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EXPRESSION OF HEME OXYGENASE-1 (HSP32) IN HUMAN PROSTATE: NORMAL, HYPERPLASTIC, AND TUMOR TISSUE DISTRIBUTION*

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ABSTRACT

Objectives. Heme oxygenase isozymes, HO-1 and HO-2, are members of the stress/heat shock (HSP) family of proteins, with the known function of cleaving the heme molecule to biliverdin, iron, and carbon monoxide. The aim of this study was to examine the pattern of tissue expression of HO-1 in the human prostate under different states of proliferation and differentiation and to investigate whether the pattern differs between these states.

Methods. Presently, we have determined the pattern of tissue expression of the stress-inducible isozyme, HO-1 (HSP32), in human prostate under normal and pathologic conditions, by immunohistochemistry, using polyclonal antibodies, and have measured HO-1 and HO-2 mRNA levels in normal prostate and benign prostatic hyperplasia (BPH) by Northern blotting. The activity of prostate to catalyze heme degradation was also assessed.

Results. In normal and BPH tissue, columnar epithelial cells of acini and ducts and cells in stroma displayed HO-1 immunoreactivity; in all cells, perinuclear staining was prominent. In BPH tissue, however, a more intense staining of the epithelial cells occurred, with notable staining of the basal cells. In undifferentiated malignant tumors, intense HO-1 staining was manifest in nearly all tumor cells, and also in the epithelial lining of blood vessels. HO-1 in the prostate tissue was found catalytically active and oxidatively cleaved the heme molecule (Fe-protoporphyrin IX) to biliverdin. Northern blot analysis shows that two forms of HO are present in the human prostate. Compared with normal tissue, predominantly hyperplastic tissue demonstrates a pronounced increase in the approximately 1.8 kb mRNA that hybridizes to the rat HO-1 probe. The levels of two transcripts, approximately 1.3 and approximately 1.7 kb, that hybridize to the rat HO-2 probe are not increased in BPH tissue.

Conclusions. The finding that HO-1 expression is increased in BPH and malignant prostate tissue is consistent with a role for this stress protein in the pathogenesis of BPH and prostate cancer; in the context of iron metabolism, an argument is made in support of this possibility. UROLOGY® **47:** 727-733, 1996.

Recent years have witnessed the accumulation of a substantial body of information on the expression and function of stress/heat shock proteins (HSP); the larger proteins, HSP70 and HSP90 in particular, have been the focus of investigation.[1-4] These HSPs each represent a family of several proteins that are similar in function, but differ in their tissue expression and regulatory mechanisms.[5-7] Stress proteins fulfill essential functions in normal and stressed cells.[1,3,8] Heme oxygenase (HO) proteins are members of the HSP30 family and consist of two forms identified to date, HO-1 and HO-2.[9,10] A well-defined function of the two proteins is the oxidation of the heme molecule.

Indeed, the voluminous data gathered on this family of HSPs has only been concerned with their heme degrading function. Moreover, for the most part, animal studies have been the primary model for experiments with heme oxygenase, and, with

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the exception of a recent report on the increased tissue levels of HO-1 in the human brain under neurodegenerative conditions,[11] nothing is known about the expression of this HSP in human tissue in either a normal state or during various states of cellular differentiation. HO-1, an exquisitely stress-responsive protein, in concert with reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase, catalyzes the isomer-specific cleavage of the heme (Fe-protoporphyrin IX) molecule to bile pigments, carbon monoxide, and iron. The heme molecule constitutes the prosthetic moiety of various hemoproteins, including cytochrome P450. These cytochromes in testes and prostate are involved in steroid biosynthesis, as well as metabolism of drugs and activation of promutagens and procarcinogens.[12–17] The second form of heme oxygenase, HO-2, is a constitutive protein.[18] HO-1 and HO-2 are evolutionarily conserved proteins, and share less than 50% similarity at the amino acid sequence level; each form, however, shows greater than 90% similarity among species, including human and the rat.[13]

Heme oxygenase serves in both a catabolic and anabolic capacity in the cell. In the former capacity, it regulates cellular heme and hemoprotein levels, including that of cytochrome P450,[19] and an inverse relationship between heme oxygenase activity and the level of cytochrome P450 has been established.[19–21] In its anabolic role, heme oxygenase produces bile pigments, carbon monoxide, and iron, all of which are biologically active: bile pigments function as antioxidants[22]; the carbon monoxide generated by HO activity has been recently suggested to function in the generation of cyclic guanosine monophosphate (cGMP)[23–26]; and iron regulates expression of various genes, including that of HO-1 itself, as well as transferrin receptors, ferritin, and NO synthase.[27–30] Transferrin, the iron source for cell growth, has in recent years been identified as a selective stimulator of human prostate carcinoma cell proliferation.[31,32]

To our knowledge, this is the first report on the expression of heme oxygenase in normal hyperplastic and malignant human prostate tissues.

