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INDUCTION OF HAEM OXYGENASE AS A DEFENCE AGAINST OXIDATIVE STRESS

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Cells respond to metabolic perturbations by producing specific stress proteins. Exposure of mammalian cells to various forms of oxidative stress induces haem oxygenase, the rate-limiting enzyme in haem degradation. This response is proposed to represent an antioxidant defence operating at two different stages simultaneously. It (i) decreases the levels of the potential pro-oxidants haem and haem proteins such as cytochrome P-450 and protoporphyrinogen oxidase, and (ii) increases the tissue concentrations of antioxidatively active bile pigments.

KEY WORDS: Oxidative stress, pro-oxidants, antioxidants, bilirubin, biliverdin, haem metabolism, haem oxygenase, induction.

ABBREVIATIONS: Bilirubin, BR; Haem oxygenase, HO; Interferon-γ, IFN-γ.

INTRODUCTION

Aerobic life is ultimately associated with the *in vivo* production of superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2).[1] In some instances these oxygen reduction products may serve a beneficial function, as in the case of $O_2^{\cdot -}$ generated by phagocytic cells during the respiratory burst to kill certain bacteria. However, uncontrolled or excessive production of oxygen free radicals can result in oxidative damage to biological material. In particular, in the presence of available transition metals such as iron or copper complexes, $O_2^{\cdot -}$ and H_2O_2 can give rise to the very reactive and toxic hydroxyl radical ($^{\cdot}OH$).[2] Various iron complexes, including haem (iron protoporphyrin IX), are present *in vivo* that can promote generation of $^{\cdot}OH$ and decompose lipid hydroperoxides with formation of alkoxyl and peroxyl radicals.[2-4]

Under normal conditions the potentially hazardous reactions initiated by oxygen free radicals are counteracted by multiple lines of antioxidant defences that operate at the level of prevention, interception and repair of oxidative damage.[5] These detoxification systems are compartmentalized (tissue, extracellular fluid, organelle, lipid or aqueous phase) and comprise enzymatic as well as non-enzymatic compounds such as the vitamins E and C.[2] There is emerging evidence that metabolic end products, such as uric acid[6] and bilirubin (BR),[7] are also important physiological antioxidants whose formation was introduced during evolution[6,8] and that may exert their protective function in a more local environment. For example, human albumin-bound BR can protect albumin-found fatty acids from oxidation[9] thereby acting as a site-specific antioxidant in human blood plasma.[10]

Responses to oxidative stress

To maintain homeostasis, cells respond to metabolic perturbations by the production of stress proteins. Exposure of cells to oxidative stress, defined as the disturbance of the cellular pro-oxidant-antioxidant balance in favour of pro-oxidants,[5] induces the specific synthesis of enzyme(s) involved in antioxidant defences. These responses have been mostly studied in procaryotic cells where a number of different defence systems can be induced.[11-14] Among lower eucaryotes, *Saccharomyces cerevisiae* responds to a shift in growth conditions from anaerobic to aerobic by an induction of manganese superoxide dismutase.[15] Expression of the same antioxidant enzyme is also drastically induced as part of the stress and pathogenesis response of the plant *Nicotiana plumbaginifolia*.[16] As in bacteria, overlaps exist among the various stress responses of eucaryotes. For example, exposure of *Drosophilia* cells to H_2O_2 leads to increased synthesis of some heat shock proteins[17] while lungs from adult rats respond to heat shock by increased synthesis of Cu, Zn-superoxide dismutase.[18] Survivors of H_2O_2 or heat-pretreated chinese hamster fibroblasts develop resistance to H_2O_2,[19] and repetitive treatment of such cells with step-wisely increasing concentrations of this oxidant can produce H_2O_2-resistent clones which contain a 10-fold higher catalase activity than the parental strain.[20] Finally, a link between gene activation of glucose-regulated proteins, heat shock proteins and oxidative injury and/or its protection has been suggested to occur in higher eucaryotes including human cells.[21-24]

HYPOTHESIS

Induction of haem oxygenase (HO; EC 1.14.99.3) provides protection against oxidative stress by decreasing the concentrations of certain potential pro-oxidants and simultaneously increasing the endogeneous concentrations of certain antioxidants.

