytoglobin, was spurred principally by the fact that, like cytoglobin, leghemoglobin is a hexacoordinate heme-based globin protein. Using highly purified samples of leghemoglobin and cytoglobin, we treated normal human dermal fibroblasts with the equivalent of 1% of each protein for 24 hours. The fibroblasts were then harvested and the RNA was retrieved, fluorescently labeled, and analyzed using a DNA microarray. It became immediately apparent when we examined the gene expression data for several important extracellular matrix proteins that, indeed, these two similar proteins could influence expression of collagen and elastin genes in very similar fashions (Table I). The apparent similarity between the fibrous protein genes upregulated by cytoglobin and those upregulated by leghemoglobin is striking and suggests that, indeed, the very close similarity of these two proteins influences dermal fibroblasts in very similar ways.

PROCOLLAGEN ASSAY RESULTS ON HUMAN DERMAL FIBROBLASTS

Examining the data from our initial studies in which we employed only the raw extract from kudzu symbiosomes, we noted that there appeared to be an increase in procollagen production attributable to the extract (Figure 2). Ascorbic acid was used as a positive control in these studies, as it has been shown in the literature to upregulate expression of Type 1A1 procollagen in human fibroblasts *in vitro* and *in vivo* (19).

This initial assay appeared to indicate that the extract of kudzu symbiosomes did stimulate procollagen production in normal human dermal fibroblasts. However, it was immediately

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Table I
Gene Expression after Treatment of Human Dermal Fibroblasts with 1% Each of Leghemoglobin and Cytoglobin

Name	ID	Ratio of medians (635/532)	Gene name
Fibroblast: 1% Leghemoglobin			
I_960109	A_23_P207521	1.9	COL1A1
I_930569	A_23_P255244	1.517	COL1A2
I_930697	A_23_P215459	1.459	ELN
Fibroblast: 1% Cytoglobin			
I_960109	A_23_P207521	1.573	COL1A1
I_930569	A_23_P255244	1.55	COL1A2
I_930697	A_23_P215459	1.323	ELN

Data shown is the ratio of medians for treated fibroblasts/untreated fibroblasts for each protein. Values of ratio of medians greater than 1.3 indicate a statistically significant upregulation of the gene.

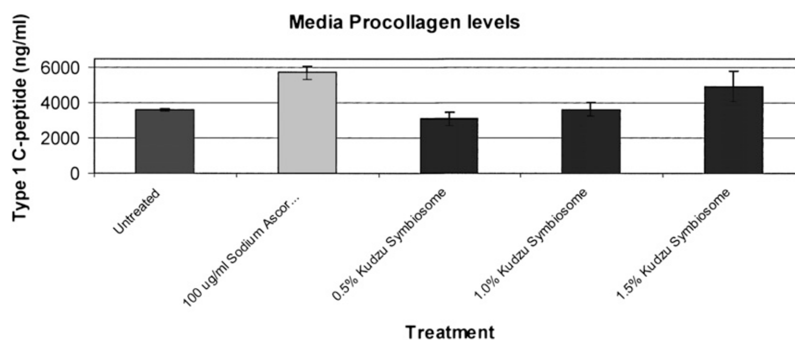

Figure 2. Type 1A1 procollagen analysis on normal human dermal fibroblasts with increasing concentrations of an extract of kudzu symbiosomes. Ascorbic acid was used as a positive control.

realized that *Pueraria lobata* extracts are known to contain also a glycosylated isoflavonoid called puerarin (Scheme 1).

While studies on puerarin have not explicitly shown that this isoflavonoid can stimulate Type 1A1 procollagen production in dermal fibroblasts, it is certainly a possibility that such an effect could be occurring. TLC and HPLC analysis of the extract from kudzu symbiosomes demonstrated that it does contain puerarin at a level of approximately 0.4 wt%. Therefore, a definitive conclusion that the procollagen stimulatory effects were related to the presence of the leghemoglobin could not be made. The experiment was repeated, but two additional control samples were tested, purified leghemoglobin and purified puerarin. Data from the second study are shown in Figure 3.

From the data available from the second study, it becomes immediately apparent that at the concentrations available in the crude extract, both puerarin and leghemoglobin provide a dose-dependent stimulatory influence on Type 1A1 procollagen synthesis in normal human dermal fibroblasts. However, it is apparent that, indeed, leghemoglobin can stimulate procollagen synthesis in support of published claims that cytoglobin, the structurally similar human protein, may play a role in oxygen transport during

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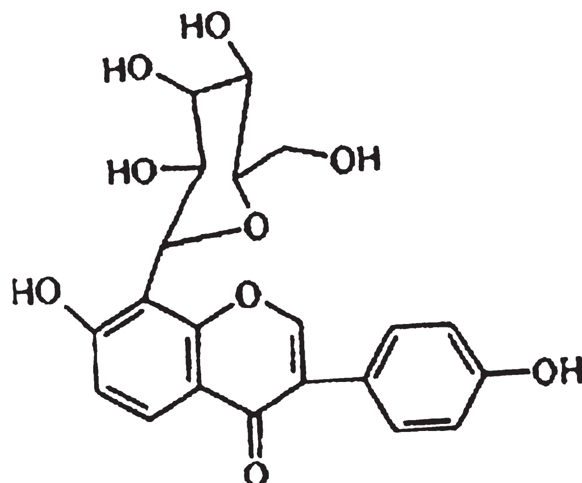

Scheme 1. Chemical structure of puerarin.

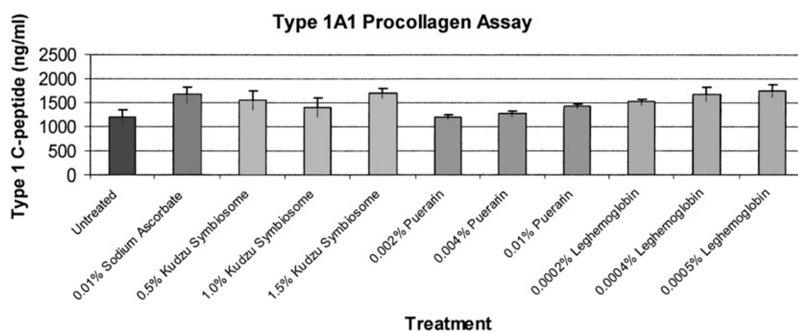

Figure 3. Type 1A1 procollagen results from kudzu symbiosome extract, ascorbic acid, and purified leghemoglobin and purified puerarin.

procollagen synthesis. Both the DNA microarray data and the *in vitro* procollagen data suggest that normal human dermal fibroblasts cannot distinguish between topically applied cytoglobin and topically applied plant-derived leghemoglobin in terms of the ability of these molecules to influence procollagen synthesis.

CONCLUSIONS

It is tempting from the results of these experiments to conclude that globin proteins can influence procollagen production via control of oxygen transportation at the cellular level. However, such conclusions cannot be made directly from the data provided in these studies. Because topical application of both cytoglobin and leghemoglobin appears to upregulate the genes responsible for Type 1A1 procollagen synthesis, the effects seen here may be related to direct transcription influences, not to fundamental oxygen transport effects. Distinguishing whether cytoglobin (and indeed leghemoglobin) affect procollagen synthesis through oxygen control will be tricky, given that these proteins can influence

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these cells at the genomic level. Proof will likely come through more intimate studies done under conditions of hypoxia, where it is known that cellular cytoglobin upregulation has been demonstrated. However, even in these studies, separating genetic influences from oxygen-shuttling influences will remain fundamentally difficult.

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