MATERIAL AND METHODS

Biochemicals were purchased from Sigma Chemical Co. (St. Louis, Mo) and were of the highest purity commercially available. Nytran sheets were obtained from Schleicher and Schuell (Keene, NH). α-^{32}P 2′-deoxycytidine 5′-triphosphate (dCTP) was obtained from DuPont-NEN Corp. (Boston, Mass). For preliminary experiments that were aimed at establishing experimental conditions, human tissue supplied by The National Disease Research Interchange (Philadelphia, Pa), frozen at −80°C, was used. Rat prostate tissue was obtained from young adult male Sprague-Dawley rats (Harlan Industries, Madison, Wis). The National Institutes of Health *Guide for the Care and Use of Laboratory Animals* was strictly followed.

Human Prostate Tissue

Prostate tissue specimens were obtained from 6 patients, 53 to 69 years of age (mean, 64 years), with clinically localized carcinoma of the prostate (CaP), who underwent radical prostatectomy. None of the patients received any hormonal therapy prior to radical prostatectomy. All tissue specimens were collected on ice. In all cases, prostate tissue specimens were cut and immediately processed. Tissue samples were initially classified by gross appearance as normal prostate tissue, benign prostatic hyperplasia (BPH), or CaP. Specimens were fixed using the AmEX method[33] to optimize antigen preservation for immunostaining. Tissues were fixed in acetone at −20°C overnight, then cleared in methyl benzoate and xylene, and embedded in paraffin at 58 to 60°C. Sections 3 to 4 μm thick were obtained. Histology was confirmed by light microscopic examination of hematoxylin and eosin (H&E) stained slides from each specimen. Tissue specimens that manifested heterogeneity in terms of mixed populations of normal, BPH, and CaP cells on the H&E sections were used for assessment of prostate heme oxygenase activity as detailed later, and only those specimens manifesting exclusively pure normal prostate, BPH, or CaP cell populations were used for the immunocytochemical and Northern blot analyses. Because of scarcity of homogenous cancerous tissue, the blot analysis was carried out only with normal and BPH samples. The human prostate samples were provided by the Urology Department of the University of Rochester.

Microsome Preparations and Enzyme Assays

The prostate was fractionated, as previously described,[15] and used for measurement of heme oxygenase activity as detailed earlier[15] in the presence of purified exogenous NADPH-cytochrome P450 reductase and purified rat liver biliverdin reductase. The latter enzyme was used to convert biliverdin to bilirubin, which was subsequently measured using an extinction coefficient of 40 mM·1 cm^{-1} between 470 and 530 nm. The protein concentration was determined by the method of Lowry and coworkers,[34] using bovine serum albumin as the standard.

Probes, RNA Preparation, and Northern Blot Analysis

The HO-2 probe used was the full length (1300 bp) HO-2 cDNA isolated from a rat testis cDNA library[35]; the HO-1 probe was a cDNA fragment corresponding to HO-1 nt 71→833.[36] The two oligonucleotide primers used to generate HO-1 probe by polymerase chain reaction (PCR) were: 5′TGGAGATCGGTGCAGAGAAT3′ homologous to HO-1 cDNA nt 71→90, and 5′AGGAAACTGAGTGTGAGGAC3′ complimentary to HO-1 cDNA nt 833→814. The PCR product was digested with *Eco*RI and *Pst*I to yield a 568 bp fragment, which was ligated into PBS+ vector predigested with the same enzymes. *Escherichia coli* XL-1 blue were transformed with the ligation products, and the sequence of the insert was confirmed to be homologous to rat HO-1. All probes used in this study were labeled according to the manufacturer's instructions with α-^{32}P dCTP, using the random priming method (random primers DNA labeling system; U.S. Biochemical Corp.), and further purified by spin column chromatography.