The physiology and regulation of haem metabolism has been reviewed extensively,[25-27] and will not be repeated here. Rather, it is the intention of the following to present our knowledge on haem metabolism and its regulation in the light of a possible function of HO as an antioxidant defence system.

DECREASE OF PRO-OXIDANTS BY HAEM OXYGENASE

Intracellular haem

Haem serves as the prosthetic group of various haemoproteins such as those that transport oxygen or electrons, activate oxygen, or those that degrade peroxides (Figure 1). Mitochondrial δ-aminolevulinic acid synthetase and microsomal HO are the rate-limiting enzymes in haem biosynthesis and degradation, respectively.[25] HO is a ubiquitous enzyme using free haem as well as haem loosely bound to proteins as in methaemalbumin, methaemoglobin, or denatured myoglobin, as substrates.[27] Recently the haem of purified cytochrome P-450b/P-420b was shown to be converted to biliverdin by a reconstituted HO system.[28]

As mentioned above, free haem is a form of low-molecular iron capable of catalysing oxygen radical reactions and hence is a powerful pro-oxidant. Beside stimulating lipid peroxidation,[4] it causes oxidative damage to DNA[29] and protein[30] and is cytotox-

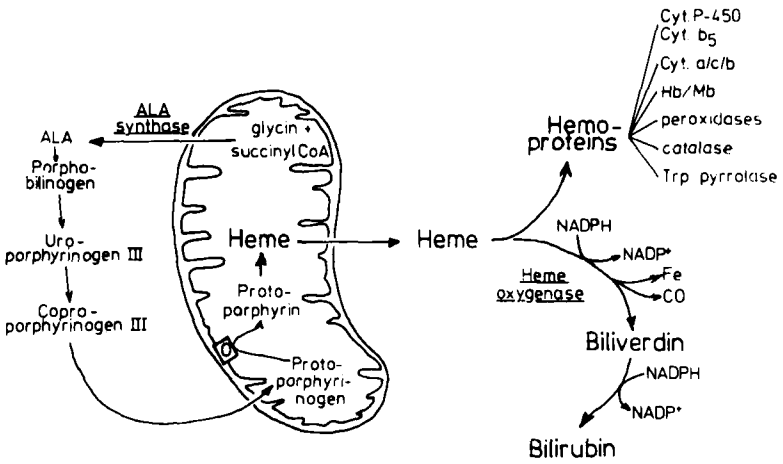

FIGURE 1 Pathway of Haem Metabolism. Mitochondrial δ-aminolevulinic acid synthetase and microsomal haem oxygenase, the rate-limiting enzymes in haem synthesis and degradation, respectively, are underlined. The H_2O_2-producing protoporphyrinogen oxidase ⊡ is an integral membrane protein of the mitochondrial inner membrane of unknown orientation.[46]

ic to various cell types.[31] Although little is known about the mechanism(s) by which haem is translocated intracellularly,[32] it is generally accepted that at least in liver parenchymal cells there is an intracellular "free haem pool" defined as free or loosely-bound haem at a concentration of 0.1–0.2 μM[33] that readily exchanges with haem of haemoproteins.

In rats two forms of isozymes of HO (HO-1 and HO-2) are present at different relative concentrations in the various tissues. These isozymes seem to represent products of distinct genes[34] and differ in their immunological and biochemical properties.[27,35] The apparent K_m values of HO-1 and HO-2 for protohaem are 0.24 and 0.67 μM, respectively,[35] indicating that at physiological haem concentrations, HO is operating at sub-maximal activity. Since exposure of animals to HO inducers is accompanied by an increase in the activity of HO-1, the more efficient form of the two isozymes, and does not affect HO-2,[27] such conditions are expected to result in significantly increased rates of haem degradation. Indeed, an increase in HO activity is generally accompanied by a decrease in the cellular concentrations of total[25,36–38] and free[33] haem, thereby lowering endogenous levels of this pro-oxidant.