Total RNA was isolated from human and rat prostate with the guanidine isothiocyanate/cesium chloride technique.[37] Poly(A$^+$) RNA was prepared by oligo(*dt*)-cellulose chromatography and was subsequently formaldehyde-denatured and fractionated on a 1.2% (wt/vol) agarose gel and transferred to Nytran. The filters were prehybridized, hybridized

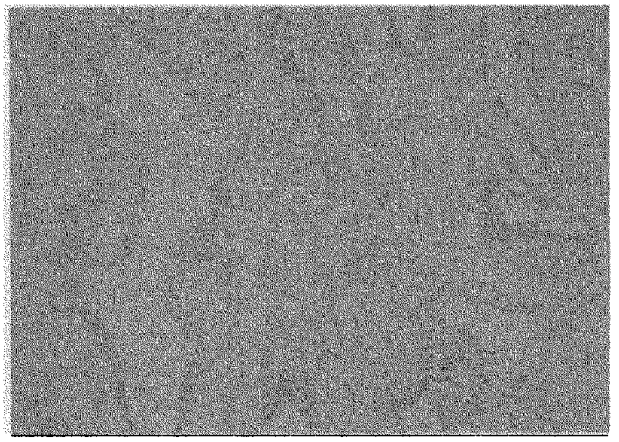

FIGURE 1. *Immunostaining with HO-1 protein antibody in normal prostate tissue. Note the perinuclear staining of the epithelial lining of the prostate acini, as well as fibroblasts and smooth muscle cells in the stromal tissue. There is also weak staining of the basal cell layer and a diffuse cytoplasmic staining throughout the tissue. (Original magnification: ×50.)*

with the appropriately labeled cDNA probe, washed, and exposed to Kodak X-OMAT-AR film at −80°C as previously detailed.[38] Signal was quantitated by densitometry, using an Ultra Scan XL densitometer (Pharmacia LKB Biotechnology, Piscataway, NJ). Northern blot analysis of normal and BPH prostate was carried out with several different preparations. However, because of required handling of tissue during surgery, some denaturation of mRNA was unavoidable and routinely noted in human samples.

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Rat liver HO-1 purified to homogeneity[9] was used to raise polyclonal antibodies in New Zealand White rabbits as previously described.[10] The antibody to HO-1 does not cross-react with HO-2.[10,18] Moreover, preabsorption of the antibody with purified HO-1 totally abolishes tissue immunostaining.[39] Immunostaining was performed on multiple, consecutive sections of AmEX-processed normal, hyperplastic (BPH), and cancerous (CaP) human prostate. The use of AmEX-processed sections yields excellent immunostaining (similar to frozen sections) and better preservation of tissue morphology, besides facilitating the handling of tissue and blocks. Slides of duplicate specimens were overlaid with normal rabbit se-

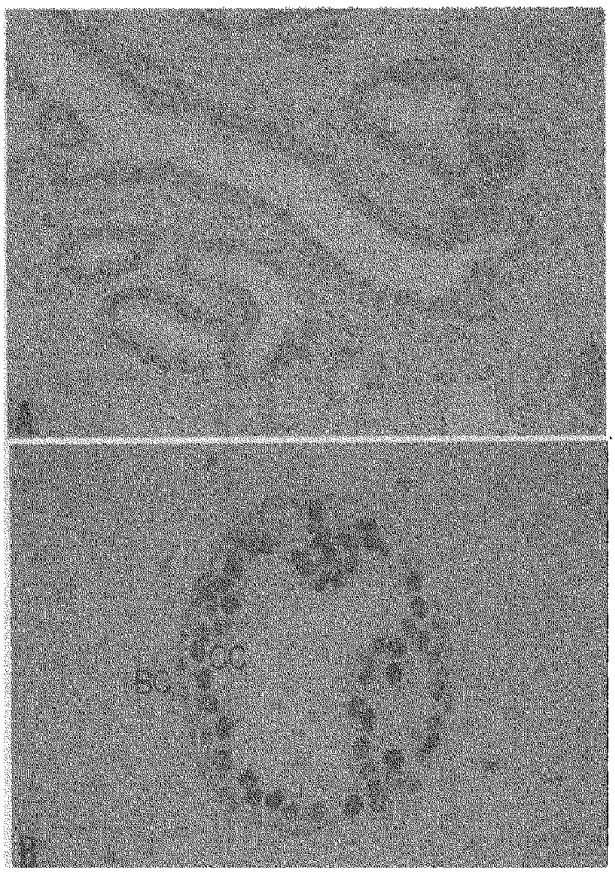

FIGURE 2. *(A,B) Immunostaining for HO-1 protein in benign prostatic hyperplasia tissue. Note that in BPH tissue, HO-1 immunostaining is most intense in the columnar epithelial cells (CC); less intense, but quite evident, staining of the basal cells (BC) lining the prostatic acini is also visible. In addition, there is HO-1 immunostaining throughout the stromal cell population on the nuclear membrane and the cytoplasm. (Original magnification: A, ×50; B, ×124.)*

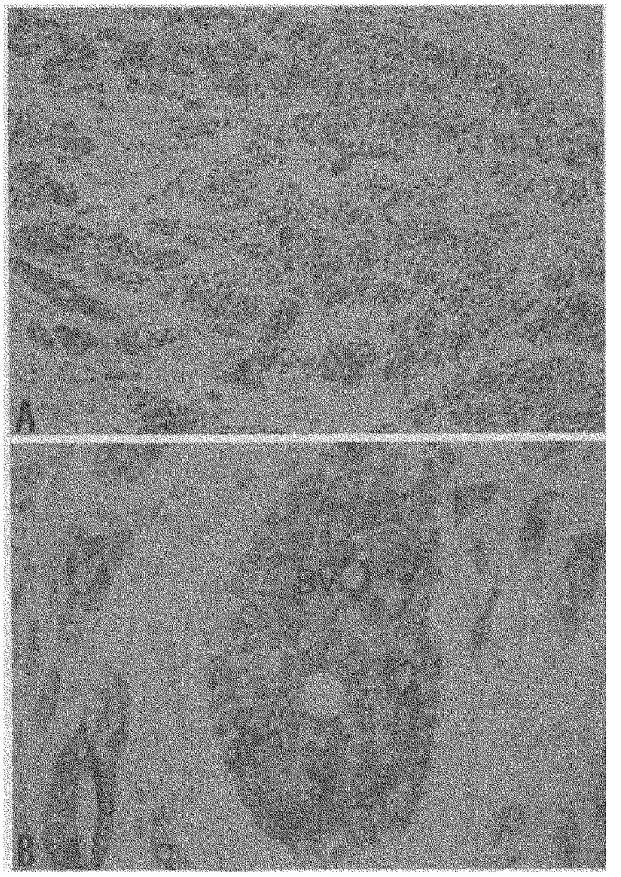

FIGURE 3. *Immunostaining for HO-1 protein in poorly differentiated adenocarcinoma of the prostate. Staining of an undifferentiated tumor tissue is shown. (A) Variable but intense HO-1 staining can be seen throughout the cancerous tissue. (B) On magnification, perinuclear and punctate staining of nearly all tumor cell nuclei is seen. Also noteworthy is the cytoplasmic staining of tumor cells and stromal cells. BV = blood vessel. (Original magnification: A, ×50; B, ×124.)*

rum (1:20), incubated at 24°C for 20 minutes, and drained. The slides were incubated overnight at 4°C with HO-1 antibody (working dilution 1:250 of antiserum). After each step, the tissue was rinsed in phosphate-buffered saline. A biotinylated goat anti-rabbit immunoglobulin G antibody (1:200) was applied and incubated for 30 minutes at 25°C. The immune complex was visualized with chromogen 3-amino-9-ethyl carbazole (AEC) (Zymed, San Francisco, Calif). After a final rinse in tap water, the slides were cover slipped. Primary antibody was diluted in 0.01 M phosphate buffer, pH 7.6 containing 137 mM sodium chloride, 2.7 mM potassium chloride, and 0.5% bovine serum albumin. No immunostaining was obtained with normal rabbit serum.

RESULTS

Immunostaining with rabbit anti-rat HO-1 protein antibody in the periurethral region of the normal prostate is shown in Figure 1. As Figure 1 shows, HO-1 immunoreactivity is more or less uniformly distributed in secretory epithelial cells, wherein it is predominantly perinuclear. In addition, staining of the fibroblast and smooth muscle cells in the stromal tissue is observed; in these cells also, staining is more intense in the perinuclear region of the cells. Cells in the basal layer of the epithelium manifested weaker staining for HO-1. Diffuse and weak cytoplasmic staining is observed in the stroma and parenchyma cells throughout the tissue.

The pattern of immunoreactivity of the antibody to HO-1 protein in BPH tissue, which is shown in Figure 2A,B, differs from the control. To elaborate, as with the normal tissue, immunostaining is observed in the epithelial columnar cells, the fibroblasts, and the smooth muscle cells of the stroma in the BPH tissue; however, staining is clearly more intense in the epithelial columnar cells. Also noted is the presence of HO-1 immunoreactivity in the epithelial basal cells of the prostate acini. A larger magnification of an acinus (Fig. 2B) clearly shows the staining of the basal epithelial cells, and also demonstrates that among all cell populations the columnar epithelial cells display the most intense HO-1 immunoreactivity. Furthermore, these cells, in addition to showing perinuclear staining, also show a discernible pattern of punctate nuclear staining.