Cytochrome P-450

It is well known that biotransformational reactions dependent on cytochrome P-450 are a major source of oxidants produced by the microsomal electron transport chain.[38,40] Most often induction of haem oxygenase results in decreased cellular cytochrome P-450 activity[36,38,41–43] through a reduction of cellular haem available for incorporation into newly synthesized cytochrome P-450 and increased degradation of the haem moiety of this haemoprotein caused by increased HO activity.[25] In many tissues cytochrome P-450 requires a great part of the total haem synthesized. This, together with its short half-life compared to other haemoproteins[43,44] and HO's ability to directly degrade some cytochrome P-450 isozymes,[28] led Maines to suggest that HO partly regulates cytochrome P-450-dependent biotransformations.[27]

Protoporphyrinogen oxidase

A decrease in the levels of cellular pro-oxidants via induction of HO may involve a third and additional mechanism. Protoporphyrinogen IX oxidase (EC 1.3.3.4), the penultimate enzyme in haem biosynthesis, catalyses a six electron transfer reaction to form protoporphyrin IX from protoporphyrinogen IX.[45] Similar to xanthine oxidase (EC 1.1.3.22), mouse liver protoporphyrinogen oxidase produces H_2O_2 during its catalytic cycle.[46] BR is a competitive inhibitor of the oxidase with a calculated K_i of 25 μM.[46] Interestingly, this K_i value is very similar to the known physiological concentration of BR in liver cytosol.[47] Thus, an increase in the cellular BR concentration may result in significant inhibition of protoporphyrinogen oxidase, thereby reducing H_2O_2 production by protoporphyrinogen oxidase.

INCREASE IN ANTIOXIDANTS BY HAEM OXYGENASE

Based on the haem degradation pathway (Figure 1) an induction of HO is expected to result in increased formation and steady state concentrations of bile pigments. Although available literature on the effect of HO induction on intracellular (hepatic) levels of bile pigments is rather scarce, BR is expected to accumulate as its formation from biliverdin occurs so rapidly in mammals that normally no appreciable levels of the latter accumulate.[48] This is supported indirectly by several lines of evidence. As mentioned earlier, induction of HO results in a decrease in cellular levels of haem and haemoproteins,[25,36-38] and this reduction is caused partly through increased haem degradation.[25] As a consequence, BR is produced at higher rates with subsequent possible increase in its steady state concentration. In addition, accumulation of bile pigments can also result from decreased rate of its removal, as is the case in hepatocytes. Thus, under conditions of oxidative stress (see below), biliary excretion of BR is markedly decreased,[49] possibly the result of decreased uridine diphosphate-glucuronyltransferase activity.[49] In non-hepatic cells, where only little "free haem" and haemoproteins are present, significant amounts of BR may still accumulate as such cells are devoid of enzymatic systems that remove the pigment formed. Present models of BR dynamics suggest that in humans circulating BR exchanges rapidly with extravascular and hepatic pools of the pigment, and the BR concentration increases from the vasculature to the liver, bile and intestine.[51] Circulating levels of BR have been reported to be increased under several conditions where HO is induced[48,52,53] (e.g. dietary restriction, haemolytic anemia or treatment with phenylhydrazin). As expected, under these conditions hepatic and biliary BR levels are elevated.[54,55]

The antioxidant activities of bile pigments and BR in particular have been discussed previously.[7,9,10,56-59] From these references and the once quoted therein, it is obvious that all physiological forms of BR present throughout the intra- and extracellular space of our body are efficient radical scavengers. This strongly supports the function of the pigment as a physiologically important antioxidant.