A different pattern of staining emerges in cancerous tissue, although HO-1 protein staining intensity varied between specimens and within a tissue section (Fig. 3A,B), with the greatest variation in poorly differentiated tumors. In all instances, however, tumors of the prostate display intense immunostaining, due to the predominance of HO-1 immunoreactive cells (Fig. 3B). Moreover, perinuclear staining remained intense. Nearly as intense HO-1 immunoreactivity was present throughout the cytoplasm of the tumor cells. Also noteworthy is a most intense staining of the epithelial lining of blood vessels.

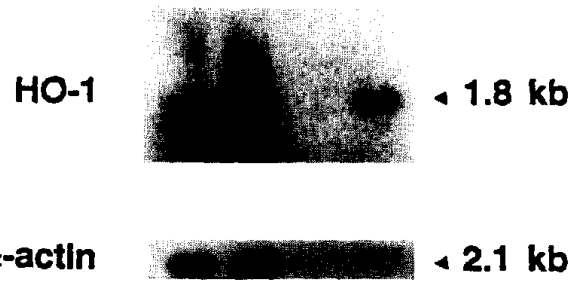

FIGURE 4. *Northern blot analysis of heme oxygenase transcripts in human prostate. Poly (A⁺) RNA was isolated from BPH and normal human prostate, and from rat prostate. Northern blot analysis was performed as detailed in Material and Methods. A total of 8 μg of human or 4 μg of rat poly(A⁺) RNA was loaded in each lane. Sequentially, a ^{32}P-labeled HO-1 cDNA fragment, a full length HO-2 cDNA, and α-actin were used as probes. Actin was used for quantitation of the signals. An increase of nearly threefold in the approximately 1.8 kb mRNA level was noted. No change in the approximately 1.3 and 1.7 kb HO-2 homologous transcripts was detected.*

Next, we examined whether an increase in the HO-1 immunoreactive protein detected in human prostate tissue was the result of an increased transcript level. Northern blot analysis was used to measure HO-1 mRNA. Because of limited tissue supply and difficulty obtaining uniform cancerous tissue, analysis was conducted using normal and BPH tissue only. Also, due to the unavailability of human hyperplastic reference points, rat prostate mRNA, for which the sizes of the HO-1 and HO-2 messages are known, was used as the means of size comparison for human prostate samples. Data in Figure 4 show the presence of a very low level of approximately 1.8 kb mRNA in the human prostate tissue that hybridizes with the rat HO-1 cDNA probe. The same transcript is also detected in the rat prostate. Moreover, when quantitated, the transcript level is found to be significantly higher (approximately threefold) in the BPH tissue; although levels of actin message confirm approximately equal loading. Figure 4 also shows that, as with the rat, two transcripts for HO-2 are detected in both normal and BPH human prostate samples when probed with rat HO-2 cDNA; the transcripts measure approximately 1.3 and approximately 1.7 kb in size. As already noted, the nucleotide sequences for each heme oxygenase isozyme in rats and humans are highly conserved (more than 90%); therefore, the hybridization signals are real and specific. The observation that, in BPH samples, the HO-1 transcript level is increased, whereas that of HO-2 is not, is consistent with the fact that HO-1 is a stress protein and HO-2 is the constitutive cognate.

To ensure that the HO-1 protein detected in human prostate is indeed catalytically active, heme oxygenase activity of the organ was measured. Because the protein was closely associated with the nuclear membrane in many cells, the nuclear pellet fraction as well as the microsomal fraction was used as enzyme source. Moreover, because of the large tissue requirement for preparation of cellular fractions, mixed (normal and BPH) tissue samples were used. When these samples were assayed, the human prostate displayed good heme degrading activity. The mean value for activities of two samples for the microsomal and nuclear enriched preparations were 1.25 and 0.95 nmol bilirubin/mg of protein/h, respectively. The heme degradation was determined to be due to heme oxygenase activity as bilirubin formation was not detected when the assay was carried out in the presence of all components in the system, except for prostate fractions. Because of the mentioned experimental tissue constraints, heme oxygenase measurements were not intended to arrive at a statistical evaluation of the relative activity of the fractions.