INDUCTION OF HAEM OXYGENASE BY OXIDATIVE STRESS

As mentioned earlier, HO-1 activity can be induced up to 100-fold by exposing the animal to inducers while HO-2 is uninducible.[27,35] A great variety of chemicals, factors and conditions have been identified by various investigators to be able to induce

TABLE 1
Induction of haem oxygenase in response to various conditions that are or can be associated with oxidative stress

Test system	Condition of oxidative stress	Ref
Human fibroblasts	Exposure to oxidants – H_2O_2, menadione	62, 63
Mouse fibroblasts	– sodium arsenite	62, 63
Human leukemic cells	– sodium arsenite	64
Mouse fibroblasts	Irradiation with Near UV light	62, 63
Mouse fibroblasts	Exposure to phorbol myristate acetate chemical carcinogens, or metal salts	65-67
Rat macrophages	Phagocytosis of antibody-coated erythrocytes	68
Rat	Whole body X-ray irradiation	27
Rat	Endotoxemia, treatment with IFN-γ inducers	69, 70
Rat	Exposure to – hemin	71
	– heavy metals	36
	– SH-reactive agents	72
	– CCl_4	25
	– phenylhydrazine	53
Mouse	Deficiency in – selenium	73
Rat	– copper	74
Guinea Pig	– ascorbic acid	75

HO[25,26,52,60] at the transcriptional[61] and, possibly, translational level.[38] Although not true for all, these inducers include various conditions that directly or indirectly can be associated with oxidative stress (Table 1).

Oxidizing agents and heavy metals

Exposure of normal human or mouse fibroblasts to either near ultraviolet radiation, H_2O_2, menadione, or the thiol-oxidizing agent diamide, induces a 32-kDa protein.[62] Tyrrell and co-workers have recently cloned this protein and identified it as HO.[63] In support of our previous suggestion[9] these authors have also proposed its induction to be a general defence mechanism against oxidative stress.[63] Induction of HO by sulfhydryl reactive agents has long been known,[72] and recent studies on its mechanism indicate that both glutathione depletion by more than 80% and formation of glutathione conjugates are required for the 32-kDa protein to be induced.[64,76] Exposure to arsenite or heavy metals specifically induces the synthesis of a 32-kDa protein in human cells[37,38] that has now been identified as HO.[64] In the light of these findings it seems likely that the yet unidentified stress protein induced in rat hepatoma cells upon exposure to ADP-iron or 4-hydroxynonenal,[77] is HO. 4-Hydroxynonenal is a highly reactive electrophilic reagent which reacts easily with thiol groups and is formed *in vivo* during lipid peroxidation.[78] In human cells a 32-kDa stress protein is also induced by certain gold-containing compounds such as auranofin that are approved for the treatment of rheumatoid arthritis.[79,80] Since aggressive oxidants seem to be partly responsible for the damage observed in rheumatoid arthritis[2] it is tempting to speculate that part of the anti-arthritic action of gold components may be due to an increased antioxidant protection provided by the induction of HO.

Some of the HO-inducing oxidants listed in Table 1 also cause haemolysis and haemoglobin denaturation, and one could argue that induction of HO may be needed simply to remove excessive haemoglobin and haem protein degradation products.

However, free haemoglobin and haem in the extracellular space can promote radical-mediated tissue damage[81] that is inhibited in the presence of the haemoglobin and haem-transporting proteins, haptoglobin and hemopexin, respectively.[82,83] In this context, induction of HO can be regarded as an additional antioxidant defence working in conjunction with haptoglobin and hemopexin by actually helping to remove haem from circulating complexes of haem/haemoglobin and their transporting proteins. Under conditions of haemoglobinemia where the plasma haptoglobin-binding capacity is exceeded, HO-activity in renal epithelial cells is induced to stabilize plasma levels of haemoglobin.[84]

Tumour promoters, carcinogens and heavy metals

Independent from Tyrrell's group, the HO gene was also cloned from cDNA coding for the 32-kDa protein that is induced in mouse fibroblasts by various tumour promoters, chemical carcinogens and metal salts.[65–67] These findings may be relevant to the known tumour promotional and carcinogenic activity of pro-oxidants and the anticarcinogenic activity of antioxidants.[85,86] The increase in HO activity in spontaneous or chemically-induced tumours[87] is in agreement with the general observation that most tumour cells have altered antioxidant defences.[88]

Erythrophagocytosis

Circulating erythrocytes which have reached the end of their physiological life-span become opsonized by antibodies to erythrocyte membrane constituents before they are removed by mononuclear phagocytes in the spleen and liver. Phagocytosis of antibody-coated erythrocytes is an Fc-receptor mediated process that results in activation of the respiratory burst of phagocytes[89] and has long been known to be accompanied by an increase in HO activity.[68] More recent studies have shown that the induction of HO during erythrophagocytosis requires the production of $O_2^{-\cdot}$ and can be inhibited by N-(2′-mercaptoethyl)-1,3-propanediamine,[90] a free thiol compound that can substitute for glutathione as hydrogen donor. The authors therefore suggested that free radical-mediated depletion of glutathione is involved in induction of HO.

Endotoxemia and interferon-γ

Acute endotoxemia, associated with systemic hypotension and multisystem failure, causes oxidative stress in experimental animals.[91,92] Through its interaction with cellular components of the immune system, endotoxin also stimulates the release of IFN-γ. Pretreatment of rats with IFN-γ inducers which is characterized by unaltered alveolar superoxide dismutase, increased HO activity and reduced levels of haem and cytochrome P-450,[41,70] reduces the mortality of the animals caused by subsequent exposure to hyperoxia,[70] IFN-γ-mediated protection against oxidative stress may not solely be due to elevated HO activity but also involve induction of metallothioneins[93] and indoleamine 2,3-dioxygenase (EC 1.13.11.17).[94] Interestingly, we have found recently that some of the products derived from the action of the dioxygenase are also very powerful peroxyl radical scavengers,[95] much in analogy to bile pigments. Furthermore, since indoleamine 2,3-dioxygenase is known to use $O_2^{-\cdot}$ as substrate and co-factor[96] the analogy between 2,3-dioxygenase and HO extends even further. Both enzymes can be regarded as antioxidant defences acting at two different stages: they

consume potential pro-oxiants while at the same time they can produce powerful antioxidants.

Parturition-induced oxidative stress

Preterm babies most often are deficient in vitamin E when compared with fullterm babvies,[97] and hence would be expected to be especially vulnerable to oxidative tissue damage caused by the sudden exposure to higher oxygen partial pressure during and shortly after birth. As erythrocytes from prematurely born babies are also low in vitamin E[97] their circulating life span is shortened,[98] and this in itself plays a contributory role to the development of hyperbilirubinemia in the early neonatal period.[99] Plasma levels of vitamin E and BR are inversely correlated in preterm babies, and administration of vitamin E decreases serum BR.[99] These findings, together with the several-fold higher HO activity in human fetal liver than in adult liver,[100] can be regarded as an antioxidant protection provided by hyperbilirubinemia against parturition-induced oxidative stress. Indeed hyperbilirubinemic preterm infants develop retinopathy, a blinding disease resulting from oxidative damage to the incompletely vascularized retina,[101] with lower frequency than preterms with normal levels of plasma BR.[102] In another *in vivo* situation, induction of hyperbilirubinemia by either a loading dose of BR or ligation of the common bile duct, markedly reduced oxygen radical-dependent, stress-induced mucosal injury in rats.[103]

CONCLUSIONS

Amongst numerous other conditions, oxidative stress has been reported in the literature to induce HO in animal and human tissues. Such induction can be regarded as an additional line of antioxidant defence under these stress conditions. The proposed protection afforded by HO induction is two-fold, i.e. it decreases the levels of certain potential pro-oxidants while increasing those of antioxidatively active bile pigments. Depsite a wealth of knowledge on HO induction, its regulation and consequences on haem metabolism, clearly more information is needed on the effects of such induction on tissue levels of the various physiological forms of BR.

The proposed antioxidant defence provided by HO induction could be tested using induced and non-induced cells and examining their sensitivity towards oxidative damage. Sn-Protoporphyrin, a potent competitive inhibitor for HO *in vitro* and *in vivo*[104,105] could be used to down-regulate HO activity in isolated cells or animals. In addition, the measurement of changes in plasma levels of biliverdin, the oxidation product of albumin-bound BR,[9] may be useful to evaluate the relative importance of circulating BR as antioxidant under conditions of oxidative stress.

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