COMMENT

This report describes, for the first time, the detection of the stress protein, HO-1 (HSP32), at the protein and transcript levels in the human prostate under normal and pathologic conditions, and it demonstrates that there is an increase in HO-1 expression in hyperplastic prostate tissue compared with that of normal tissue. Using the criteria of immunoreactivity, we found HSP32 to be normally present in the cellular components of the stroma and epithelium of the prostate gland. Using this method of analysis, along with Northern blotting, a notable increase in the protein in the epithelial cells and transcript level for the stress protein was found in BPH tissue. At this time, it is not clear whether the increase in HO-1 mRNA in BPH tissue reflects a general increase in transcript level throughout the prostate or is related to differences in normal and BPH tissue in the composition of the stroma and epithelial cell layer. Moreover, the expression of HO-1 was found increased under the malignant condition, as suggested by the detection of the protein in nearly all cells comprising the tumor nodules in the cancerous specimens.

It is noteworthy that HO-1 is the only HSP for which a catalytically based mechanism of action of cellular proliferation or transformation might be proposed in relation to iron and transferrin, as discussed later. At present, the role of other HSPs in cellular proliferation and cancer is not understood, although numerous reports have documented expression of various HSPs in proliferating tissue. In general the reports encompass a description of the occurrence of HSPs in tumor tissue[40-42] and provide a correlation between their increased expression and that of other proteins known to be involved in cellular transformation, such as p53[43] and estrogen receptor.[44] For the most part, however, an increased expression of HSPs is interpreted as relevant to the progression of cancer.[44]

Admittedly, the increase in HO-1 expression in benign and cancerous prostate tissue does not constitute unequivocal evidence for the existence of a relationship, in the prostate, between the stress protein and abnormal cellular proliferation or cellular transformation; nonetheless, the observed positive correlation, plus the known activity of the enzyme to degrade the heme molecule and generate biologically active products, permits speculation on such possibilities. Accordingly, the following line of reasoning is used to propose a role for increase in HO-1 expression in prostate cell proliferation or transformation, which is based on the function of the heme oxygenase system in the catalysis of the heme molecule and release of the chelated iron.

The sensitivity of prostate carcinoma to the mitogenic activity of transferrin, which functions as an iron donor to cells, has been shown.[31,32] Because iron chelates can donate iron to proliferating cells without using transferrin receptor pathways,[45,46] it is evident that iron is the active component of the iron-transferrin complex, and in proliferating cells, the requirement for iron is increased, as the metal is a component of numerous enzymes.[47] With these considerations in mind, heme oxygenase, using hemoglobin heme as the substrate, could likely supply high levels of iron to the cancerous tissue, promoting cellular proliferation. The observed increase in HO-1 immunoreactivity of the epithelial lining of the blood vessels in tumor tissue is supportive of this suggestion. In addition, iron released by heme oxygenase activity could also regulate prostate tissue transferrin levels—a suggestion that is in line with the proposed model for iron transport in the testis.[48] According to this model, apotransferrin synthesized in the endoplasmic reticulation is secreted in the Golgi vesicles; iron in the form of Fe^{2+} or Fe^{3+} then interacts with the apotransferrin before, during, and after secretion.

The above argument is not intended to suggest that increased heme oxygenase may always stimulate all stages of prostate proliferation or cellular transformation. The dependence on androgens for prostate gland development and function is commonly known. Specifically, 5α-dihydrotestosterone (DHT), the product of testosterone reduction by cytochrome P450-dependent activity,[13,17] is the active form of androgen produced in the prostate, wherein it binds to androgen receptors and,

subsequent to nuclear translocation and DNA binding, promotes transcriptional activation of steroid-regulated gene expression and prostate proliferation. To elaborate, as noted earlier, an established function of heme oxygenase is its role in the regulation of cellular levels of cytochrome P450-type hemoproteins, including those involved in steroidogenesis.[19] Accordingly, increased HO-1 activity may result in a decrease in DHT production, as the result of enhanced heme degradation. However, it would be reasoned that as the malignant tissue becomes androgen independent, this potential advantage of activation of the heme oxygenase activity is lost.

In conclusion, the data presented here, which constitutes the first report regarding detection of HO-1 (HSP32) in human prostate, suggest an association of the stress protein's expression and pathogenesis of BPH and prostate cancer. In the context of the catalytic activity of HO-1, the increase in its expression in benign and cancerous tissue is consistent with a role for the protein in cellular proliferation and transformation. Specifically, it is proposed that the enzyme, by releasing iron chelated in hemoglobin heme, could supply high levels of iron, which is essential for growth of the tissue.

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